Research paper

Title: Role of AMPK signaling in mediating the anti-cancer effects of silibinin in esophageal squamous cell carcinoma

Running title: AMPK mediates anti-cancer effects of silibinin

Keywords: AMPK; silibinin; cancer progression; esophageal cancer

Abstract

Objective: Emerging evidence suggests that activation of adenosine monophosphate-activated protein kinase (AMPK) may suppress cancer growth. Identification of novel AMPK activators is therefore crucial to exploit AMPK as a potential target for cancer prevention and treatment.

Research design and methods: We determined the expression status and role of AMPK in esophageal squamous cell carcinoma (ESCC) and investigated whether silibinin, a non-toxic natural product, could activate AMPK to inhibit ESCC development.

Results: Our results from 49 pairs of human ESCC and normal tissues showed that AMPK was constitutively inactive in the majority (69.4%) of ESCC. We found that silibinin induced apoptosis, and inhibited ESCC cell proliferation *in vitro* and tumorigenicity *in vivo* without any adverse effects. Silibinin also markedly suppressed the invasive potential of ESCC cells *in vitro* and their ability to form lung metastasis in nude mice. The anti-cancer effects of silibinin were abrogated by the presence of compound C or shRNA against AMPK. More importantly, silibinin enhanced the sensitivity of ESCC cells and tumors to the chemotherapeutic drugs, 5-fluorouracil and cisplatin.

Conclusions: This preclinical study supports that AMPK is a valid therapeutic target and suggests that silibinin may be a potentially useful therapeutic agent and chemosensitizer for esophageal cancer.

1. Introduction

Esophageal squamous cell carcinoma (ESCC) is the predominant type of esophageal malignancy in the world. Surgery is the gold standard for ESCC therapy, but it is inadequate in treating patients with locally advanced tumor or distant metastasis. On the other hand, the effectiveness of multimodality therapy may be hampered by adverse side effects and presence of treatment-resistant cancer cells [1,2]. Therefore, it is important to identify novel non-toxic agents that have inhibitory effects on ESCC cells or sensitize them to chemotherapy [3].

Adenosine monophosphate-activated protein kinase (AMPK) acts as a "fuel gauge" in cells which can be activated by energy stress. The active form of AMPK, i.e. phosphorylated AMP-activated protein kinase (p-AMPK), can downregulate the energy consuming process as well as increase adenosine triphosphate (ATP) generation [4,5]. In this study, we determined the expression status of p-AMPK in human ESCC, and found significantly lower expression in ESCC tumor tissues compared with paired adjacent normal tissues. The role of p-AMPK in ESCC is unclear, but it was reported that treatment with metformin, an anti-diabetic drug and an activator of AMPK, can inhibit the proliferation of acute myeloid leukemia cells [6] and hepatocellular carcinoma cells [7], as well as suppress invasion of melanoma cells [8]. However, metformin is known to cause lactic acidosis as a rare but serious side effect. Here, we report for the first time that silibinin (or silybin), a naturally occurring product with no known toxic effect, can activate AMPK and thereby exert significant anti-cancer effects on ESCC. Our data also showed that silibinin can sensitize ESCC cells to traditional chemotherapeutic drugs. Silibinin is the major component of silymarin which is a naturally-occurring flavonoid derived from milk thistle (*Silybum marianum*). Thus, silibinin which has long been used to treat hepatic diseases

such as chronic hepatitis and liver cirrhosis [9-11] may be a novel and promising therapeutic agent in esophageal cancer therapy.

However, there is as yet no report on the effect of silibinin on esophageal cancer. Moreover, little is known about the effects of silibinin on AMPK pathway. In this study, we investigated the anti-cancer effects of silibinin on ESCC *in vitro* and *in vivo*, and proved that these effects are dependent on the activation of AMPK.

2. Materials and Methods

2.1 Drugs and cell lines

Silibinin and Compound C were purchased from Sigma (St. Louis, MO, USA) and EMD Chemicals (La Jolla, CA, USA), respectively. Cisplatin and 5-fluorouracil (5-FU) were purchased from Calbiochem (San Diego, CA, USA). These chemicals were diluted in culture medium before use to obtain the desired concentration. In most of the *in vitro* experiments, silibinin was used at a concentration of 100 μM which was of the same order of magnitude as the silibinin concentration detected in the plasma of SENCAR mice after 5 weeks of treatment with oral silymarin [12]. The ESCC cell lines KYSE270, KYSE510 (obtained from DSMZ, Braunschweig, Germany) [13], and T.Tn (from Dr. Hitoshi Kawamata, Dokkyo University School of Medicine) [14] were maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Gaithersburg, MD, USA) at 37°C in 5% CO_2. Immortalized normal esophageal epithelial cell line NE2-hTERT [15] was maintained in a 1:1 mixture of defined keratinocyte serum free medium and EpiLife (Invitrogen).

2.2 Esophageal cancer patient tissue samples

Human ESCC samples and the corresponding adjacent normal esophageal tissues were collected with informed consent and Institutional Review Board approval from 49 patients undergoing surgical resection of primary ESCC at Queen Mary Hospital in Hong Kong from 2011 to 2014, and at the First Affiliated Hospital, Zhengzhou University in Zhengzhou from 2008 to 2010. All specimens were snap-frozen immediately in liquid nitrogen and stored at -80°C [16].

2.3 Transfection and AMPK knockdown

Transient transfection was performed using Lipofectamine (Invitrogen) following the manufacturer's protocol. About 2 x 10^5 cells per well were seeded in a 6-well plate in RPMI medium containing 10% FBS. The cells at >70% confluency were serum-starved for at least 30 minutes before transfection with plasmid containing 0.5 mg full-length shRNA against AMPK (5'-CTTTCTGGCTTCCGCCGATAGAA-3') or vector shRNA (5'-CGGTGTCGAAGAATTTTT-3') [17]. Cells were collected 48 h after transfection for subsequent analysis.

2.4 Cell proliferation assay

The proliferative ability of ESCC cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay as described previously [18].

2.5 Colony forming assay

Cancer cells were seeded in 6-well plates (about 5,000 cells per well) and desired drugs were added 24 h later. After incubation for 14 days, the cells were fixed in 70% ethanol then stained with 10% (v/v) Giemsa. Colonies consisting of >50 cells were counted [18].

2.6 Soft agar assay

About 5×10^5 cells per well were seeded in 6-well plates and were treated with different concentrations of silibinin 24 h later. After treatment for 24 h, the cells were trypsinized and suspended in RPMI 1640 containing 0.33% agar and 10% FBS. The cell mixture was layered on consolidated gel consisting of a mixture of 0.6% agar and 10% FBS in RPMI 1640. Photographs of colonies were taken 20 days later, and the ratio between the number of colonies formed by treated cells and untreated cells was calculated [18].

