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Eupatilin Suppresses Pancreatic Cancer Cells *via* Glucose Uptake Inhibition, AMPK Activation, and Cell Cycle Arrest

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Abstract. *Background/Aim: Pancreatic cancer is one of the most devastating malignancies worldwide. Because of the disappointing outcome of traditional treatment, new drug candidates are being investigated. This study analysed the effect of eupatilin on pancreatic cancer cells. Materials and Methods: Cell viability assay, western blot, siRNA transfection, 2-deoxyglucose uptake assay, AMP/ADP/ATP assay, and fluorescent activated cell sorting were performed. Results: Eupatilin decreased cell viability and activated AMPK in MIA-PaCa2 cells. Eupatilin decreased glucose uptake in pancreatic cancer, which led to cell starvation and AMPK activation. It is well known that AMPK induces p21 and cell cycle arrest by activating p53. In MIA-PaCa2 cells, p53 is mutated and wild-type p53 protein is suppressed. Treatment with eupatilin induced p21 expression but inhibited the expression of mutated p53. Eupatilin activated Tap73, a p53 family member, which can substitute wild-type p53's role. Conclusion: Eupatilin shows an anticancer effect against pancreatic cancer cells via glucose uptake inhibition, AMPK activation, and cell cycle arrest.*

Pancreatic cancer is one of the most aggressive and devastating malignancies, with a 5-year survival rate of 10% in the USA. Approximately 80-85% of the cases are unresectable or metastatic and for those individuals, the main treatment is chemotherapy including gemcitabine, nab-paclitaxel, and FOLFIRINOX (5-fluorouracil, folinic acid, irinotecan, and oxaliplatin) regimen (1). However, prognosis remains poor; the median overall survival was 11.1 months for metastatic pancreatic cancer patients who received FOLFIRINOX and 21.6 months at best for resectable or less advanced tumor (2, 3). Because of the disappointing outcome, several adjuvants are being investigated. Paracalcitol, metformin, statin, curcumin, and beberin showed anticancer effects (4, 5). Of note, metformin, the first-line drug of type II diabetes mellitus, has been shown to be beneficial as adjuvant therapy for pancreatic cancer patients (6).

AMP-activated protein kinase (AMPK) is an evolutionary conserved protein, which acts as a sensor of cellular energy status (7, 8). AMPK exists as a heterotrimeric complex consisting of the catalytic subunit (α) and two regulatory subunits (β and γ), and its activation requires phosphorylation at Thr^{172} of subunit α (7, 9). AMPK has been studied primarily as a target for type II diabetes but recently is emerging as a promising target for cancer prevention and treatment. AMPK shows its anticancer property by mediating various cell signaling pathways: promoting cell cycle arrest by activation of p53, repressing mammalian target of Rapamycin (mTOR), which induces cell proliferation, and changing lipid metabolism by inhibition of Acetyl-CoA carboxylase (ACC) (10). According to epidemiological studies, treatment with metformin, an AMPK activator, reduces the incidence of cancer (4, 11, 12).

Eupatilin (5,7-Dihydroxy-3',4',6-trimethoxyflavone) is a phytochemical derived from *Artemisia Asiatica* and known as binding agonist of peroxisome proliferator-activated receptor α (PPARα) (13). Eupatilin is used as a drug (Stillen™) for gastritis and peptic ulcer since it protects the gastric mucosa against inflammation and oxidative stress (14). In recent years, eupatilin was identified as a suppressing agent of tumors such as prostate cancer, ovarian cancer, renal cell carcinoma, glioma, gastric cancer, and endometrial cancer (15-20). However, it is still not elucidated whether eupatilin affects pancreatic cancer.

In our study, we found that eupatilin had inhibitory effects on pancreatic cell proliferation. It has been shown that a diversity of natural products shows anticarcinogenic effects *via* AMPK (8, 21-25), and several drugs for pancreatic cancer induce cell death by AMPK activation (26-28). Furthermore, one study indicated that eupatilin activates AMPK in mouse intestinal epithelial cells (29). It is

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noteworthy that several gene mutations in liver kinase 1 (LKB1)-AMPK signaling pathway are significantly associated with an increased pancreatic cancer risk (30). Our finding and these existing results, collectively, suggest that eupatilin ameliorates pancreatic cancer by activating AMPK.

