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Sumoylation of eukaryotic elongation factor 2 is vital for protein stability and anti-apoptotic activity in lung adenocarcinoma cells

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Abstract

By screening mouse monoclonal antibody libraries for Kelch repeats, we serendipitously identified monoclonal antibodies to eukaryotic elongation factor 2 (eEF2). Interestingly, eEF2 was highly expressed in lung adenocarcinoma (LADC), but not in the neighboring non-tumor lung tissue. Normally, eEF2 is involved in the peptidyl-tRNA translocation during protein synthesis. Overexpression of eEF2 would implicate an association with disease progression of LADC. In this study, we investigated the prognostic significance of eEF2 in patients with LADC. Expression of eEF2 was detected by immunoblotting, immunohistochemistry and confocal immunofluorescence microscopy. Our results show that patients with high eEF2 expression had significantly higher incidence of early tumor recurrence (67.8% vs. 18.2%, $p = 0.016$), and a significantly worse prognosis ($p < 0.001$). In an *in vitro* study, silencing of eEF2 expression increased mitochondrial elongation, cellular autophagy and cisplatin sensitivity. Moreover, eEF2 was sumoylated in LADC cells, and eEF2 sumoylation correlated with drug resistance. These results suggest that eEF2 is an anti-apoptotic marker in LADC. However, biological function and involvement of eEF2 in the disease progression of LADC require further studies.

Keywords: autophagy, cisplatin sensitivity, eEF2, mTOR, SAE2, sumoylation

Abbreviations used are: ATAD3A, the ATPase family, AAA domain containing 3A; Bcl-xL, Bcl-2-like 1; DRP1, dynamin-related protein 1; eEF2, eukaryotic elongation factor 2; LADC, lung adenocarcinoma; Mfn-2, mitofusin-2; mTOR, mammalian target of rapamycin; SAE2, SUMO-1 activating enzyme subunit 2; SUMO, small ubiquitin-related modifier

Introduction

High metastatic potential and cell growth rate, as well as resistance to irradiation and anticancer chemotherapeutic agents are major characteristics of lung adenocarcinoma (LADC).[1] To find out whether the malignant characteristics correlated with specific gene expression, we had used different methods to screen gene expression profiles, and we identified several genes of interest, such as dihydrodiol dehydrogenase, hepatocyte growth factor (HGF), HGF receptor (or c-Met, a product of proto-oncogene c-*met*), dynamin-related protein 1 (DRP1) and ATPase family, AAA domain containing 3A (ATAD3A).[2-6] Among these genes, protein level, but not mRNA level, of ATAD3A was increased when LADC cells were exposed to the hypoxic condition, suggesting that translation efficacy was altered under such circumstance.[6,7]

Protein translation factors, e.g., eukaryotic protein initiation factor 4E (eIF4E) and protein elongation factor 1A2 (eEF1A2), have been shown to be associated with oncogenesis.[8,9] Their expression correlates with tumor cell growth, invasion, metastasis and hence the poor prognosis in lung, breast, ovarian cancer and acute myeloid leukemia.[10-13] When screening mouse monoclonal antibody libraries for Kelch repeats,[14] we serendipitously found monoclonal antibodies to eukaryotic elongation factor 2 (eEF2), a 95-kDa protein that catalyzed translocation of peptidyl-tRNA on the ribosome during the elongation phase of translation.[15] The effect of eEF2, however, has not been determined in the lung adenocarcinoma.

In this report, we studied eEF2expression in the LADC specimens by immunoblotting and immunohistochemistry. We statistically evaluated the correlation between eEF2 expression and the clinicopathological parameters as well as the prognostic value of eEF2 in LADC patients. *In vitro*, we determined how eEF2 expression influenced cisplatin resistance in LADC cell lines and characterized the cytoprotection function of eEF2. Our results showed that eEF2 was highly

expressed in the LADC. Interestingly, eEF2 was sumoylated, and sumoylation was essential for the protein stability and anti-apoptotic function of the eEF2 in LADC cells.

Materials and methods

Tissue specimens and lung cancer cell lines. From January 2001 to December 2005, we enrolled 372 patients, who were diagnosed having lung adenocarcinoma (LADC). Disease stage was re-classified according to the new international staging system.[16] The intramural Medical Ethics Committee of China Medical University Hospital approved the protocol (DMR99-IRB-203), and written informed consent to donate biopsy specimens was obtained from each patient. All patients had undergone surgical resection and radical N2 lymph node dissection, followed with cisplatin-based chemotherapy. After treatment, patients were routinely followed every 3 to 6 months in outpatient department. Immunohistochemical staining was carried out using a single-blinded procedure.

Seven lung cancer cell lines (H23, H226, H838, H1437, H2009, H2087 and A549) were used for the evaluation of gene expression. H23, H838, H1437, H2009, H2087 and A549 are LADC, and H226 is epithelial type. Human type II pneumocytes (ATII) and bronchial epithelial cells (BEC) were obtained using previously reported methods.[3,17,18] Identity of the BEC and ATII cells was confirmed by immunoreactivity to cytokeratin 18 or surfactant protein B.[3,17] Cells were grown at 37°C in a monolayer in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100 I.U./ml penicillin and 100 μg/ml streptomycin.

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR had been described previously.[2] The primers were selected by Primer3 (http://frodo.wi.mit.edu/). For eEF2, the primers are: eEF2s: 5'-GGTCTACTCAGCCAAGAAT-3' (sense primer, nts 1523-1543, NM_001961) and eEF2a: 5'-CACTTCCTCCCGTAGTCAAA-3' (antisense primer, nts 2116-2133). The estimated cDNA fragment is 611 base-pair (bp). For SUMO-1 activating enzyme subunit 2 (SAE2), the primers are: SAE2s: 5'-TTCAAATAATGCCGACGTCA-3' (nts

1861-1880, NM_005499.2) and SAE2a: 5'-TCTCATGACTGGTTATGCAC-3' (nts 2232-2213). The amplified fragment is 372 bp. The primers for β-actin are as follows: 5'-AGAGCTACGAGCTGCCTGAC-3' (nts 797-816, NM_001101.3) and 5'-CACCTTCACCGTTCCAGTTT-3' (nts 1375-1356). The amplified β-actin fragment is 579 bp.

Immunoprecipitation, gel electrophoresis and protein analysis by MALDI-TOF. The procedure for immunoprecipitation, gel electrophoresis and protein analysis by MALDI-TOF was published previously.[5,6] Briefly, cell lysate was added to protein G sepharose™ (PGS) (Amersham, Uppsala, Sweden) to remove non-specific protein bindings. The supernatant was reacted with 5 μg of monoclonal antibodies and fresh PGS at 4°C for 18 hrs. The reaction mixture was centrifuged at 800 × g for 1 min, and the precipitate was dissolved in a loading buffer (50 mM Tris, pH 6.8, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.01% bromophenol blue, 10% glycerol, 5% β-mercaptoethanol and 1% SDS). Electrophoresis was carried out in two 10% polyacrylamide gels. One gel was stained with Coomassie blue. Protein bands on Coomassie-stained gel, which corresponded to the immunoblotting-positive bands, were extracted for identification by MALDI-TOF (Applied Biosystems, Milpitas, CA). Peptide fingerprints were matched to SwissProt database by MS-fit.

Immunoblotting, immunological and immunofluorescent staining. The procedure for immunoblotting has been described previously.[2] Briefly, proteins separated on a polyacrylamide gel were transferred to a nitrocellulose membrane. The membrane was probed with specific antibodies. The protein was visualized by exposing the membrane to an X-Omat film (Eastman Kodak, Rochester, NY) with chemiluminescent reagent (NEN, Boston, MA). Antibodies to eEF2 were raised in the laboratory (Supplementary Fig. S1-S6). For

immunocytochemistry, the cells were grown on slides, and fixed with methanol/acetone at 4°C for 10 minutes before staining. Immunological staining was performed by an immunoperoxidase method.[2] For immunofluorescence staining, MitoTracker® Red CMXRos (Molecular Probes, Inc., Eugene, OR) was used to label mitochondria, and nuclei were stained with 4',6-Diamidino-2-phenylindole (DAPI). Slides were examined under a laser scanning confocal microscope (LSM510 Zeiss, Chicago, IL).

Slide evaluation of eEF2 expression by immunohistochemical staining. In each pathological section, non-tumor lung tissue (NTLT) served as internal negative control. Slides were evaluated by two independent pathologists blinded to the clinicopathological knowledge. The ImmunoReactive Scoring system was adapted for this study:[19] a specimen was considered having strong signals when more than 25% of cancer cells were positively stained ($eEF2^+$); and negative, if less than 25% of the cells were stained ($eEF2^-$).[3-6]

Statistical analysis. Correlation of eEF2 levels with clinicopathological factors was analyzed by the Chi-Square test. Survival curves were plotted using the Kaplan-Meier estimator.[20] Statistical difference in survival between different groups was compared by a log rank test.[21] Statistical analysis was performed using GraphPad Prism5 statistics software (San Diego, CA). Statistical significance was set at $p < 0.05$.