2.7 Wound healing assay

A wound was created in a confluent monolayer of cancer cells using the tip of a micropipette. Phase contrast microscopy was used to document the speed of wound closure by taking photographs immediately after wound creation and 24 h later [18].

2.8 In vitro cell invasion assay

In vitro cell invasion assays were performed with the use of BD BioCoat Matrigel Invasion Chambers (BD Biosciences, Bedford, MA, USA) as described previously [19]. Images of three different fields were captured from each membrane and the chambers were dissolved in 70 μl DMSO. The optical density (OD) at 570 nm was measured on a Labsystems Multiskan microplate reader.

2.9 Western blot

Western blot analysis of cell and tissue lysates was performed as described previously [20]. The primary antibodies used include: p-AMPK (Thr172), AMPK, p-ACC (Ser79), ACC, p-mTOR (Ser2448), mTOR, Bax, Bcl-2, Bcl-xl, caspase 3 and cleaved caspase 3 from Cell Signaling Technology (Beverly, MA, USA); N-cadherin and actin antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and E-cadherin from BD Biosciences. After incubation with corresponding secondary antibodies, the signals were detected using ECL plus Western blotting system (Amersham, Piscataway, NJ, USA). The bands of p-AMPK and AMPK were quantified using ImageJ, and the expression level of p-AMPK relative to that of AMPK (i.e. relative expression level) was analyzed.

2.10 Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling

Apoptotic cells were detected using the In Situ Cell Death Detection kit Fluorescein (Roche Diagnostics, Mannheim, Germany) based on terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) as described previously [21]. The stained slides were visualized under fluorescence microscopy with ×40 objective. Representative areas were randomly selected and at least 5000 DAPI-positive cells were scored. The apoptotic index was expressed as the ratio between the number of TUNEL-positive cells and the total number of DAPI-positive cells.

2.11 In vivo tumorigenesis experiments

Tumor xenograft experiments were performed as described previously [22]. About 1×10^6 KYSE270 cells were suspended in a mixture consisting of equal volumes of PBS and matrigel (BD Biosciences), then injected subcutaneously into the left flank of nude mice (6 to 8 weeks old). The mice were randomly separated into treatment and control groups (n = 10 per group) when the tumor diameter reached about 5 mm. To study the inhibitory effect of silibinin alone on tumor growth, the treatment group received oral gavage of silibinin at a dose of 100 mg/kg daily, while the control group received the vehicle. To study the synergistic effects of silibinin and chemotherapeutic drugs, mice were given oral silibinin daily (25 mg/kg) and twice weekly intraperitoneal injection of 5-FU (20 mg/kg) or cisplatin (2 mg/kg) dissolved in DMSO/PBS buffer (1:1, v/v); the control group received vehicle only. Tumor size was measured every 3 days with calipers, and tumor volume was calculated according to the equation Volume = (length × width^2)/2. At the end of the experiments, tumors, as well as liver, lung and kidney tissues, were harvested for Western blot and histologic analyses. All the animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong.

2.12 In vivo experimental metastasis experiment

The inhibitory effect of silibinin on metastasis of ESCC cells was determined by *in vivo* bioluminescent imaging [23]. Briefly, luciferase-expressing KYSE150 cells (~1×10^6 cells in PBS) were injected through the lateral tail vein of nude mice. After 24 h, the mice were treated with silibinin (100 mg/kg, oral gavage daily) or vehicle for 8 weeks (n = 5 per group). Metastatic activity was assessed by bioluminescent imaging with an IVIS Imaging System (Xenogen,

Alameda, CA, USA) after intraperitoneal injection of D-luciferin (Gold Biotechnology, St Louis, MO, USA).

2.13 Statistical analysis

All *in vitro* experiments were repeated at least three times. SPSS (Aspire Software International, Leesburg, VA, USA) was used to analyze the results. The data from each experiment (expressed as the mean ± SD) were compared by ANOVA. *P*-values < 0.05 were deemed significant.

3. Results

3.1 AMPK signaling pathway is frequently inactivated in human ESCC

Since the significance of the AMPK signaling pathway in esophageal cancer is still unknown, Western blot was performed to determine the expression level of p-AMPK and AMPK in 49 pairs of human ESCC and adjacent normal esophageal tissues (**Figure 1A)**. A lower p-AMPK/total AMPK ratio was observed in the majority of primary esophageal tumors examined (34 of 49; 69.4%), compared to the paired normal tissue (**Figure 1B)**. As shown in **Figure 1C**, the mean p-AMPK/total AMPK ratio in the tumor tissues was significantly lower than that in the corresponding normal tissues ($P < 0.01$). These data suggest that AMPK may be dysfunctional in a vast majority of ESCC and that it may serve as a therapeutic target of cancer.

3.2 Silibinin induces apoptosis and inhibits proliferation and tumorigenicity of ESCC cells

To determine the anti-cancer effects of silibinin in ESCC cells, TUNEL assay was used to examine the effect of silibinin on apoptosis of ESCC cells. After incubation with silibinin at 100 μM and 150 μM for 48 h, there was a dose-dependent and significant increase in apoptotic cells, compared to less than 2% in cells without silibinin treatment (**Figure 2A**). The downregulation of pro-survival protein Bcl-2, and the increase in the pro-apoptotic protein Bax and apoptotic marker cleaved caspase-3, confirmed that silibinin had marked apoptotic effect on ESCC cells (**Figure 2B**). Treatment with silibinin inhibited the proliferation of KYSE270 and T.Tn cells in a dose-dependent manner, with IC_{50} values of 133 μM and 95 μM, respectively, after 96 h of incubation (**Figure 2C**). Notably, silibinin had no significant cytotoxic effect on immortalized normal esophageal epithelial cells (**Figure 2C**). The ESCC cells treated with silibinin showed

dose-dependent decrease in anchorage-dependent and -independent growth in both cell lines (**Figure 2D and E**). Furthermore, we explored the anti-tumorigenic effect of silibinin *in vivo* using a tumor xenograft model. Our data showed that oral silibinin at a daily dose of 100 mg/kg was very effective in suppressing the growth of KYSE270 tumor xenografts (**Figure 2F**). Moreover, no significant difference was found in the body weight (**Figure 2G**) or morphology of the vital organs (**Supplementary Figure S1**) between the control and treatment groups, suggesting that silibinin did not have any obvious toxic effect on the animals.