Materials and Methods

Reagents. Antibodies against p53 and phosphorylated p53 were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibody against p21 was purchased from Abcam (Cambridge, UK), while antibody against β-actin was obtained from Enogene Biotech (Washington Heights, NY, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and goat anti-mouse IgG secondary antibodies were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Eupatilin, GW-6471, and propidium iodide were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. MCF-7 human breast cancer cells, MIA-PaCa2 human pancreatic cancer cells, and SH-SY5Y human neuroblastoma (ATCC, Rockville, MD, USA) were maintained in Dulbecco's high glucose (25 mM glucose) modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were cultured at 37˚C in a humidified incubator with 5% CO_2.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell viability was measured by MTT assay. MCF-7, MIA-PaCa2, and SH-SY5Y cells were seeded into 96-well plates and allowed to grow for 24 h. The medium was replaced by serum-free DMEM 24 h prior to treatment. Subsequently, MTT (0.5 mg/ml) was added and the cells were incubated for 2 h at 37˚C. Afterward, the cells were lysed with dimethyl sulfoxide (DMSO) and the absorbance at 540 nm was measured by a microplate reader (BioTek, Seoul, Republic of Korea).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted using TRIzol (Sigma-Aldrich) according to the manufacturer's protocol. mRNA was eluted in DEPC-treated water and quantified with NanoDrop ND-100 device (Thermo Fisher Scientific, Waltham, MA, USA). Avian myeloblastosis virus reverse transcriptase (AMVRT) and Oligo (dT) (Promega, Madison, WI, USA) were used to synthesize cDNA from 2 μg of mRNA. The cDNA was synthesized with 1 μg/μl mRNA, 2 μl 10 mM dNTP, 11.1 μl DEPC-treated water, 0.2 μl Rnasin, 0.2 μl AMVRT, 0.5 μl 500 nM oligo (dT), and 4 μl 5×RT buffer in PCR tubes by incubating at 42˚C for 1.5 h. cDNA was diluted with 20 μl dH_2O and stored at –80˚C until use. Primer3 version 0.4.0 was used to design primers. The following primers were used for amplification: β-actin (NM_001101.5), 5'-AGA GCT ACG AGC TGC CTG AC -3'(sense) and 5'-AGC ACT GTG TTG GCG TAC AG-3' (antisense)), Tap73 (NM_00116240.3), 5'-GCA CCT ACT TCG ACC TTC CC-3' (sense) and 5'-GTA GTC ATG CCC TCC AGG TG -3' (antisense). The RT-PCR program used was the following: 95˚C for 10 min, 40 cycles at 95˚C for 15 s, 60˚C for 30 s, 72˚C for 30 s, followed by 10 min at 72˚C. Ten microliters of product were visualized with agarose gel electrophoresis.