Electron microscopy. Cells were fixed with 2.5% glutaraldehyde (EM grade, Sigma, St Louis, MO) at 4°C overnight, and then with 1% osmium tetroxide. The cells were suspended in 2% molten agar, which was dehydrated and embedded in LR white (Agar Scientific Ltd., Essex, UK). The cell blocks were cut with ultramicrotome (Leica Ultracut R, Vienna, Austria). The thin sections were transferred to copper grids, and stained with 2% uranyl acetate for 30 min, and 2.66% lead citrate (pH 12.0) for 10 min, before electron microscope observation (JEM1400,

JEOL USA, Inc., Peabody, MA, USA). For gene silencing experiments, cells were harvested 48 hours following siRNA treatment.

Transwell migration and invasion assays. H1437 cells were transfected with vector or pcDNA3-eEF2, and seeded at 15,000 cells per well into collagen-coated invasion chambers (5 μm pore size, Corning Inc. Life Sciences, MA). The wells were fixed with 4% paraformaldehyde after 3 h for invasion assays. The wells were r stained with 0.1% crystal violet. The number of cells that had migrated was visualized with an Olympus CKX41 inverted microscope. Number of the cells that had migrated was quantified using GraphPad Prism5 analysis (GraphPad Software, Inc., La Jolla, CA).

Results

Expression of eEF2 in LADC cells. Expression of eEF2 was detected in 7 lung cancer cell lines by RT-PCR (Fig. 1a). In 22 pairs of lung cancer biopsies, eEF2 mRNA was detected in all lung adenocarcinoma and the corresponding non-tumor lung tissues (NTLT) (Fig. 1b). No significant difference was detected between tumor and NTLT. DNA sequence of the amplified fragments matched that of eEF2: NM_01961, *Homo sapiens* eukaryotic translation elongation factor 2 (http://blast.ncbi.nlm.nih.gov/Blast.cgi). No polymorphism or mutation was detected.

Our antibodies recognized a 110 kDa protein band (Fig. 2a). Levels of the 110-kDa protein varied among cancer cells: high in H23, H226, H2009, H2087 and H838cells, and moderate in A549, H1437 and HeLa cells. Using specific shRNA (Table 1) to silence eEF2 ($eEF2^{kd}$) expression reduced eEF2-positive protein band (Fig. 2b), validating that monoclonal antibodies raised in our lab recognized eEF2. However, silencing of eEF2 expression reduced cell growth (Fig. 2c) and increased cisplatin sensitivity (Fig. 2d), as well as partial degradation of β-actin. To examine whether the effect of eEF2 silencing was a general phenomenon, we used dynamin-related protein 1 (DRP1), human homolog of yeast RAD23 A (hHR23A), 70-kDa heat shock protein (Hsp70), procaspase 3 and α-tubulin as cytoplasmic markers; the ATPase family, AAA domain containing 3A (ATAD3A), Bcl-xL, mitofusin (Mfn)-2, optic atrophy 1 (OPA1) and programmed cell death protein 8 (PCD8) as mitochondrial markers; and poly (ADP-ribose) polymerase (PARP) as nuclear marker. Although silencing of eEF2 markedly reduced protein levels of β-actin and DRP1 (Fig. 2e), and to a lesser extent in Mfn-2, OPA1 and PARP, it did not cause marked change in other proteins.

Expression of eEF2 correlates with patients' survival. Compared to NTLT (Fig. 3a), eEF2 overexpression was detected in 295 (79.3%) of lung adenocarcinoma (LADC) (Fig. 3b-3d).

Overexpression of eEF2 (13/16, 81.25%) was verified by immunoblotting (Fig. 3e). Statistical analysis showed that eEF2 expression correlated with tumor staging and cigarette smoking (Table 2). Smokers and patients with earlier LADC stages are more likely to express eEF2.

Among the 295 patients who had high eEF2 levels, 200 (67.8%) patients had tumor recurrence. Among 77 patients who had low eEF2, 14 (18.2%) patients had tumor recurrence. All 214 patients developed new tumors within 18 months after operation. The recurrence rate in patients with high eEF2 was 3.73-fold higher than that in patients with low eEF2. The difference was significant ($p = 0.016$). A significant difference was also found in cumulative survival of all the patients enrolled (Fig. 3f, $p < 0.001$), and in patients with stage I disease (Fig. 3g, $p = 0.0129$). When patients were divided into groups by each of clinicopathological parameters, significant difference by univariate analysis was found in cigarette smoking, cell differentiation, tumor stage, lymph node involvement, eEF2 expression and gender (Table 2). In multivariate analysis, cigarette smoking ($p = 0.021$), cell differentiation ($p = 0.037$), tumor stage ($p < 0.001$), lymph node involvement ($p = 0.029$) and eEF2 expression ($p = 0.046$) remained significant.

Sumoylation of eEF2 is essential for protein stability and cell survival against cisplatin in lung adenocarcinoma cells. Because eEF2 appeared as a 110-kDa protein, and the amino acid sequence of the 110-kDa protein matched that of the 95-kDa eEF2 (Fig. S2A and 2B), the data suggested that the 110-kDa eEF2 could be post-translationally modified. However, the 110-kDa eEF2 was insensitive to calf alkaline phosphatase, excluding a possibility of protein phosphorylation. Since ubiquitylation accelerates eEF2 degradation, prediction of ubiquitylation may not be applicable.[22] When eEF2 antibodies-precipitated proteins were probed with rabbit antibodies to SUMO-1 (Cell Signaling Technology, Inc., Danvers, MA), the 110-kDa protein was positive for SUMO-1 (Fig. 4a), indicating that the 110-kDa eEF2 was sumoylated. Although

other higher molecular weight proteins were detected by antibodies to SUMO-1, but not antibodies to eEF2, the data suggested that eEF2 might interact with other proteins that were sumoylated.

Our previous studies had shown that nicotine increased cytoprotection against anticancer drugs of the cancer cells, possibly in part by strengthening mitochondrial function and in part by increasing protection of nucleoli.[2-6] Interestingly, addition of nicotine increased expression of 110-kDa eEF2 and DRP1 (Fig. 4b), as well as resistance to cisplatin (Fig. 4c). Moreover, ectopic expression of eEF2 (Fig. 4d) not only increased cisplatin resistance (Fig. 4e), but also invasion ability of lung adenocarcinoma (LADC) cells as determined by transwell assays (Fig. 4f). Because 110-kDa eEF2 was detected in 81.25% of LADC biopsies and its expression was positively correlated with patient's cigarette smoking habit, we expected that enzymes for sumoylation might be highly expressed in LADC. SUMO-1 activating enzyme subunit 2 (SAE2)[23] was indeed highly expressed in both cancer cell lines and tumor biopsies (Fig. 4g, 4h and 4i) as detected by RT-PCR or immunoblotting. Treatment with nicotine increased SAE2 expression and protein levels of 110-kDa eEF2 (Fig. 4j). Silencing of SAE2 expression, on the other hand, reduced protein levels of eEF2 (Fig. 4k) and drug resistance (Fig. 4l), which were consistent with our previous data and indicated that sumoylation was crucial for eEF2 stability and drug resistance.

The effect of eEF2 silencing on mitochondrial morphology. Since silencing of eEF2 concurrently reduced DRP1 levels, and previous studies showed that reduced DRP1expression induced dilatation of endoplasmic reticulum (ER), mitochondrial elongation and formation of autophagic vesicles.[5] Silencing of eEF2 ($eEF2^{kd}$) also provoked these phenomena (Fig. 5a), which were similar to those found in $DRP1^{kd}$ cells (Fig. 5b). We then examined the gene

silencing effect of the other mitochondrial transport-related proteins.[6] Knockdown of ATAD3A increased the number of autophagic vesicles, and mitochondrial fragmentation (Fig. 5c). In Mfn-2^{kd} cells, although a marked increase in the number of peroxisomes as well as the tethering between the ER and the mitochondria was observed, no extensive mitochondrial elongation was detected (Fig. 5d). These results corresponded well with our immunoblotting data.

Discussion

Our results show that the eEF2 is highly expressed in lung adenocarcinoma (LADC). Expression of eEF2 in LADC patients correlated with significantly higher incidence of tumor recurrence and increased cisplatin resistance, which ultimately reflected in worse prognosis.