3.3 Silibinin suppresses invasive and metastatic potential of ESCC cells

We first investigated the ability of silibinin to suppress the metastatic potential of ESCC cells was *in vitro*. Enhanced motility, invasiveness and epithelial-mesenchymal transition are phenotypes associated with metastasis. The results of the wound healing assay showed that treatment with 100 μM silibinin for 24 h inhibited the ESCC cells from migrating into the wound (**Figure 3A**). Silibinin also significantly suppressed the invasive potential of ESCC cells *in vitro*, as evidenced by the results of the matrigel invasion assay (**Figure 3B**). Western blot analysis of the cell lysates showed that silibinin treatment induced E-cadherin expression and suppressed N-cadherin in ESCC cells suggesting reversed epithelial-mesenchymal transition (EMT) (**Figure 3C**). We then investigated the anti-metastatic effect of silibinin *in vivo* using experimental metastasis assay. Our data showed that oral silibinin at a daily dose of 100 mg/kg significantly suppressed the metastasis of ESCC cells, as indicated by the lower bioluminescence in the lungs of the silibinin-treated mice compared to the control group (**Figure 3D)**.

3.4 Anti-cancer effects of silibinin are mediated by activation of AMPK

Western blot analysis showed that silibinin increased p-AMPK expression and decreased the phosphorylation of its downstream targets, mTOR and acetyl-CoA carboxylase (ACC) in ESCC cells. No obvious changes were detected in total AMPK, ACC and mTOR expression levels after silibinin treatment (**Figure 4A**). To determine the significance of p-AMPK in mediating the anti-cancer effects of silibinin, KYSE270 cells were treated with Compound C, an inhibitor of AMPK. The data showed that treatment with 0.5 μM Compound C successfully reduced the basal level of p-AMPK in KYSE270 and T.Tn cells, and was also highly effective in attenuating the effect of silibinin on p-AMPK expression **(Figure 4B).** Compound C treatment also rescued the ESCC cells from the inhibitory effects of silibinin on cell proliferation, colony formation, cellular invasion, and E- to N-cadherin switching (**Figure 4C-F**).

We also used shRNA knockdown to confirm the importance of AMPK in mediating the effects of silibinin. Successful knockdown of AMPK, even in the presence of silibinin, was verified 48 hours after transfection using Western blot assay (**Figure 5A**). Compared to the ESCC cells transfected with shRNA control vector, the AMPK-knockdown cells were significantly less responsive to the inhibitory effects of silibinin on proliferation, colony formation and invasion (**Figure 5B-E**). Taken together, these *in vitro* data strongly support that the anti-cancer effects of silibinin were mediated through activation of AMPK.

3.5 Silibinin sensitizes ESCC cells to traditional chemotherapeutic drugs

Cisplatin and 5-FU are commonly used chemotherapeutic agents for patients with advanced esophageal cancer. To determine if silibinin has synergistic effects with these drugs and if it can

increase chemosensitivity, *in vitro* and *in vivo* experiments were performed using combinations of low-dose silibinin and low-dose 5-FU/cisplatin. KYSE270 cells were treated with silibinin *in vitro* at a concentration of 15 μM, which was much lower than the IC_{50} value of 133 μM. Cisplatin and 5-FU were used at 10 μM and 2.5 μM, respectively, which were concentrations shown to have negligible effect on the proliferation of ESCC cells in our previous study [18]. Our data showed that low-dose silibinin, cisplatin or 5-FU alone had no anti-proliferation effect on KYSE270 cells. However, a combination of low-dose silibinin and low-dose cisplatin or 5-FU had synergistic suppressive effect on the proliferation of ESCC cells *in vitro*, with combination indices of 0.480 and 0.348, respectively (**Figure 6A** and **B**). *In vivo* experiments were performed to confirm this synergistic effect. The tumor xenografts in nude mice treated with a combination of silibinin and cisplatin, or silibinin and 5-FU were significantly smaller than those in the control group and in the groups receiving single drugs at low dosages (**Figure 6C**). The combination index of silibinin plus cisplatin was 0.273, and that of silibinin plus 5-FU was 0.262, indicating synergistic effects. More importantly, the body weight of mice receiving combined treatments was comparable to that of the control group (**Figure 6D**), suggesting that low dosage cisplatin or 5-FU may shrink tumors with minimal toxicity and side effects when used in combination with silibinin.

4. Discussion

We have demonstrated for the first time the inactivation of AMPK signaling pathway in esophageal cancer. Recent studies have shown that activation of AMPK may induce anti-cancer effects in cancer cells [24-27], but relatively little is known about the expression status and role of p-AMPK in human cancers. Our Western blot showed that the ratio of p-AMPK to total AMPK was much lower in ESCC compared with adjacent normal tissue. This suggests that AMPK inactivation may be a common phenomenon in ESCC, and that AMPK activators may be potentially useful in cancer therapy. Currently, metformin is being tested in clinical trials as a therapeutic agent against breast cancer [28]. However, metformin is known to have serious side effects such as lactic acidosis in diabetic patients [29], which may limit its application in cancer patients. In this regard, silibinin which also activates AMPK, but is a non-toxic natural product, may be a promising alternative.

There was a lack of information on the effects of silibinin on the AMPK signaling pathway in esophageal cancer prior to this study. We have shown, for the first time, that silibinin decreased the expression of p-mTOR in esophageal cancer cells through the activation of AMPK. It was reported previously that silibinin inhibits mTOR as well as activates the PI3K/AKT pathway in cervical and hepatoma cancer cell lines [30]. PI3K/AKT is a major upstream activator of mTOR, but there are conflicting reports on whether silibinin inhibits [31,32] or activates AKT [33]. Taken together, it is likely that other factor/pathways may be involved in mediating the suppressive effect of silibinin on mTOR. A recent study shows that silibinin activates AMPK and thereby targets SREBP1 (sterol response element binding protein 1), leading to inhibition of aberrant lipid metabolism and proliferation of prostate cancer cells [34]. Here, our observation that silibinin treatment increased p-AMPK expression in ESCC cells, and that the anti-cancer

effects of silibinin could be abolished by pharmacologic and genetic knockdown of p-AMPK, confirms that the AMPK/mTOR pathway also mediates the anti-cancer effects of silibinin in human cancer. It is well documented that most cancer cells rely primarily on aerobic glycolysis (i.e. Warburg effect) [35,36] to support the energy requirements for survival, proliferation and metastatic activities. Since silibinin could activate AMPK which is a negative regulator of Warburg effect [37,38], it is possible that silibinin exerts ant-cancer activities by reversing the Warburg phenotype.