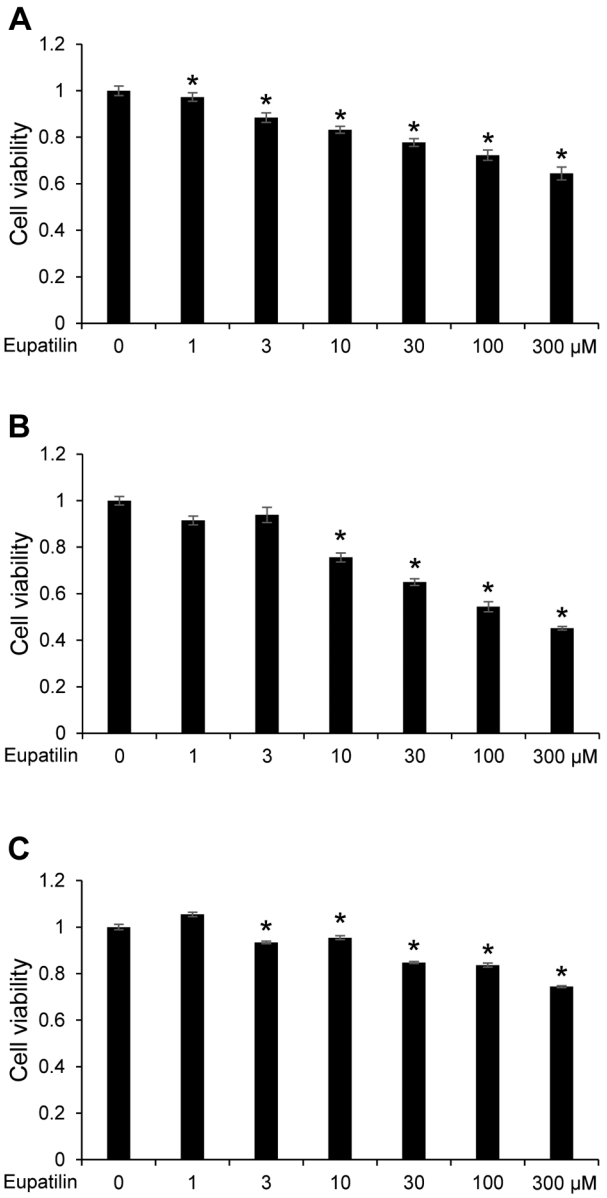


Figure 1. *Eupatilin exerts anticancer effects in pancreatic cancer, neuroblastoma, and breast adenocarcinoma. (A, B, C) Three kinds of cancer cells were treated with eupatilin (1~300 μM) for 24 h (A. MIA-PaCa2; pancreatic cancer, (B) SH-SY5Y; neuroblastoma, (C) MCF-7; breast adenocarcinoma). Cell viability was expressed as the ratio of the number of viable cells after treatment with eupatilin vs. the number of untreated control cells.*

Western blot analysis. The cells were grown in six-well plates. At 80% confluency, the cells were serum-starved for 24 h before treatment with selected agents. After treatment, the medium was aspirated and the cells were washed twice with ice-cold phosphate-buffered saline (PBS). Afterward, the cells were lysed in 30 μl lysis buffer [50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM EDTA, 1 mM sodium orthovanadate

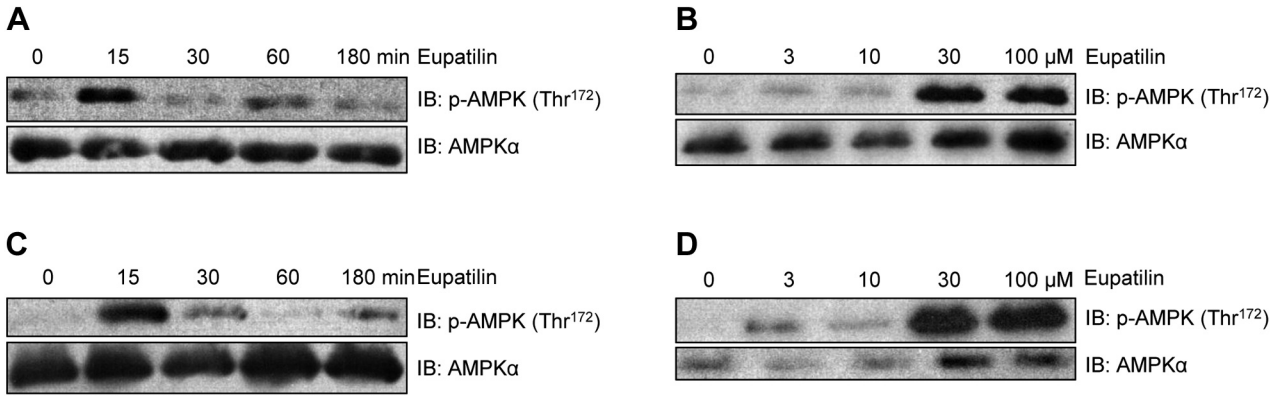


Figure 2. *Eupatilin activates AMPK in MIA-PaCa2 and SH-SY5Y cells in a time- and concentration-dependent manner. (A, B) After serum starvation for 12 h, MIA-PaCa2 cells were treated with eupatilin (30 μM) for the indicated time periods (A) or treated with the indicated concentrations of eupatilin for 1 h (B). (C, D) Identical experiments were performed with SH-SY5Y cells. Cell lysates were analyzed by western blotting using antibodies against phospho-AMPK (Thr^{172}), with AMPKα serving as a control.*

(Na_3VO_4), 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The lysate was resolved on SDS-PAGE gels and then transferred to nitrocellulose membranes. The membranes were blocked in 5% dry milk (w/v) for 1 h and then washed three times with tris-buffered saline (TBS) containing 1% Tween-20. The membranes were incubated overnight at 4˚C with primary antibodies and then probed with an HRP-conjugated secondary antibody for 1 h. The blots were visualized with Amersham Biosciences ECL Detection System (Amersham plc, GE healthcare, Chicago, IL, USA).