By showing that the 110-kDa protein, which was detected in LADC cell lines and tumor biopsies, was positive for SUMO-1, our results suggested that the 110-kDa eEF2 was sumoylated. SUMO-1 is a 101-amino-acid protein (about 12-kDa)[24,25] and the estimated molecular weight of the naked eEF2 is 95-kDa; therefore, the 110-kDa molecular weight is approximately a product of one naked eEF2 with one SUMO-1 residue.

Sumoylation was first identified on ras-like GTPase activating protein (RanGAP1).[24] Sumoylation facilitates RanGAP1 to react with the Ran GTP binding protein RanBP2. In contrast, desumoylation reduced the binding ability of RanGAP1.[26] However, the emerging evidence shows that extranuclear proteins, e.g., glucose transporters (GLUT1 and GLUT4), the type I transforming growth factor-β receptor (TβRI), and protein tyrosine phosphatase 1B (PTP1B), are sumoylated as well.[23,27] Interestingly, these proteins are integral membrane proteins. The hydrophobic stretches in eEF2 suggested that the protein might also be located on the membrane (Supplementary Fig. S6). Besides, eEF2 contains a stretch of coiled-coil, which may interact with proteins that also contain coiled-coil, e.g., DRP1 and Mfn-2,[5,6] during protein synthesis and the subsequent protein transportation (Supplementary Fig. S7). These features further implicate that to perform adequate SUMOylation, the enzyme machinery should be in the vicinity of substrate proteins. Interestingly, SAE2 contained a hydrophobic domain and could be placed in the near vicinity of membrane proteins (Supplementary Fig. S8). Moreover, as determined by RT-PCR, SAE2 was highly expressed in lung adenocarcinoma specimens (LADC)

and cell lines. It is possible that SAE2 is concomitantly expressed with eEF2 to increase the efficacy of sumoylation and to maintain eEF2 stability as well as the synthesis of cancer-specific proteins. The protein level and the intracellular location of SAE2 in LADC, however, are yet to be resolved.

Using autoantibody-mediated identification of antigens (AMIDA or serological proteome approach, SERPA),[28] Suzuki *et al*. found that eEF2 was one of the significant tumor-associated antigens in melanoma patients.[29] Using an immunohistochemical method, Nakamura *et al*. showed that eEF2 was overexpressed in gastric and colorectal cancers.[30] Our results supported their observations, indicating that cancer cells could express eEF2 to facilitate cell growth and metastasis.[31] Silencing of eEF2 expression, on the other hand, inhibited cell growth as well as increased apoptosis and drug sensitivity, suggesting that eEF2 could be an anti-apoptotic factor.

However, eEF2 silencing did not evidently affect apoptosis-associated proteins, e.g., procaspase 3, Bcl-xL or PCD 8. Silencing of eEF2, on the other hand, induced ER dilatation, mitochondrial elongation and formation of autophagic vacuoles, phenomena that were similar to cells with loss of function in DRP1, ATAD3A or OPA1 gene,[5,32,33] supporting that the anti-apoptotic effect of eEF2 could be on mitochondrial shaping. Elegant studies by Rolland *et al*. and Fannjiang *et al*. suggested that anti-apoptotic activity of Bcl-2-like protein CED-9 of *Caenorhabditis elegans* was *via* FZO-1 [ortholog of mammalian mitofusin (Mfn-1 and Mfn-2)] or EAT3 (OPA1 homolog of *C. elegans*) to inhibit DRP1-related cell lethality.[32-35] Interestingly, stability of ATAD3A and OPA1 was maintained by PKC phosphorylation,[6] whereas that of DRP1 was mediated by AMP-activated protein kinase (AMPK) and nutrient deficiency-mediated kinases.[5] Because eEF2 silencing markedly reduced DRP1 level, but not that of ATAD3A or OPA1, our results suggested that eEF2silencing might not affect AMPK or eEF2 kinase

(eEF2K),[36] and the autophagy induced by eEF2 silencing could be independent of mammalian target of rapamycin (mTOR).[5,36,37]

As noted above that overexpression of protein translation factors, e.g., eIF4E and eEF1A2, was associated with tumor growth, invasion and metastasis of lung cancer.[10-13] Our study showed that eEF2 expression correlated with tumor cell growth and poor prognosis in lung adenocarcinoma patients as well. However, post-translational modification of eEF2 is sumoylation, whereas that of eIF4E and eEF1A2 is phosphorylation, which is regulated by hypoxia and mTOR response to inhibit global protein synthesis.[36,37] It is worth noting that hypoxia increased expression of hepatocyte growth factor (HGF), interleukin-8 and prostaglandin $F_{2\alpha}$.[38] Serum starvation, however, increased protein level of ATAD3A and mRNA levels of HGF, matrix metalloproteinases and histone deacetylase 5.[6] Since accelerated cancer cell proliferation frequently generated local hypoxia and nutrient deficiency in tumor nest, which could then shut down protein synthesis to conserve energy consumption *via* inhibition of the mTOR system,[37] our data offered a reasonable explanation for how oxygen or nutrients deprivation-induced genes concert their efforts for the survival of cancer cells. More importantly, our results indicated that cancer cells might take an alternative means to sustain biosynthesis of vital proteins while cutting down translation efficacy for non-essential proteins.

In conclusion, our results showed that sumoylated eEF2 was frequently detected in the lung adenocarcinoma, and was associated with poor prognosis. These data suggested that eEF2 and sumoylation-related enzyme SAE2 could be potential oncogenes, and targets of chemotherapeutics.

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The authors declare no conflict of interest.

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37 Yang Q, Guan KL. Expanding mTOR signaling. *Cell Res* 2007;**17**:666-81.

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Figure Legends

Fig. 1. Expression of eEF2 in lung cancer cells. a, Expression of eEF2 was detected by RT-PCR in lung cancer cell lines. b, In lung biopsies, expression of eEF2 was detected in all of lung tissues. No significant difference was found between cancer and non-tumor lung tissue (NTLT). Expression of β-actin was used as a standard. N: NTLT, T: tumor fraction of surgical resections.

Fig. 2. Characterization of monoclonal antibodies to eEF2. a, Monoclonal antibodies recognized a 110-kDa protein band by immunoblotting. Expression of eEF2 was detected in all the cancer cell lines. b, Silencing of eEF2 expression by shRNAs ($eEF2^{kd}$) reduced protein level of eEF2 and β-actin as determined by immunoblotting. c, cell proliferation decreased in $eEF2^{kd}$ cells. d, Silencing of eEF2 increased cisplatin sensitivity in lung cancer cells. ○, H838, wild-type; ■, H838, $eEF2^{kd}$ with shRNA1; ●, H838, $eEF2^{kd}$ with shRNA2. F-test, $p < 0.01$; e, The effect of eEF2 silencing on protein stability. DRP1, hHR23A, Hsp70, procaspase 3 and α-tubulin were cytoplasmic markers; ATAD3A, Bcl-xL, Mfn-2, OPA1 and PCD8 were mitochondrial markers; PARP was nuclear marker. Besides β-actin, eEF2 silencing reduced protein level of DRP1. Although there was a decrease in Mfn-2, OPA1 and PARP, the reduction was not as dramatic.

Fig. 3. Correlation between eEF2 expression and survival in patients with lung adenocarcinoma (LADC). Representative examples of eEF2 expression in pathological specimens as detected by immunohistochemistry (crimson precipitates). Expression of eEF2 was not detected in (a) NTLT, but in lung adenocarcinoma cells (b-d). e, Expression of eEF2 was confirmed by immunoblotting. Expression of β-actin was used as a monitoring standard. N: NTLT, T: tumor fraction. f, Comparison of Kaplan-Meier product limit estimates of survivals in LADC patients. Patients

were divided based on eEF2 expression. Survival difference was compared by a log rank test. $p < 0.001$; g, Patients with stage I disease were stratified according to eEF2 expression. $p = 0.0129$

Fig. 4. Protein eEF2 is sumoylated, and sumoylation maintains eEF2 stability and drug resistance. a, eEF2 antibodies-precipitated 110-kDa protein was positive for SUMO-1 (detected by rabbit antibodies to SUMO-1), indicating that the 110-kDa eEF2 was sumoylated. In addition to 110-kDa eEF2, other higher molecular weight protein bands were positive for SUMO-1, but not positive for antibodies to eEF2, suggesting that eEF2 might interact with proteins which were sumoylated as well. b, Treatment with nicotine increased 110-kDa eEF2. c, Addition of nicotine increased cisplatin resistance in lung adenocarcinoma (LADC) cells H838. ○, H838, control; ■, H838, 2 μM nicotine; ▲, H838, 10 μM nicotine; The effect of ectopic expression of eEF2 on cell behavior. (d) Ectopic expression of eEF2 increased (e) cisplatin resistance, and (f) invasion ability of H1437 cells. Expression of SAE2 in LADC cells as determined by RT-PCR (g & h) and immunoblotting (i). Compared to the non-tumor cells (NTC), BEC and ATII, SAE2 was highly expressed in (g) lung cancer cell lines and (h & i) tumor biopsies. * indicated a potentially phosphorylated SAE2. j, Treatment of H1437 cells with 10 μM nicotine for 24 h increased SAE2 expression and protein levels of 110-kDa eEF2. Silencing of SAE2 expression, however, (k) reduced protein levels of eEF2 and (l) drug resistance.