Silibinin was found to have anti-cancer effects in several different kinds of cancer such as lung cancer [39], bladder cancer [40], prostate cancer [41], and colon cancer [42,43]. To our knowledge, the present study is the first one to provide evidence that silibinin produces anti-cancer effects in esophageal cancer. Esophageal cancer is the sixth most common cause of cancer-related deaths worldwide. The historically low survival rates of esophageal cancer patients are partly attributed to late presentation and early metastasis. Systemic chemotherapy is standard treatment for esophageal cancer patients with distant organ metastases. However, these patients often have poor general medical status which makes them unsuitable for aggressive chemotherapy. Our *in vitro* and *in vivo* data showed that that silibinin had no cytotoxic effect on normal esophageal epithelial cells, but could suppress the expression levels of p-AMPK and p-mTOR and the key events of tumor development, including cell proliferation, invasion and tumor growth. Moreover, our data show that silibinin treatment can increase the sensitivity of ESCC cells or tumors to 5-FU and cisplatin, thus support the use of silibinin in esophageal cancer therapy, either alone or in combination with conventional chemotherapeutic drugs. Notably, silibinin has not shown any severe side effects over its long-history of application, and is currently tested in clinical trials for its anti-viral effect and protective effect against chronic

hepatitis C virus infection [11,44]. In addition, a phase I study of silybin-phytosome in prostate cancer patients showed that the drug could be given at high doses with acceptable toxicity [45]. Further clinical trials are needed to substantiate the therapeutic effect of silibinin in cancer patients.

5. Conclusion

In summary, this preclinical study explores the role of AMPK signaling pathway in esophageal cancer, and the results suggest that pharmacological activation of AMPK by silibinin, alone or in combination with conventional chemotherapeutic drugs, may be a potentially a safe and effective strategy in treatment of esophageal cancer.

Legends

Figure 1. Inactivation of AMPK signaling pathway in ESCC. (**A**) Expression levels of p-AMPK and total AMPK were determined in 49 pairs of ESCC and adjacent normal tissues by Western blot, and results of 6 representative tumor tissues (T) and their matched normal tissues (N) were shown. Actin was included as loading control. (**B**) p-AMPK/total AMPK ratio in 49 tumor tissues relative to matched normal esophageal tissues. (**C**) Comparison of p-AMPK/total AMPK ratios between tumor tissues and normal tissues. The boxes contain the values between 25^{th} and 75^{th} percentiles of the 49 cases, and the whiskers extend to the highest and lowest values. The lines across the boxes indicate the median values, and the dots inside the boxes represent the mean values.

Figure 2. Inhibitory effects of silibinin on apoptosis, proliferation and tumorigenicity of ESCC cells. (**A**) Detection of apoptosis by TUNEL assay**.** Treatment with silibinin (100 μM or 150 μM) significantly increased the apoptotic index. (**B**) Western blot analysis showing changes in Bcl-2, Bax, and cleaved caspase-3 expressions after silibinin treatment. (**C**) Effects of different concentrations of silibinin (up to 300 μM) on proliferation of ESCC cells and immortalized normal esophageal cells were determined using MTT assay. (**D**) Treatment with silibinin inhibited colony formation of ESCC cells–in a dose-dependent manner. (**E**) Silibinin reduced the anchorage-independent colony formation of ESCC cells in soft agar. (**F**) Ttumor growth curves of nude mice treated with silibinin or vehicle control. (**G**) Body weight curves of mice. Bars, SD; ** $P < 0.01$, *** $P < 0.001$ compared with control cells or vehicle-treated mice.

Figure 3. Silibinin suppressed invasive and metastatic potential of ESCC cells. (**A)** The silibinin-treated or untreated esophageal cancer cells were subjected to the wound healing assay. (**B**) Matrigel-coated Boyden chamber assay was applied to determine the invasive ability of the esophageal cancer cells. Treatment with silibinin resulted in significant reduction of cellular invasiveness. (**C**) Western blot of E-cadherin and N-cadherin in silibinin-treated cells. **(D)** Bioluminescent images of nude mice and quantitative bioluminescent indices showing significant effect of silibinin on suppressing lung metastasis. Bars, SD; * $P < 0.05$, *** $P < 0.001$ compared with control cells or vehicle-treated mice.

Figure 4. Inhibition of p-AMPK by Compound C abolished the anti-cancer effects of silibinin. (**A**) Western blot analysis showed that silibinin activated AMPK pathway in ESCC cells as evidenced by the increase in p-AMPK, and decrease in p-mTOR and p-ACC expressions. (**B**) Western blot showed that the presence of Compound C abolished the activating effect of silbinin on AMPK in ESCC cells. (**C**) Combined treatment with 100 μM silibinin and 0.5 μM Compound C rescued ESCC cells from the inhibitory effect of silibinin as demonstrated in the MTT assay. (**D**) The inhibitory effect of silibinin on colony formation of ESCC cells was abrogated by Compound C. (**E**) Compound C reduced the anti-invasive effect of silibinin on ESCC cell lines using matrigel-coated Boyden chamber. (**F**) Western blot of E-cadherin and N-cadherin expressions in ESCC cells treated with silibinin, Compound C or their combination. Bars, SD; ** $P < 0.01$, *** $P < 0.001$ compared with control cells.

Figure 5. Knockdown of AMPK using shRNA abolished the anti-cancer effects of silibinin. (**A**) AMPK-shRNA successfully decreased the expression of AMPK in ESCC cells, as indicated by Western blot analysis. (**B**) MTT assay showed that knockdown of AMPK by shRNA abrogated the inhibitory effect of silibinin on proliferation of ESCC cells. (**C**) Knockdown of AMPK attenuated the inhibitory effect of silibinin on the colony formation of ESCC cells. (**D**) Matrigel invasion assay showing that the anti-invasive effect of silibinin in ESCC cells was abolished by AMPK knockdown. (**E**) Western blots of E-cadherin and N-cadherin showing that AMPK-shRNA abrogated the effects of silibinin on EMT reversal in ESCC cells. Bars, SD; ** $P < 0.01$, *** $P < 0.001$ compared with control cells.

Figure 6. Silibinin sensitized ESCC cells to traditional chemotherapeutic drugs. (**A**) Silibinin (15 μM) rendered ESCC cells more responsive to low-dose 5-FU (2.5 μM) treatment, as demonstrated in the MTT assay. (**B**) Low dose silibinin (15 μM) also exerted synergistic effect with cisplatin (10 μM) on inhibiting proliferation of ESCC cells. (**C**) Growth curves of subcutaneous tumors formed by inoculation of KYSE270 in nude mice. Animals treated with a combination of silibinin and cisplatin or 5-FU showed significant reduction in tumor size, compared with groups receiving vehicle or single drug therapy. (**D**) No significant difference in body weight was observed among the different groups throughout this experiment. Bars, SD; ** $P < 0.01$, compared with ESCC cells or mice treated with 5-FU or cisplatin alone.