2-deoxy-D(^{3}H)-glucose assay. Glucose uptake was determined by measuring the uptake of 2-deoxy-D(^{3}H)-glucose (2-DG). The cells were rinsed with warm PBS and starved in serum-free DMEM for 3 h. After treatment with eupatilin, the cells were incubated in KRBH buffer [20 mM HEPES (pH 7.4), 130 mM NaCl, 1.4 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 1.2 mM KH_2PO_4, 2 mM $NaHCO_3$] containing 0.5 μCi 2-DG at 37˚C for 15 min. The reaction was terminated by washing twice with ice-cold PBS. Cells were lysed in 0.5 N NaOH with 0.5% SDS, and 500 μl cell lysate was mixed with 500 μl scintillation cocktail. Radioactivity was measured using a scintillation β-counter.

AMP/ATP assay. The cellular AMP and ATP was extracted by the boiling water method. Cells were seeded and starved for 3 h. After treatment with eupatilin for 1 h, the cells were washed twice with cold PBS. The cells were collected and sonicated, followed by measurement of the protein level using Bradford assay. The remaining lysate was boiled at 105˚C for 10 min for deproteinization and centrifuged. The supernatant was stored at –20˚C before use. AMP and ATP concentrations were measured using ATP/ADP/AMP Assay Kit (Biomedical Research Service Center, University at Buffalo, NY, USA) by detecting bioluminescence using Enspire Multimode Plate Reader (Perkinelmer, Waltham, MA, USA), according to the manufacturer's instructions. AMP/ATP ratio was normalized with the total protein level.

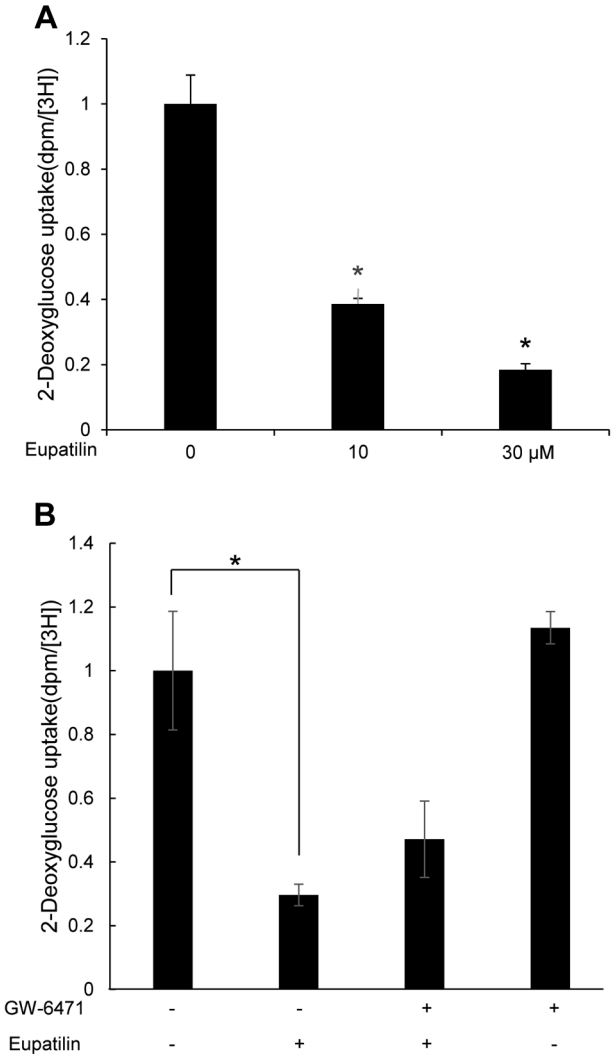
Cell cycle analysis. The cells were seeded and treated with or without eupatilin. Cells were harvested and fixed in 75% ethanol at –20˚C for 12 h. Afterward, cells were incubated with 1 mg/ml Rnase A at 37˚C for 30 min and stained with propidium iodide. The cell cycle was analyzed using BD FACSCanto II Flow Cytometry System (BD, Franklin Lakes, NJ, USA).

Statistical analysis. Results are expressed as mean±standard error of the mean (SEM). Differences within a group were analyzed by Student's *t*-test. One-way analysis of variant (ANOVA) was performed to compare multiple groups followed by Bonferroni's *post hoc* test. Statistical significance was set at $p<0.05$. All analyses were performed using SPSS (IBM, Armonk, NY, USA).

Results

Eupatilin exerts anticancer effect in pancreatic cancer, neuroblastoma, and breast adenocarcinoma. In recent studies, eupatilin was found to suppress several types of cancer (15-20). To examine the anticancer effect of eupatilin, we measured viability of pancreatic cancer (MIA-PaCa2), neuroblastoma (SH-SY5Y), and breast adenocarcinoma (MCF-7) cells. Cells were exposed to various concentrations (1-300 μM) for 24 h and cell viability was measured using the MTT assay. Eupatilin inhibited the growth of MIA-PaCa2, SH-SY5Y, and MCF-7 in dose-dependent manner, and the ratios of live cells compared to control (30 μM, 24 h) were 84.8%, 77.8%, and 65.0% respectively (Figure 1).