Fig. 5. The effect of eEF2 silencing on the mitochondrial morphology. Silencing of eEF2 ($eEF2^{kd}$) expression induced (a) mitochondrial elongation (white arrowheads), dilation of the endoplasmic reticulum (ER), and formation of autophagic vesicles (white arrow). b, Silencing of DRP1expression ($DRP1^{kd}$) induced ER dilation, formation of autophagic vesicles and

mitochondrial elongation. The elongated mitochondria, which looked like being formed by a stack of smaller subunits, were similar in both eEF2^{kd} and DRP1^{kd} cells. c, Silencing of ATAD3A (ATAD3A^{kd}) expression increased the number of autophagic vesicles. However, it did not induce mitochondrial elongation, but mitochondrial fragmentation. d. In Mfn-2^{kd} cells, though an increase in the number of peroxisomes (arrows) and the tethering between ER and the mitochondria (arrowheads) was observed, no extensive mitochondrial elongation was detected. The results corresponded well with the immunoblotting data, in which besides the reduced levels of DRP1 and β-actin, silencing of eEF2 expression affected expression of Mfn-2 and OPA1 as well, only with lesser extent.

List of the supporting information

Characterization of monoclonal antibodies to eEF2

Supplementary Figures

S1. Monoclonal antibodies to eEF2 raised in the laboratory recognized a unique protein band in an immunoblotting.

S2A. Results summary of MALDI-TOF analysis of 110-kDa eEF2

S2B. The matched sequences of eEF2, P13639**,** Elongation factor 2

S3. Immunocytochemical staining showed that eEF2 was present in the cytoplasm of cells.

S4. The granularly subcellular structures of eEF2 suggested that the presence on endoplasmic reticulum or mitochondria.

S5. As revealed by immune-gold electron microscopy, most of the eEF2 was present on the ER (arrows), and some signals were detected in the cytosol or on the mitochondria.

S6. The major characteristics and the possible subcellular localization of eEF2, which was predicted by the web pSORT II program (http://psort.ims.u-tokyo.ac.jp/).

S7. A sketch of mitochondrial protein synthesis and subsequent protein transport.

S8. Using the TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) to predict a transmembrane domain in SAE2.

Table 1. Position and sequences of siRNA oligonucleotide to eEF2

Name and access number	position	GC%	Orientation	Sequence
eEF2-s1 (NM_001961)	nts 1255-1277	42	Sense5’	5’-GGCCCUCUUAUGAUGUAUA dTdT-3’
			cDNA	5’-AAGGCCCTCTTATGATGTATATT-3’
			Antisense3’	5’-dTdT CCGGGAGAAUACUACAUAU-3’
eEF2-s2 (NM_001961)	nts 1416-1438	47	Sense5’	5’-GCCAAAUCCAGAGAACAAUC dTdT-3’
			cDNA	5’-AAGCCAATCCAGAGAACAATCTT-3’
			Antisense3’	5’- dTdT CGGUUAGGUCUCUUGUUAG-3’
eEF2-s3 (NM_001961)	nts 137-159	47	Sense5’	5’-UCCGCAACAUGUCUGUCAC dTdT-3’
			cDNA	5’-AATCCGCAACATGTCTGTCACTT-3’
			Antisense3’	5’- dTdT AGGCGUUGUACAGACAGUA-3’

Table 2. Correlation of eEF2 expression with clinicopathological parameters in LADC patients

Parameter	Expression of eEF2		Univariate	Multivariate
	High (n = 295)	Low (n = 77)	*p* value/correlation[§]	*p* value
Gender				
Male (n = 288)	235	53	0.043[†]/0.105	0.092
Female (n = 84)	60	24		
Cigarette Smoking				
Smoker (n = 202)	171	31	0.005[†]/0.144	0.016
Non-smoker (n = 170)	124	46		
Stage				
I (n = 127)	116	11	< 0.001[†]/0.492	< 0.001
II (n = 152)	127	25		
III (n = 93)	52	41		
Cell differentiation				
Well (n = 79)	51	28	0.001[†]/-0.191	0.0027
Moderate (n = 209)	170	39		
Poor (n = 84)	74	10		
Lymphovascular invasion				
Positive (n = 277)	236	41	< 0.001[†]/0.249	0.0018
Negative (n = 95)	59	36		

[†]Two-sided *p* value determined by χ^2 test

[§]The value was determined by the Spearman Correlation, which was based on normal approximation, and not assuming the null hypothesis.

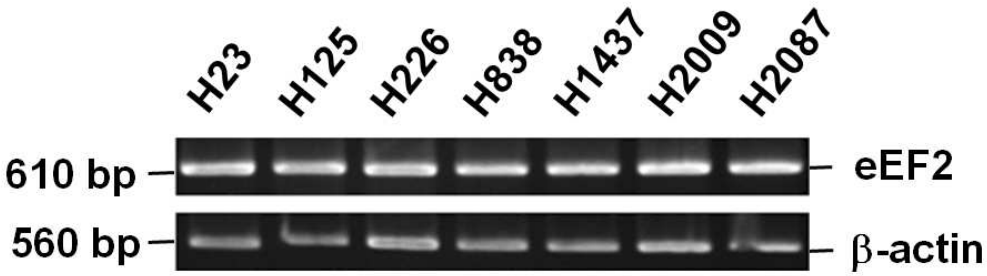

Fig 1a Expression of ATAD3A detected by RT-PCR in lung cancer cells. a, Expression of eEF2 mRNA was detected by RT-PCR in seven lung cancer cell lines.
76x22mm (300 x 300 DPI)

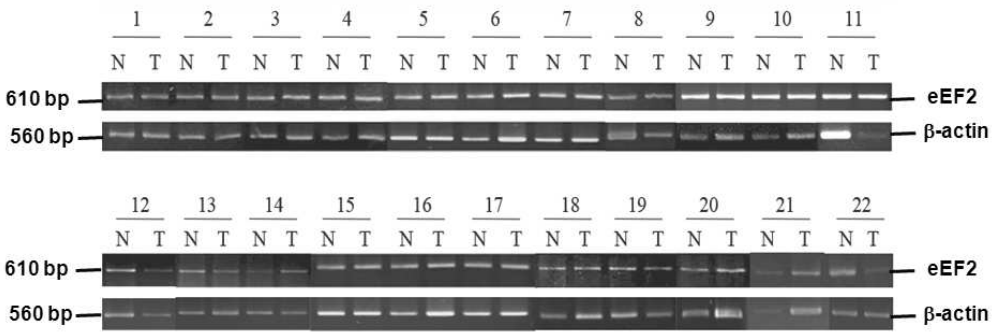

Fig 1b, In 22 pairs of lung cancer biopsies, expression of eEF2 mRNA was detected in all of Lung tissues. No significant difference was found between cancer and non-tumor lung tissue (NTLT). Expression of β-actin was used as a monitoring standard for the relative expression ratio of eEF2 mRNA. N: NTLT, T: tumor fraction of surgical resections.
79x27mm (300 x 300 DPI)

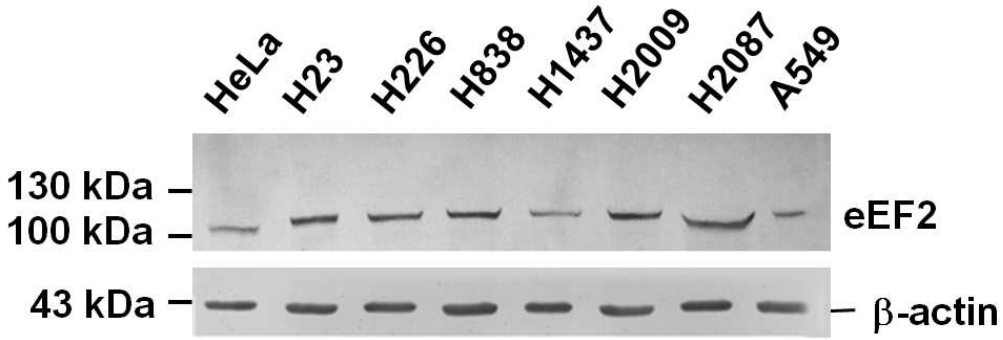

Fig 2a Characterization of monoclonal antibodies to eEF2. Immunoblotting revealed that monoclonal antibodies recognized one protein band of approximately 105 to 110-kDa. Expression of the eEF2 was detected in all seven human lung cancer cell lines.
80x28mm (300 x 300 DPI)