Bibiography

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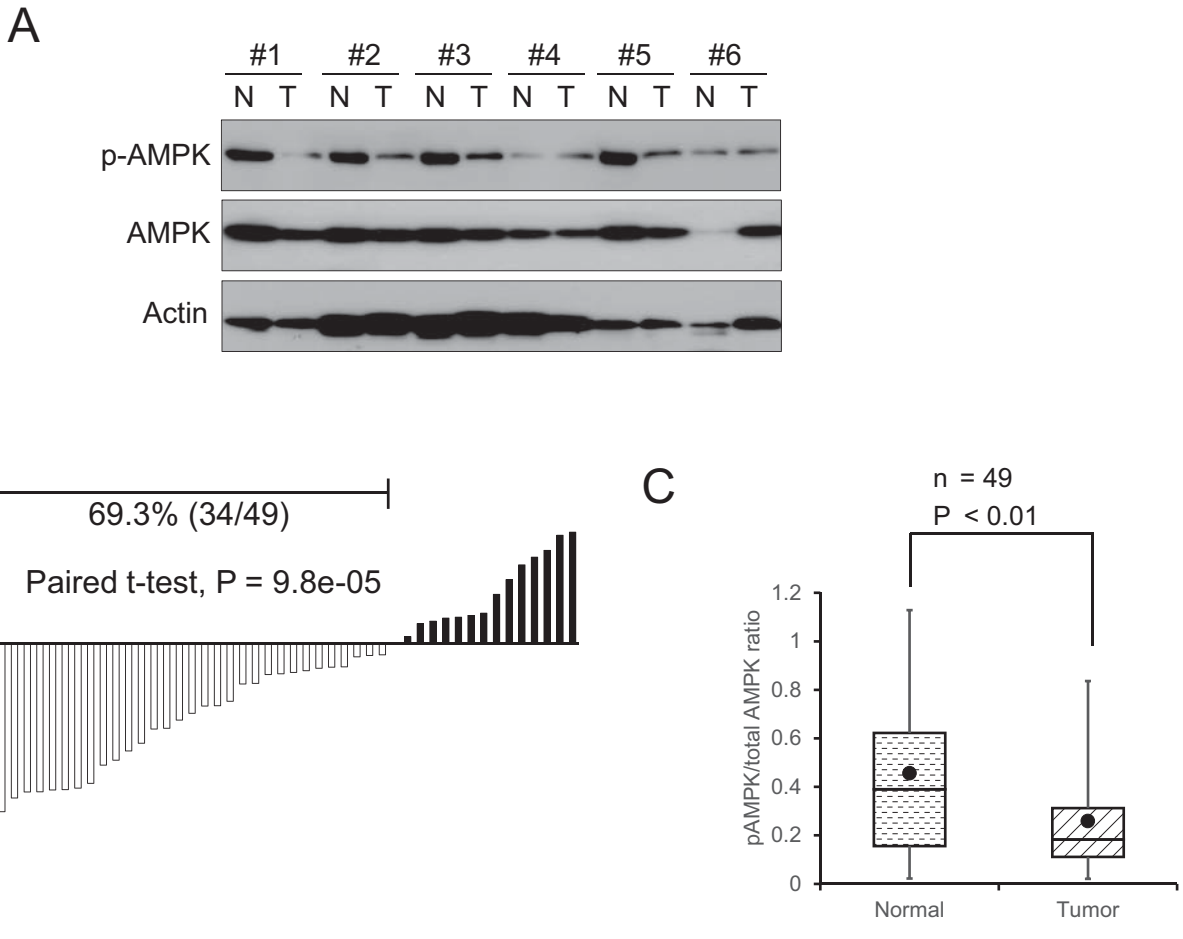

A
#1
#2
#3
#4
#5
#6
N T N T N T N T N T N T
p-AMPK
AMPK
Actin
B
69.3% (34/49)
Paired t-test, P = 9.8e-05
Log2 relative pAMPK/total AMPK
(normalized to normal tissue)
2
1
0
-1
-2
-3
-4
-5
C
n = 49
P < 0.01
pAMPK/total AMPK ratio
1.2
1
0.8
0.6
0.4
0.2
0
Normal
Tumor