Treatment of MIA-PaCa2 and SH-SY5Y cells with eupatilin activates AMPK in a time- and concentration-dependent manner. The AMP-activated protein kinase (AMPK) has an anticancer effect *via* various pathways. To elucidate the role of AMPK in cancer cells, its levels were assayed by western blotting. Treatment of MIA-PaCa2 cells with eupatilin increased AMPK phosphorylation in a time- (Figure 2A) and dose-dependent manner (Figure 2B). Similar results were



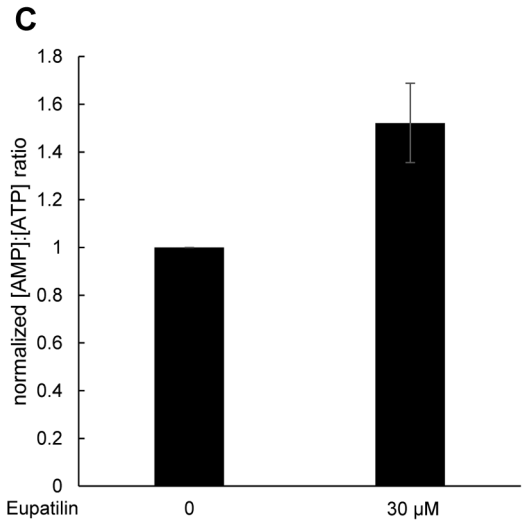


Figure 3. *Eupatilin induces energy depletion in pancreatic cancer cells. (A) After 1 h of treatment with eupatilin (10 μM, 30 μM), 2-deoxy-d[H3]-glucose (2-DG) uptake was measured in pancreatic cancer cells. (B) MIA-PaCa2 cells were co-treated with the PPARα inhibitor, GW-6471 (1 μM), and eupatilin (30 μM) for 30 min. 2-deoxy-d[H^3]-glucose (2-DG) uptake was measured. (C) Cells were starved for 4 h and then treated with eupatilin (30 μM, 1 h). Cell lysates were deproteinized and the levels of AMP, ADP, and ATP were measured. The results were normalized with the total protein level and are presented as ratios compared to control.*

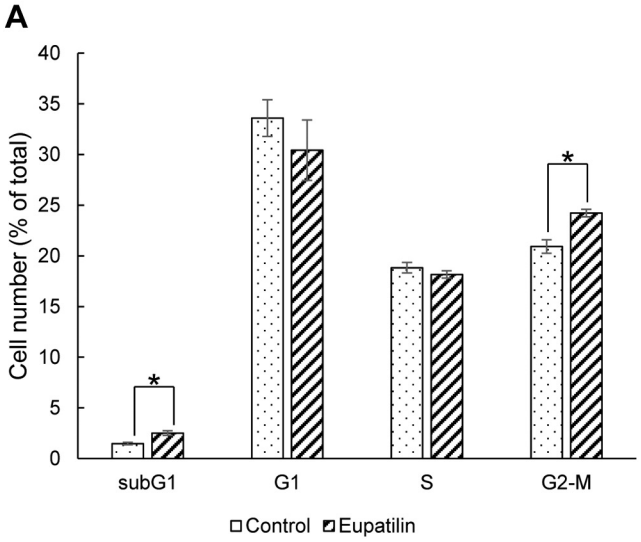
obtained in SH-SY5Y cells (Figure 2C and D). These results demonstrate that eupatilin stimulates AMPK in pancreatic cancer and neuroblastoma cells.

Eupatilin induces energy depletion in pancreatic cancer cells. Since, AMPK plays a pivotal role in cell metabolism, the effect of eupatilin may be translated in changes in cellular metabolism. To address this hypothesis, we examined the effect of eupatilin on 2-deoxyglucose (2-DG) uptake. Treatment of MIA-PaCa2 cells with eupatilin inhibited 2-DG uptake in a dose-dependent manner (Figure 3A). Only 18.46% (30 μM, 1 h) of glucose uptake was measured compared to control. This result suggests that cancer cells utilize less glucose upon eupatilin treatment.