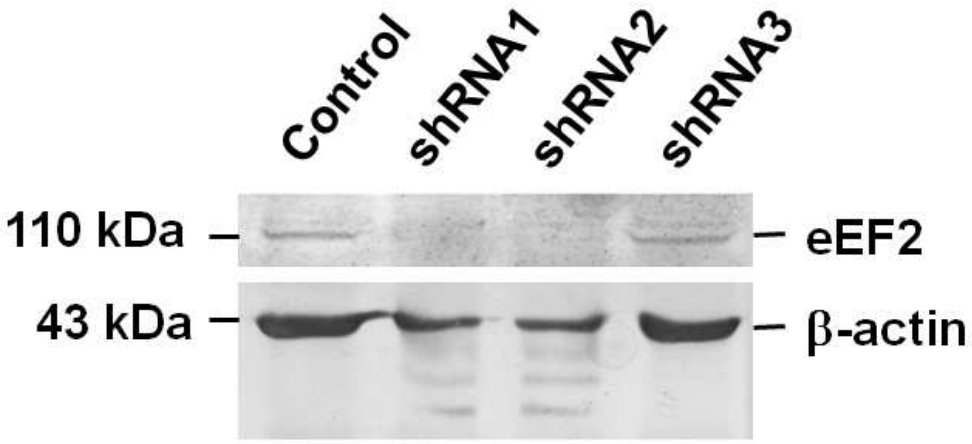

Fig. 2b, Silencing of eEF2 expression by shRNAs (eEF2^{kd}) reduced protein level of eEF2 and β-actin as determined by immunoblotting.
54x26mm (300 x 300 DPI)

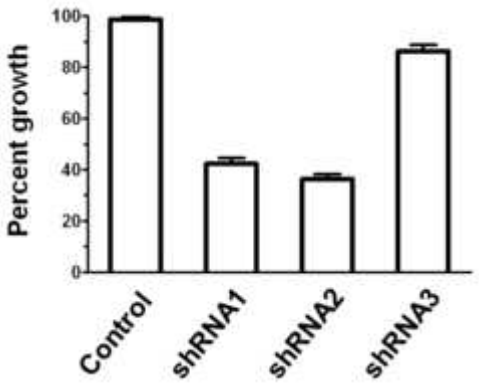

Fig 2c, Cell proliferation decreased in eEF2*kd* cells.
10x8mm (600 x 600 DPI)

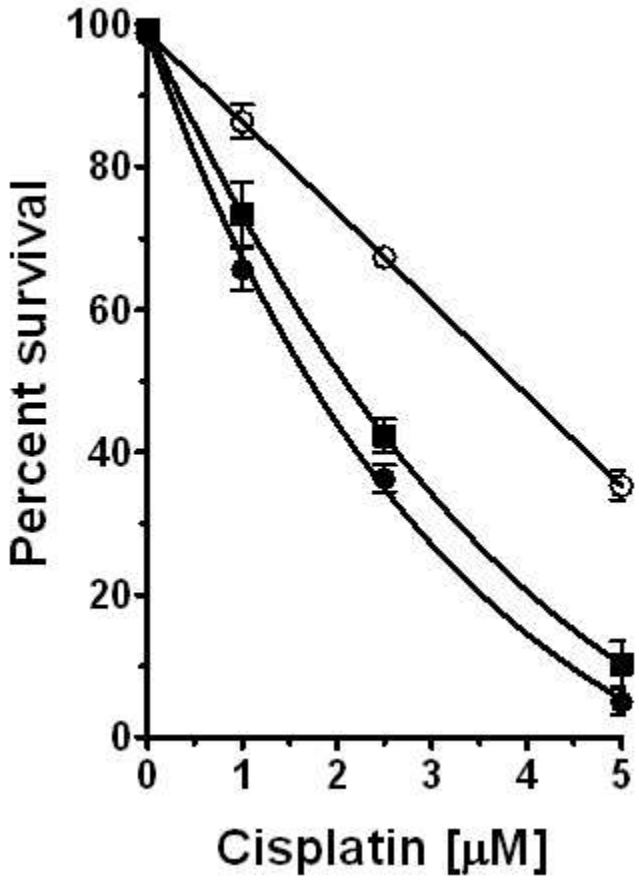

Fig. 2d, Silencing of eEF2 increased cisplatin sensitivity in lung cancer cells. ○, H838, wild-type; ■, H838, eEF2^{kd} with shRNA1; ●, H838, eEF2^{kd} with shRNA2. F-test, $p < 0.01$
29x39mm (300 x 300 DPI)

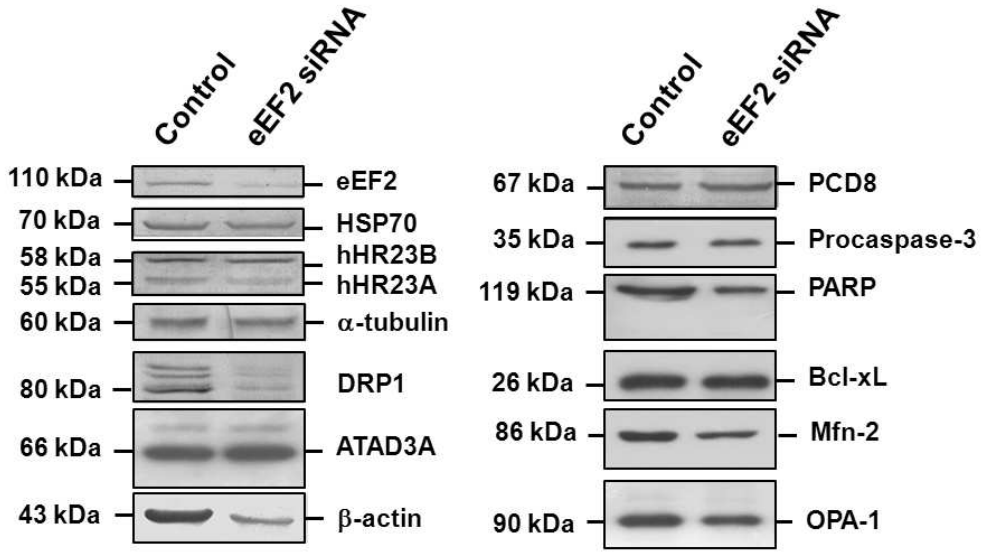

Fig 2e, The effect of eEF2 silencing on protein stability. DRP1, hHR23A, Hsp70, procaspase 3 and α-tubulin were cytoplasmic markers; ATAD3A, Bcl-xL, Mfn-2, OPA1 and PCD8 were mitochondrial markers; PARP was nuclear marker. Besides β-actin, eEF2 silencing reduced protein level of DRP1. Although there was a decrease in Mfn-2, OPA1 and PARP, the reduction was not as dramatic.
75x42mm (300 x 300 DPI)

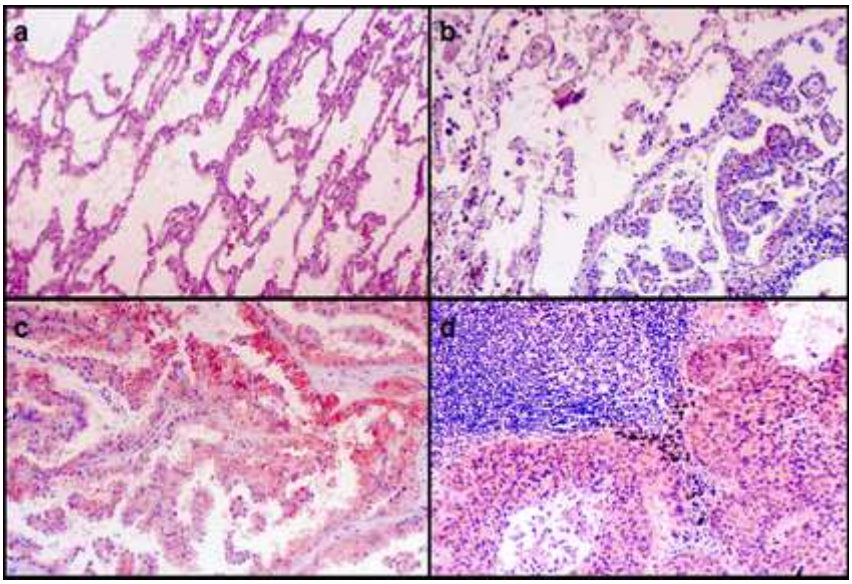

Fig 3a-d, Representative examples of eEF2 expression in pathological specimens as detected by immunohistochemistry (crimson precipitates). Expression of eEF2 was not detected in (a) NTLT, but in lung adenocarcinoma cells (b-d).
17x12mm (600 x 600 DPI)