Figure 1

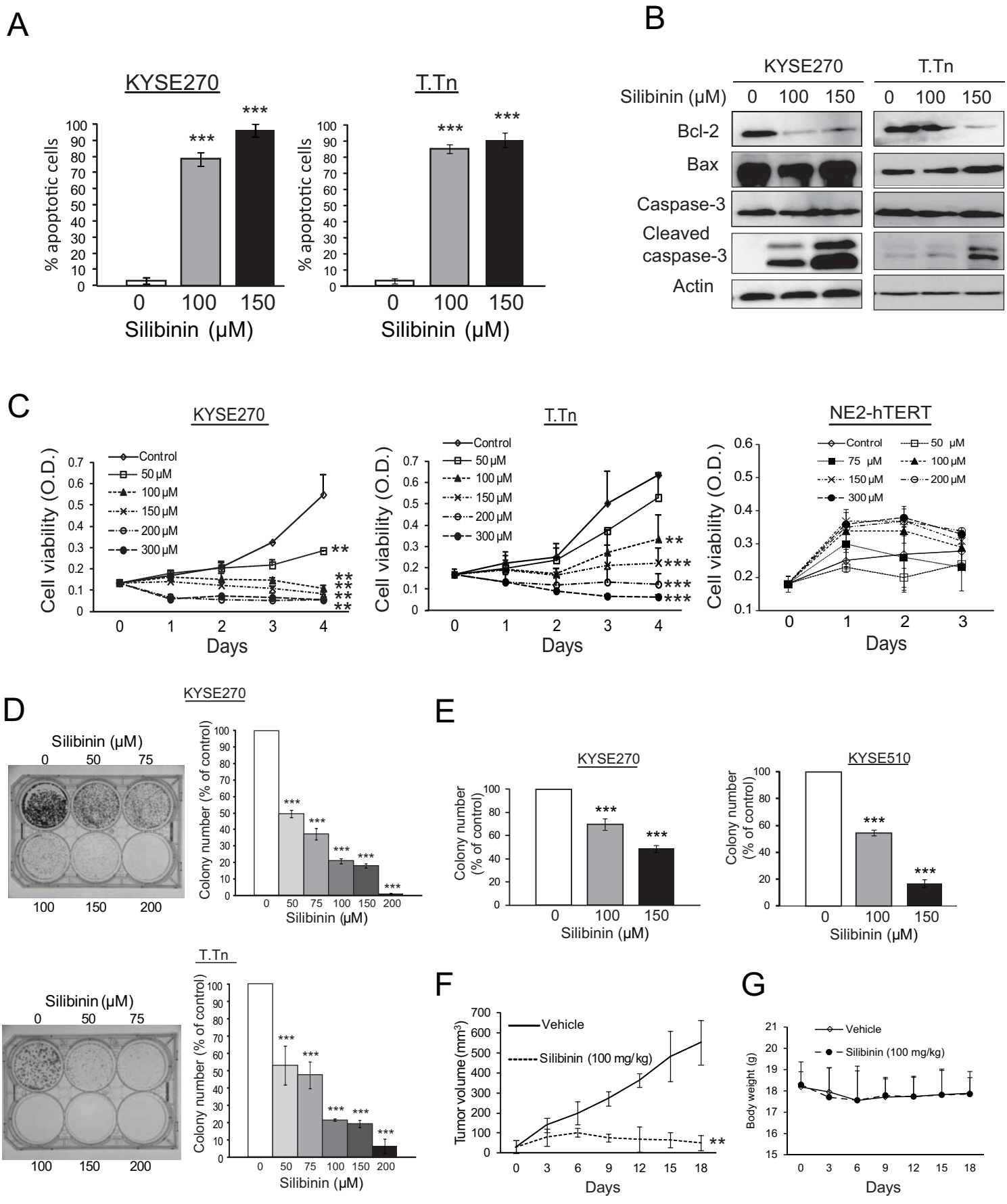

A
KYSE270
T.Tn
% apoptotic cells
Silibinin (μM)
B
KYSE270
T.Tn
Silibinin (μM)
Bcl-2
Bax
Caspase-3
Cleaved caspase-3
Actin
C
KYSE270
T.Tn
NE2-hTERT
Cell viability (O.D.)
Days
Control
50 μM
100 μM
150 μM
200 μM
300 μM
D
KYSE270
T.Tn
Silibinin (μM)
Colony number (% of control)
E
KYSE270
KYSE510
Colony number (% of control)
Silibinin (μM)
F
Tumor volume (mm3)
Vehicle
Silibinin (100 mg/kg)
Days
G
Body weight (g)
Vehicle
Silibinin (100 mg/kg)
Days

Figure 2

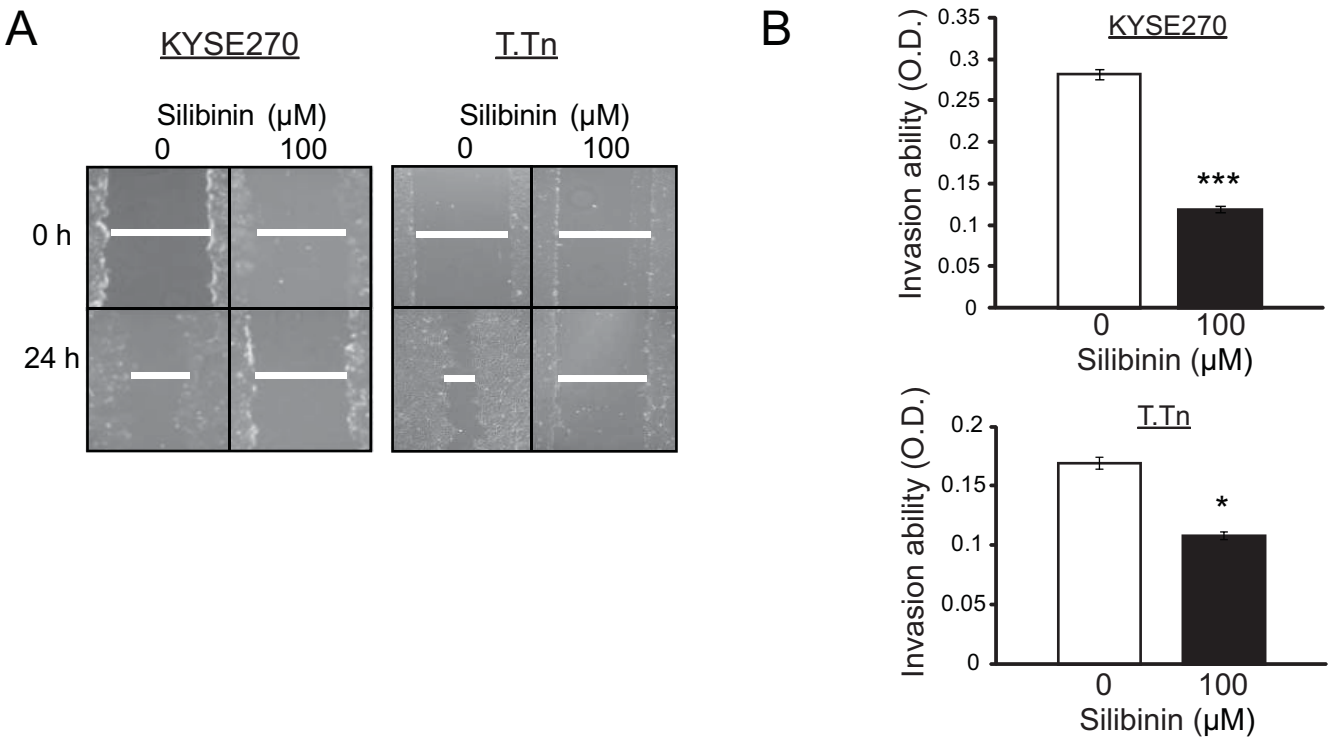

A
KYSE270
T.Tn
Silibinin (μM)
0
100
0 h
24 h
B
KYSE270
Invasion ability (O.D.)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
***
0
100
Silibinin (μM)
T.Tn
Invasion ability (O.D.)
0.2
0.15
0.1
0.05
0
*
0
100
Silibinin (μM)
C
T.Tn
Silibinin (μM) 0 100 150
E-cadherin
N-cadherin
Actin
D
Vehicle
Silibinin
Bioluminescence (% of control)
140
120
100
80
60
40
20
0
***
Vehicle
Silibinin