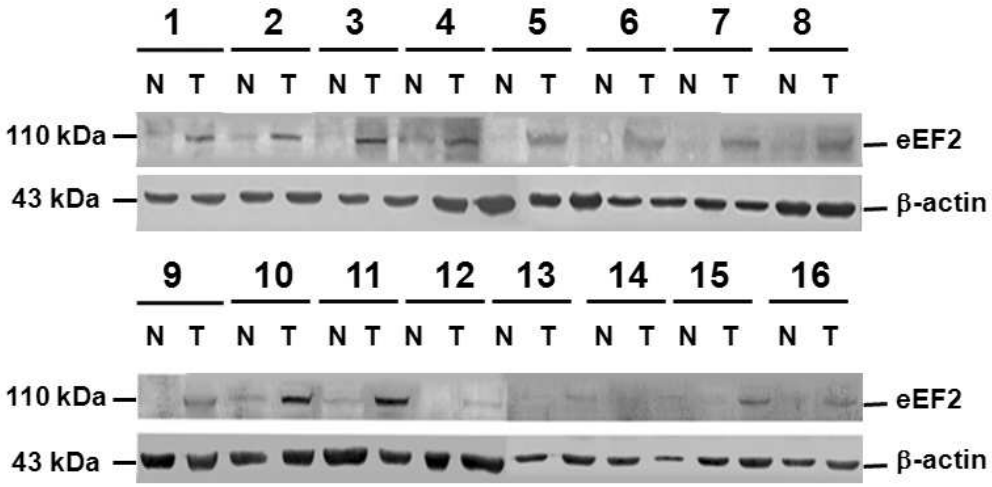

Fig 3e, Expression of eEF2 was confirmed by immunoblotting. Expression of β-actin was used as a monitoring standard. N: NTLT, T: tumor fraction.
55x27mm (350 x 350 DPI)

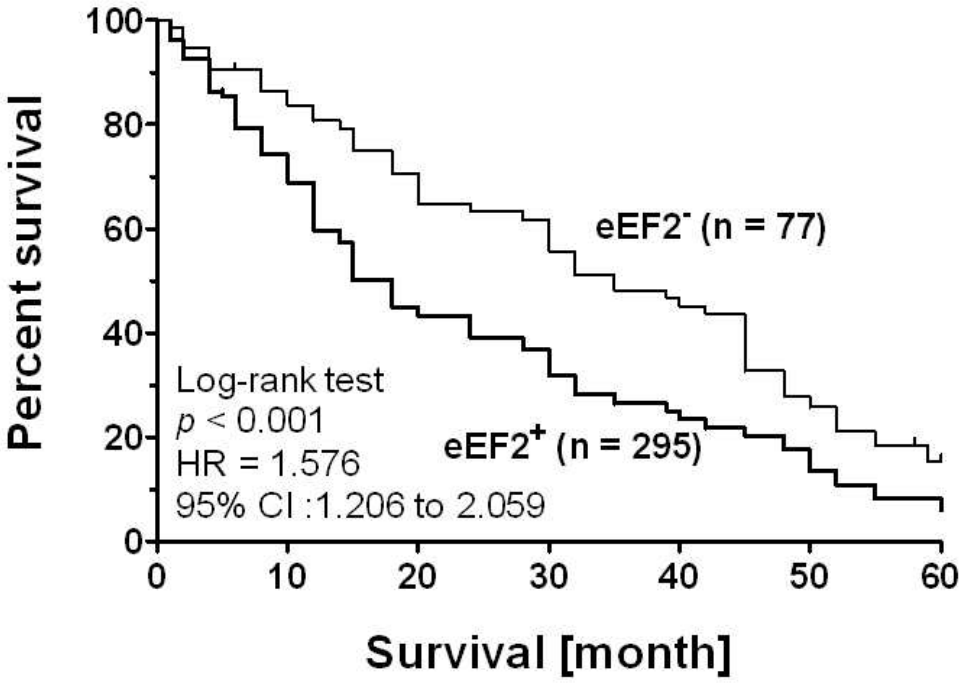

Fig 3f, Comparison of Kaplan-Meier product limit estimates of survivals in LADC patients. Patients were divided based on eEF2 expression. Survival difference was compared by a log rank test. $p < 0.001$
58x41mm (300 x 300 DPI)

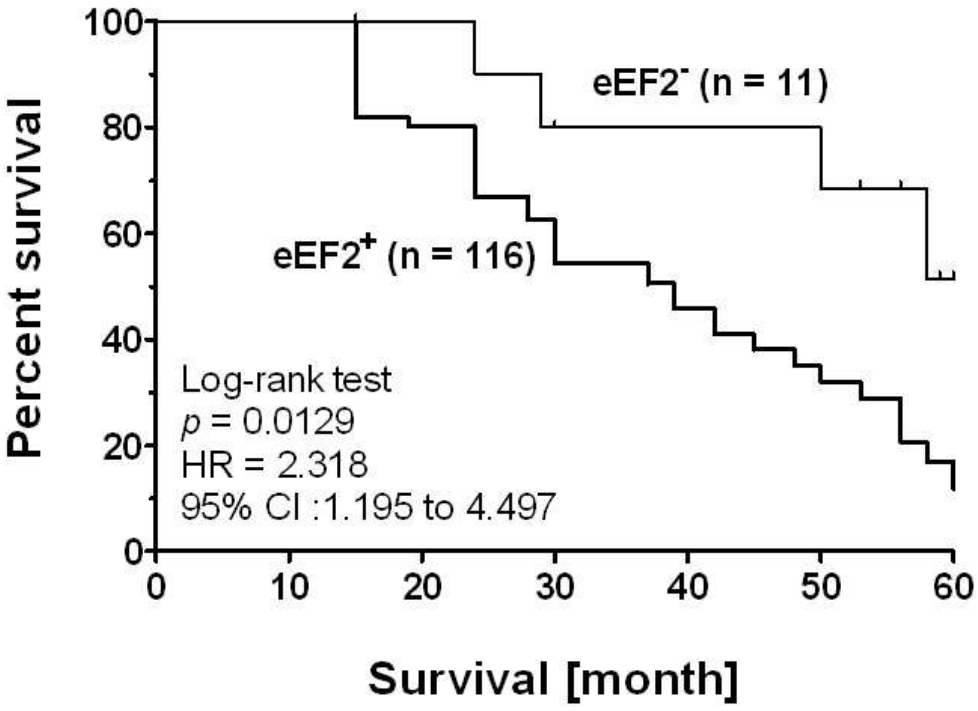

Fig 3g, Patients with stage I disease were stratified according to eEF2 expression. p = 0.0129
56x40mm (300 x 300 DPI)

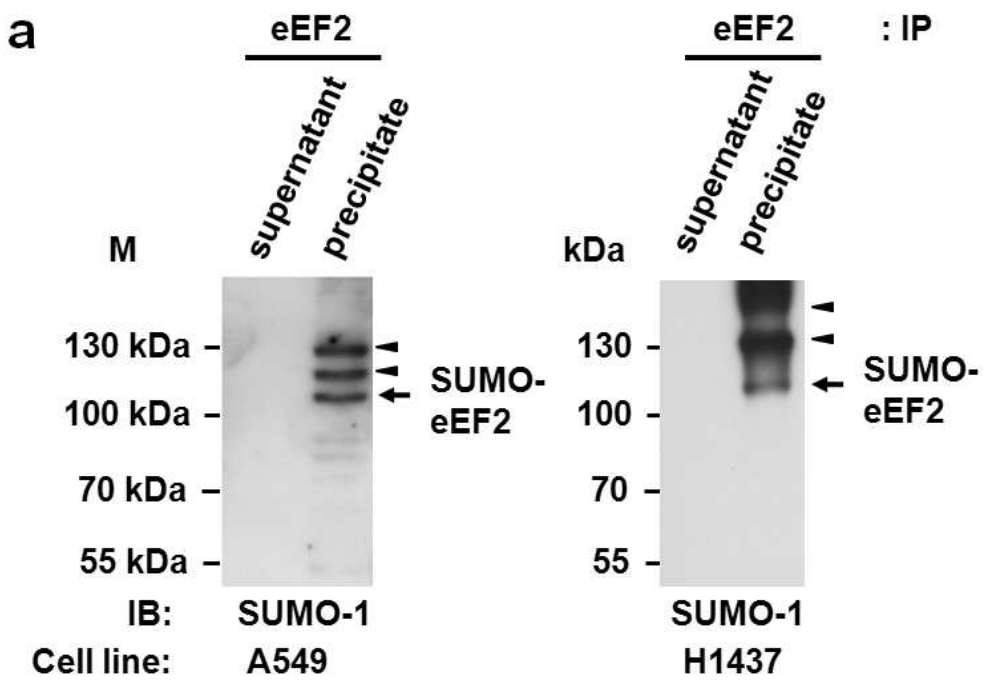

Fig 4a, Protein eEF2 is sumoylated, and sumoylation maintains eEF2 stability and drug resistance. a, eEF2 antibodies-precipitated 110-kDa protein was positive for SUMO-1 protein, which was detected by rabbit antibodies specific to SUMO-1 (Cell Signaling Technology, Inc., Danvers, MA), indicating that the 110-kDa eEF2 was sumoylated. In addition to 110-kDa eEF2, other higher molecular weight protein bands were also detected by antibodies to SUMO-1, but not by antibodies to eEF2, suggesting that eEF2 might interact with other proteins which were sumoylated as well.
63x43mm (300 x 300 DPI)