Figure 3

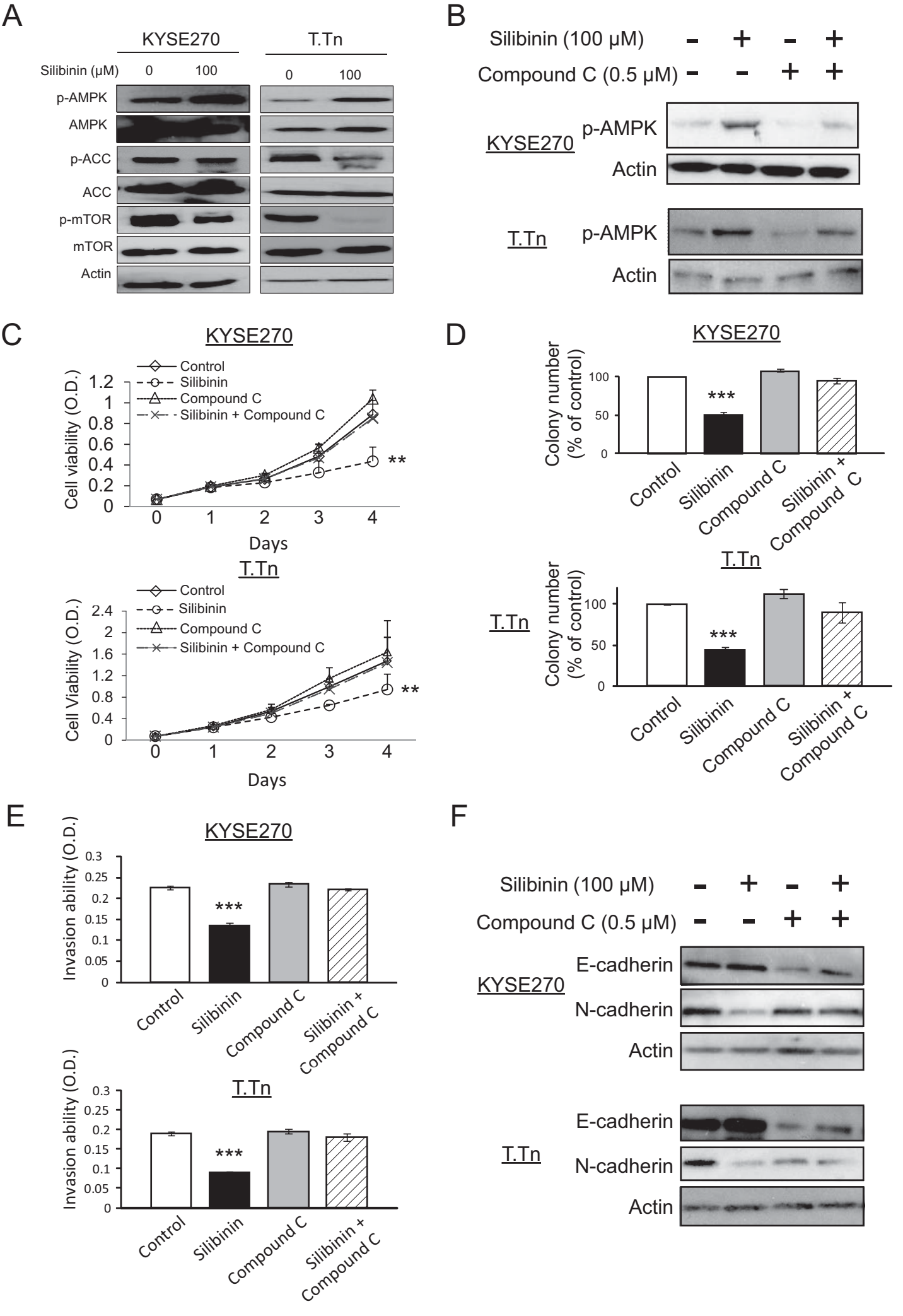

A
KYSE270
T.Tn
Silibinin (μM)
0
100
0
100
p-AMPK
AMPK
p-ACC
ACC
p-mTOR
mTOR
Actin
B
Silibinin (100 μM)
Compound C (0.5 μM)
KYSE270
p-AMPK
Actin
T.Tn
p-AMPK
Actin
C
KYSE270
Control
Silibinin
Compound C
Silibinin + Compound C
Cell viability (O.D.)
Days
**
T.Tn
Cell Viability (O.D.)
D
KYSE270
Colony number (% of control)
***
Control
Silibinin
Compound C
Silibinin + Compound C
T.Tn
E
KYSE270
Invasion ability (O.D.)
T.Tn
F
Silibinin (100 μM)
Compound C (0.5 μM)
KYSE270
E-cadherin
N-cadherin
Actin
T.Tn

Figure 4

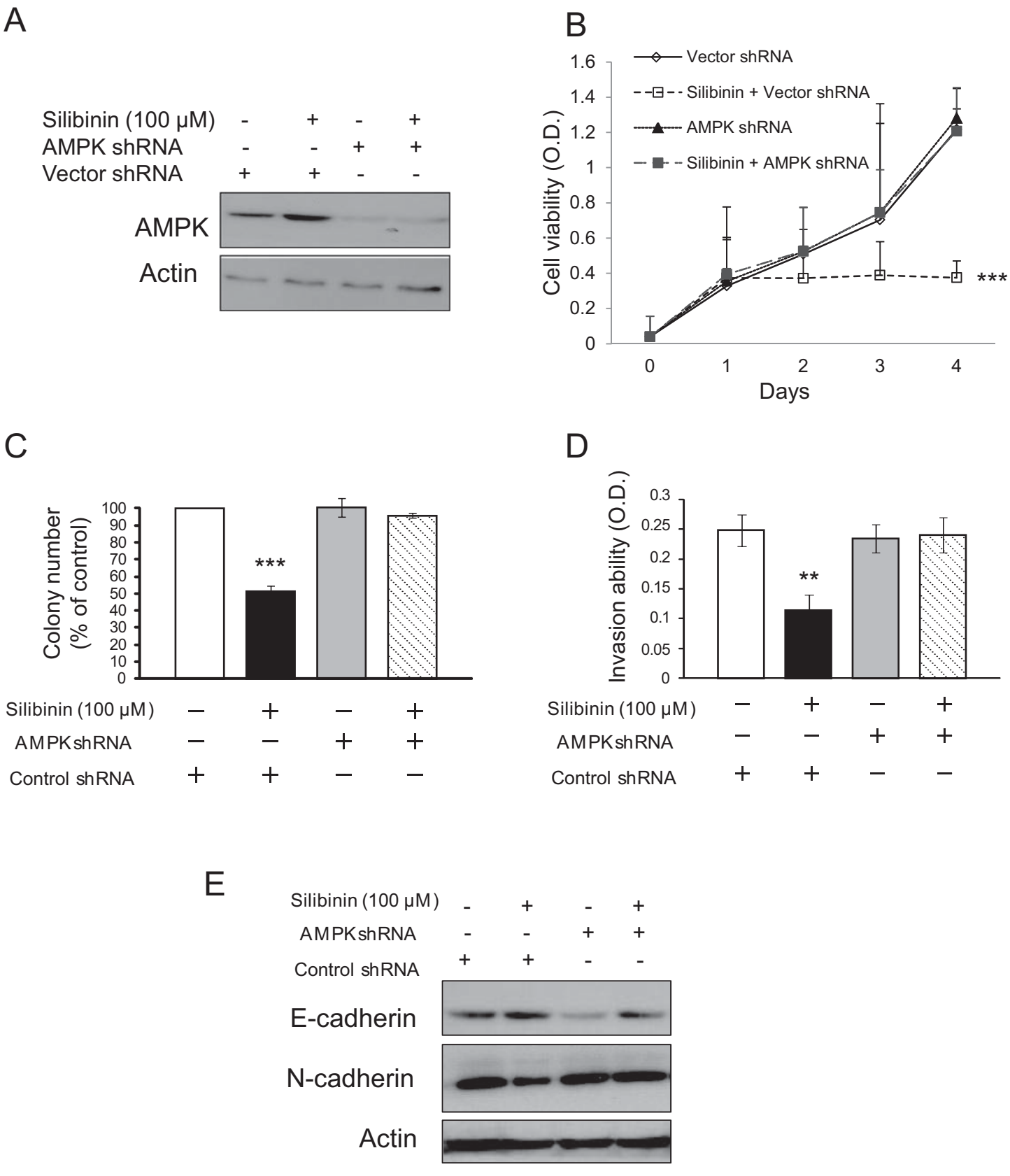
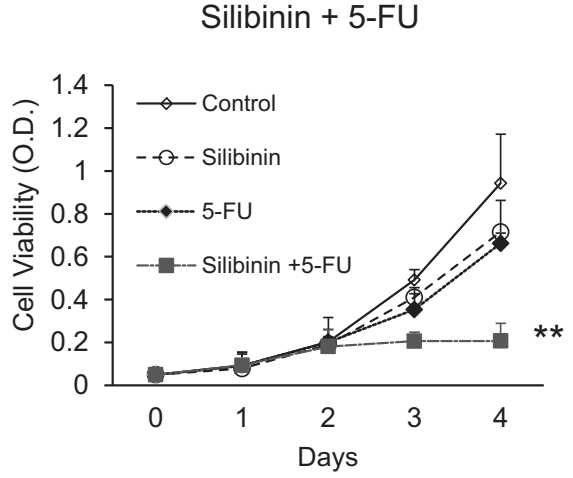
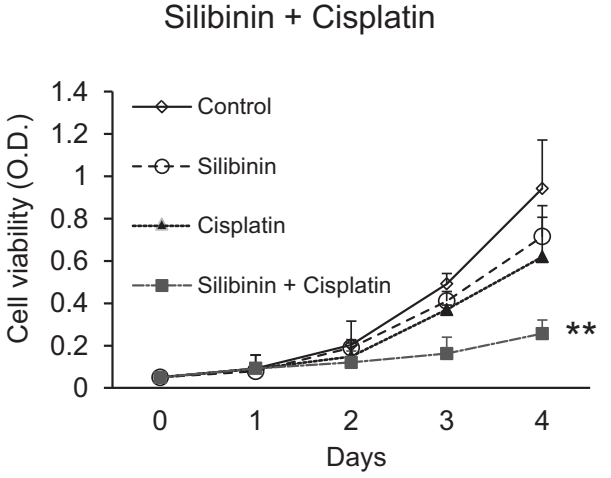
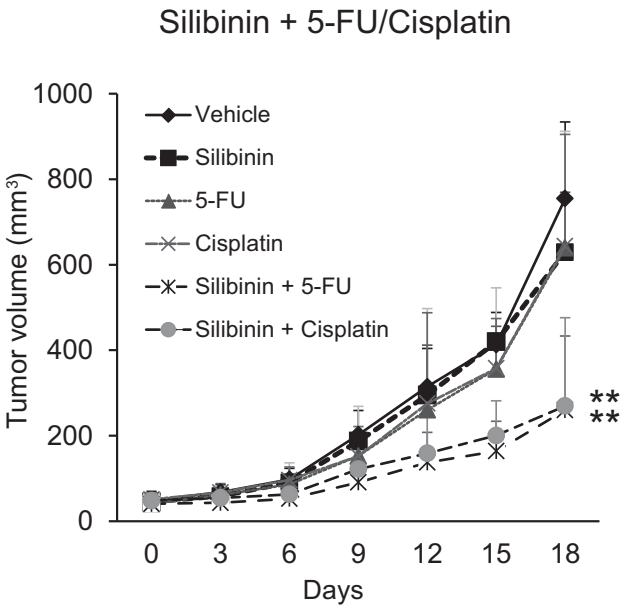

A
Silibinin (100 μM) - + - +
AMPK shRNA - - + +
Vector shRNA + + - -
AMPK
Actin
B
Vector shRNA
Silibinin + Vector shRNA
AMPK shRNA
Silibinin + AMPK shRNA
Cell viability (O.D.)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4
Days
***
C
Colony number (% of control)
100
90
80
70
60
50
40
30
20
10
0
***
Silibinin (100 μM) – + – +
AMPKshRNA – – + +
Control shRNA + + – –
D
Invasion ability (O.D.)
0.3
0.25
0.2
0.15
0.1
0.05
0
**
Silibinin (100 μM) – + – +
AMPKshRNA – – + +
Control shRNA + + – –
E
Silibinin (100 μM) - + - +
AMPKshRNA - - + +
Control shRNA + + - -
E-cadherin
N-cadherin
Actin

Figure 5


Silibinin + 5-FU
Cell Viability (O.D.)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Silibinin
5-FU
Silibinin +5-FU
**
0
1
2
3
4
Days


Silibinin + Cisplatin
Cell viability (O.D.)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Silibinin
Cisplatin
Silibinin + Cisplatin
**
0
1
2
3
4
Days


Silibinin + 5-FU/Cisplatin
Tumor volume (mm3)
1000
800
600
400
200
0
Vehicle
Silibinin
5-FU
Cisplatin
Silibinin + 5-FU
Silibinin + Cisplatin
**
**
0
3
6
9
12
15
18
Days

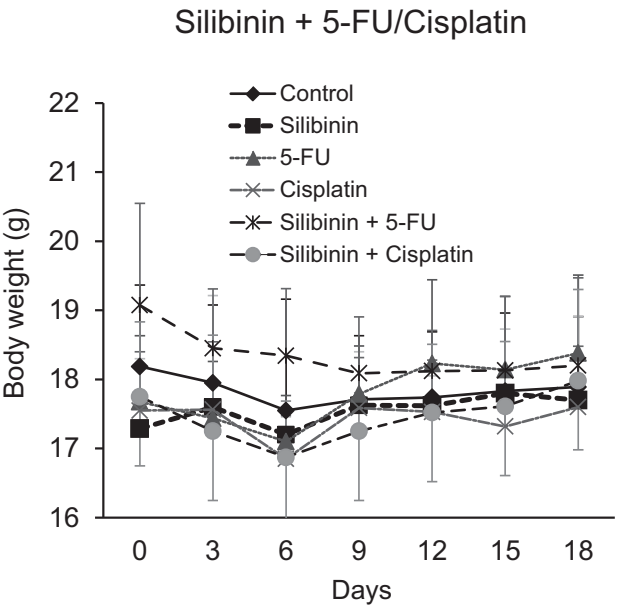

Silibinin + 5-FU/Cisplatin
Body weight (g)
22
21
20
19
18
17
16
Control
Silibinin
5-FU
Cisplatin
Silibinin + 5-FU
Silibinin + Cisplatin
0
3
6
9
12
15
18
Days

Figure 6