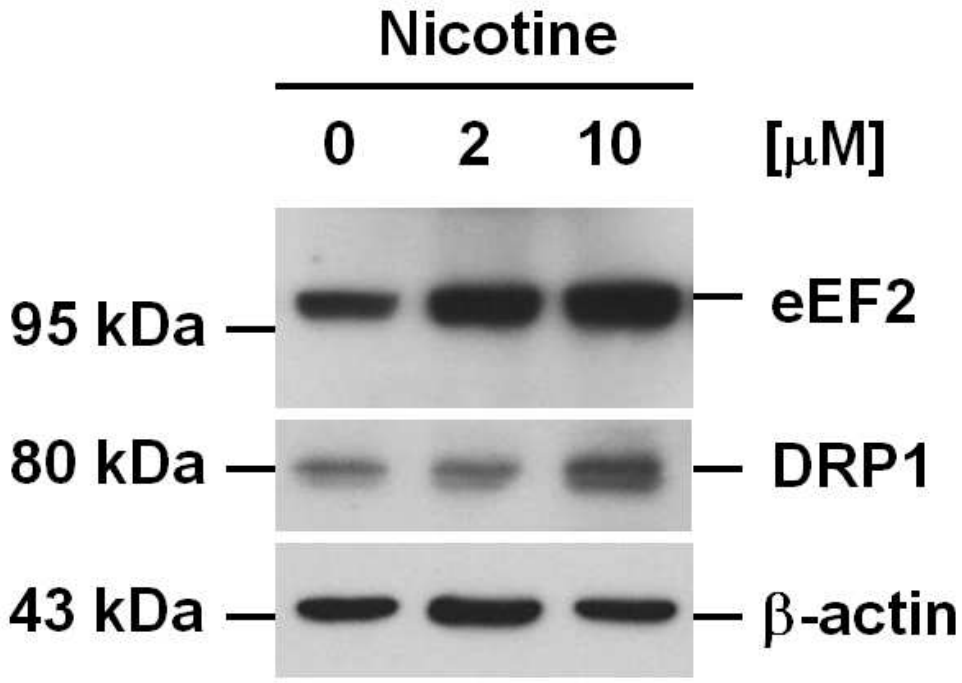

Fig 4b, Treatment with nicotine increased 110-kDa eEF2.
51x36mm (300 x 300 DPI)

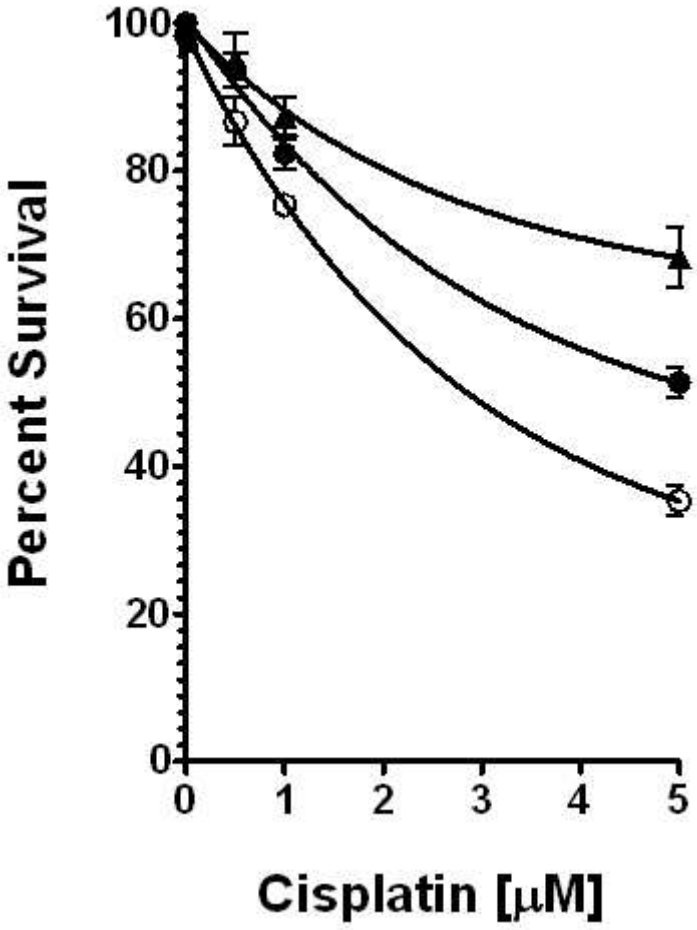

Fig 4c, Addition of nicotine increased cisplatin resistance in lung adenocarcinoma (LADC) cells H838. ○, H838, control; ■, H838, 2 μM nicotine; closed triangle, H838, 10 μM nicotine
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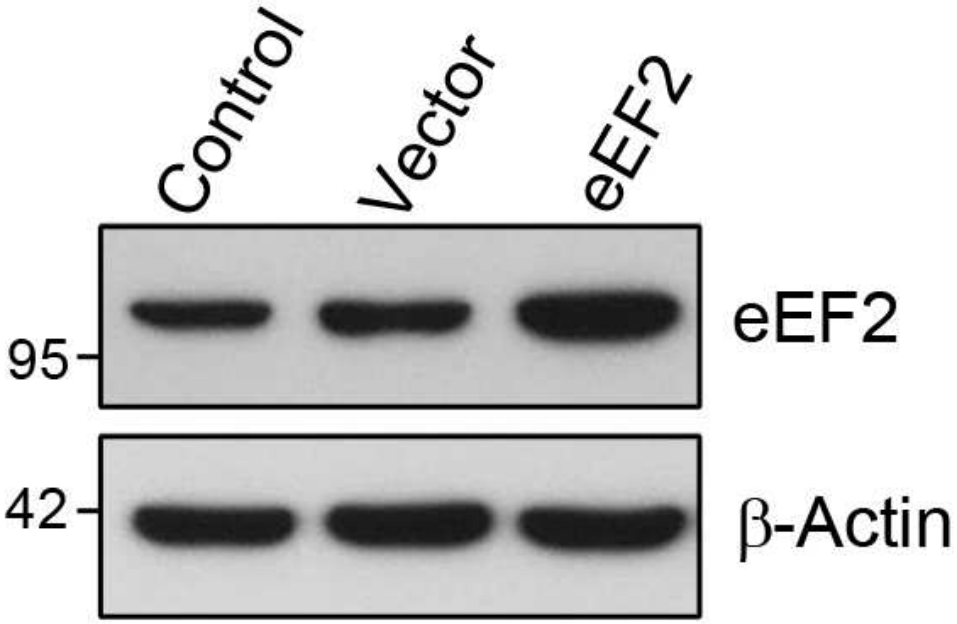


Fig 4d, Ectopic expression of eEF2 in H1437 cells.
50x39mm (300 x 300 DPI)

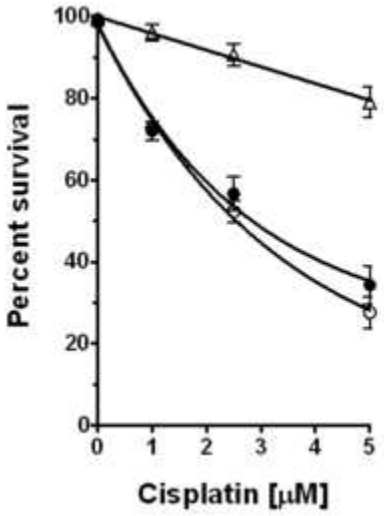


Fig 4e, Ectopic expression of eEF2 increased cisplatin resistance.
8x11mm (600 x 600 DPI)

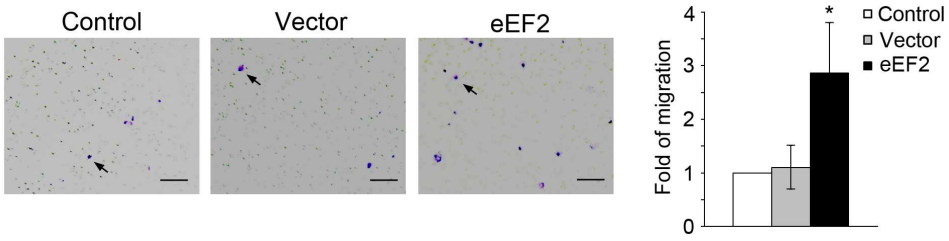


Fig 4f, Ectopic expression of eEF2 increased invasion ability of H1437 cells.
168x44mm (300 x 300 DPI)

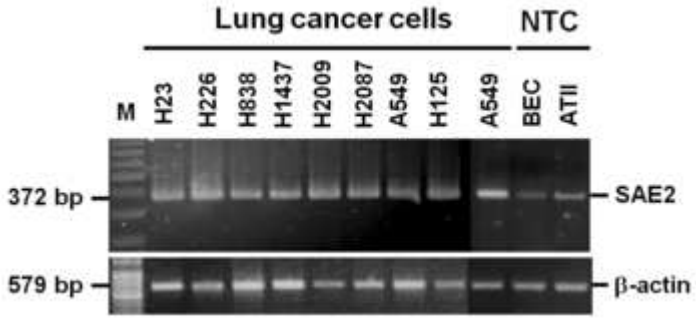


Fig 4g, Compared to the non-tumor cells (NTC), BEC and ATII, SAE2 was highly expressed in lung cancer cell lines.
14x6mm (600 x 600 DPI)

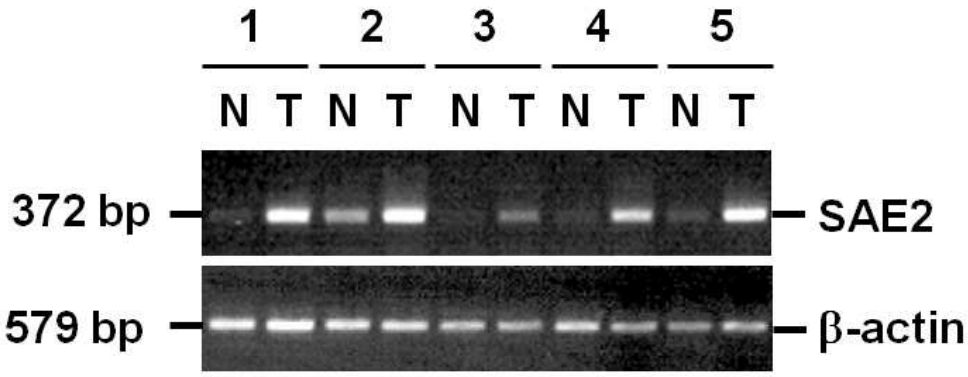


Fig 4h, SAE2 was highly expressed in tumor biopsies as determined by RT-PCR.
60x24mm (300 x 300 DPI)

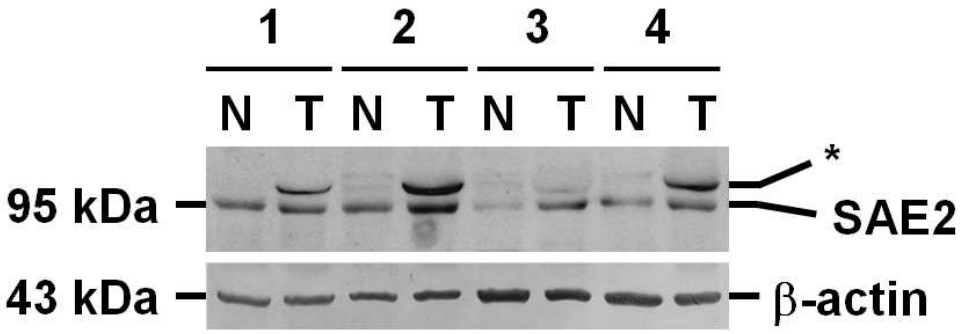


Fig 4i, SAE2 was highly expressed in tumor biopsies as determined by immunoblotting. * indicated a potentially phosphorylated SAE2.
73x26mm (300 x 300 DPI)

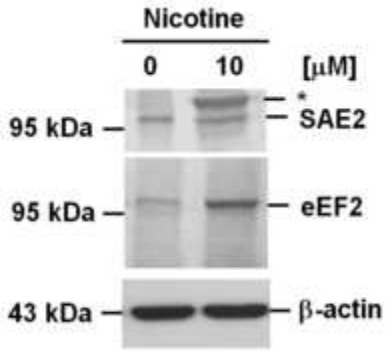


Fig 4j, Treatment of H1437 cells with 10 μM nicotine for 24 h increased SAE2 expression and protein levels of 110-kDa eEF2.
8x7mm (600 x 600 DPI)

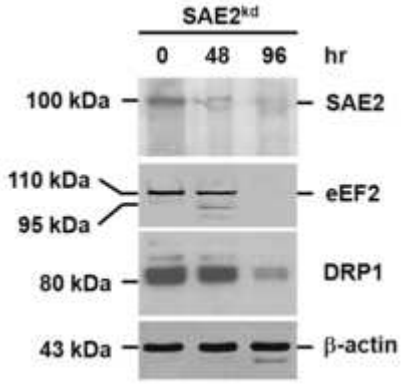


Fig 4k, Silencing of SAE2 expression, however, reduced protein levels of eEF2.
8x8mm (600 x 600 DPI)

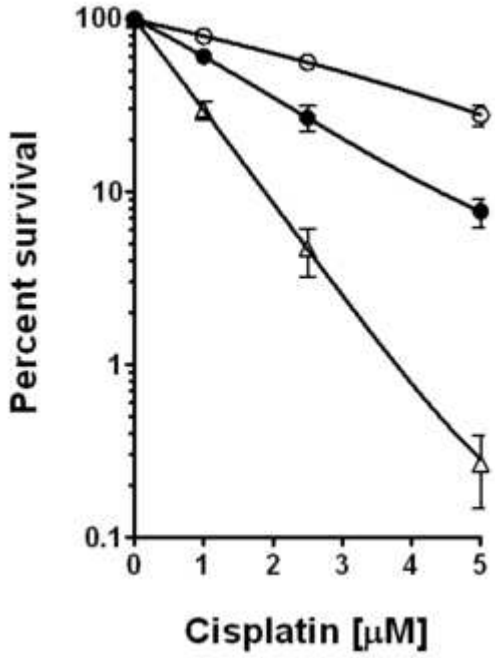
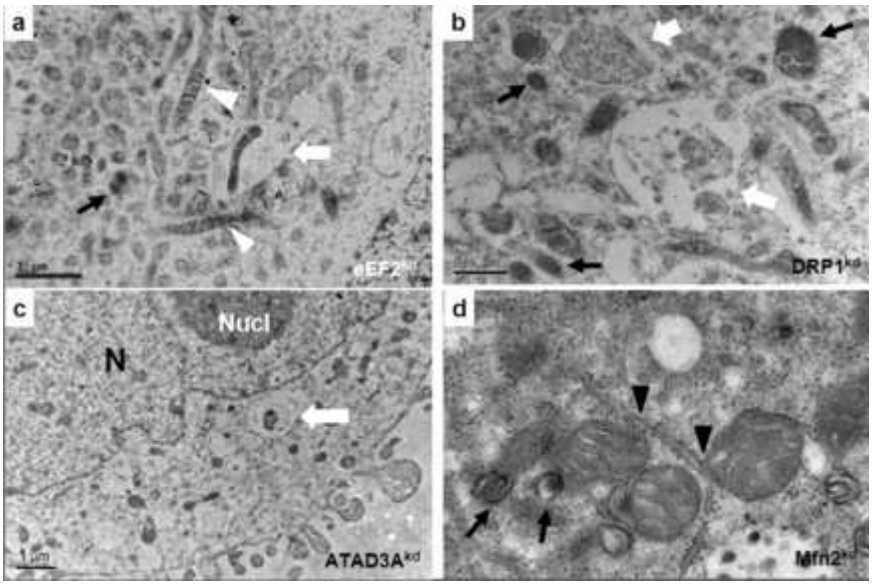


Fig 4l, Silencing of SAE2 expression, however, reduced drug resistance.
10x14mm (600 x 600 DPI)



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18x12mm (600 x 600 DPI)