Moreover, eupatilin is known as a specific agonist of peroxisome proliferator-activated receptor α (PPARα) (13). We treated cells with the PPARα inhibitor GW-6471 to examine whether eupatilin inhibits glucose uptake *via* PPARα activation. Addition of GW-6471 partially reversed the effect of eupatilin on glucose uptake but the result was not statistically significant (Figure 3B). Glucose transporter 1 (GLUT1) is the main glucose transporter of MIA-PaCa2 and up-regulation of PPARα inhibits glut1 gene expression (31). We obtained data indicating that the mRNA levels of *glut1* were not decreased upon treatment with eupatilin (data not shown). Further elucidation of the mechanism of the inhibition of glucose uptake by eupatilin is needed.

AMPK, as its name suggests, is activated by a rise in the AMP:ATP ratio, which reflects the energy status of a cell. Eupatilin was found to increase the AMP:ATP ratio, which leads to direct activation of AMPK (Figure 3C) (data not significant, p=0.07).

Eupatilin induces cell cycle arrest in pancreatic cancer cells. We found earlier that eupatilin inhibits the growth of cancer cells. To elucidate the mechanism of cancer cell suppression,



	subG1	G1	S	G2-M
Control	1.48	33.6	18.83	20.93
Eupatilin	2.51	30.43	18.17	24.23

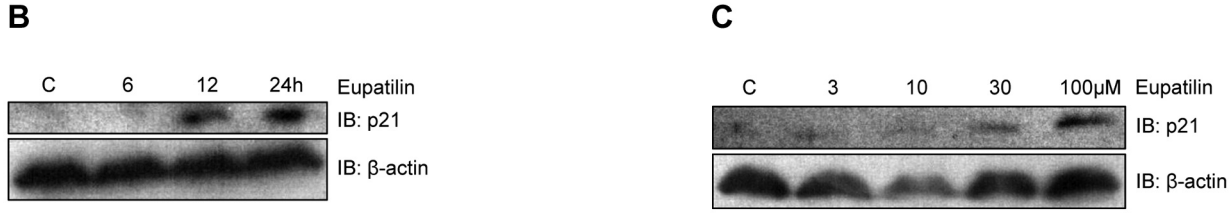


Figure 4. *Eupatilin induces cell cycle arrest. (A) MIA-PaCa2 cells were treated with eupatilin (30 μM) for 24 h. Distribution of cells in each phase of the cell cycle was analyzed with flow cytometry. (B, C) After serum starvation for 24 h, cells were treated with eupatilin (100 μM) for the indicated time periods (B) or treated with different concentrations of eupatilin for 24 h (C). Cell lysates were analyzed by western blotting using antibodies against p21, with β-actin serving as a control.*

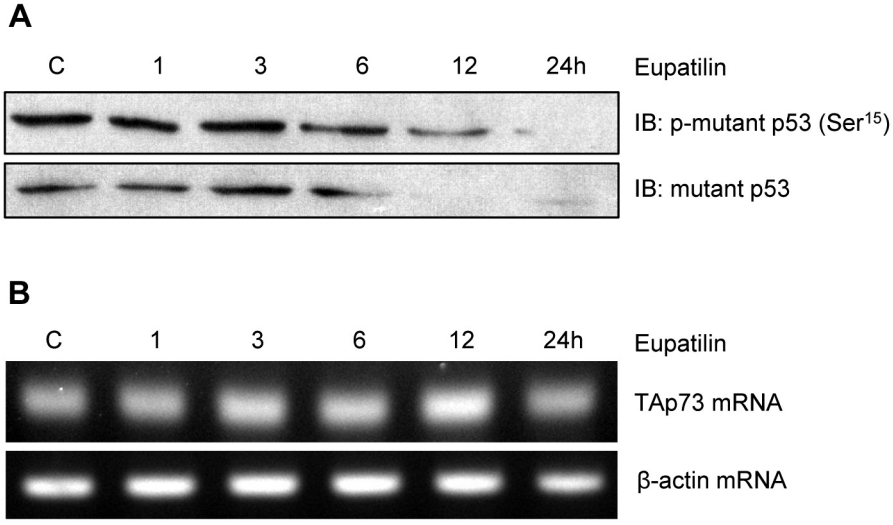


Figure 5. *Eupatilin overcomes the effect of p53 mutation in pancreatic cancer cells. MIA-PaCa2 cells were treated with eupatilin (30 μM) for the indicated time periods. (A) Levels of mutant p53 and phosphorylated mutant p53 were analyzed using western blotting. (B) Expression of Tap73 was analysed using RT-PCR and agarose cell electrophoresis.*

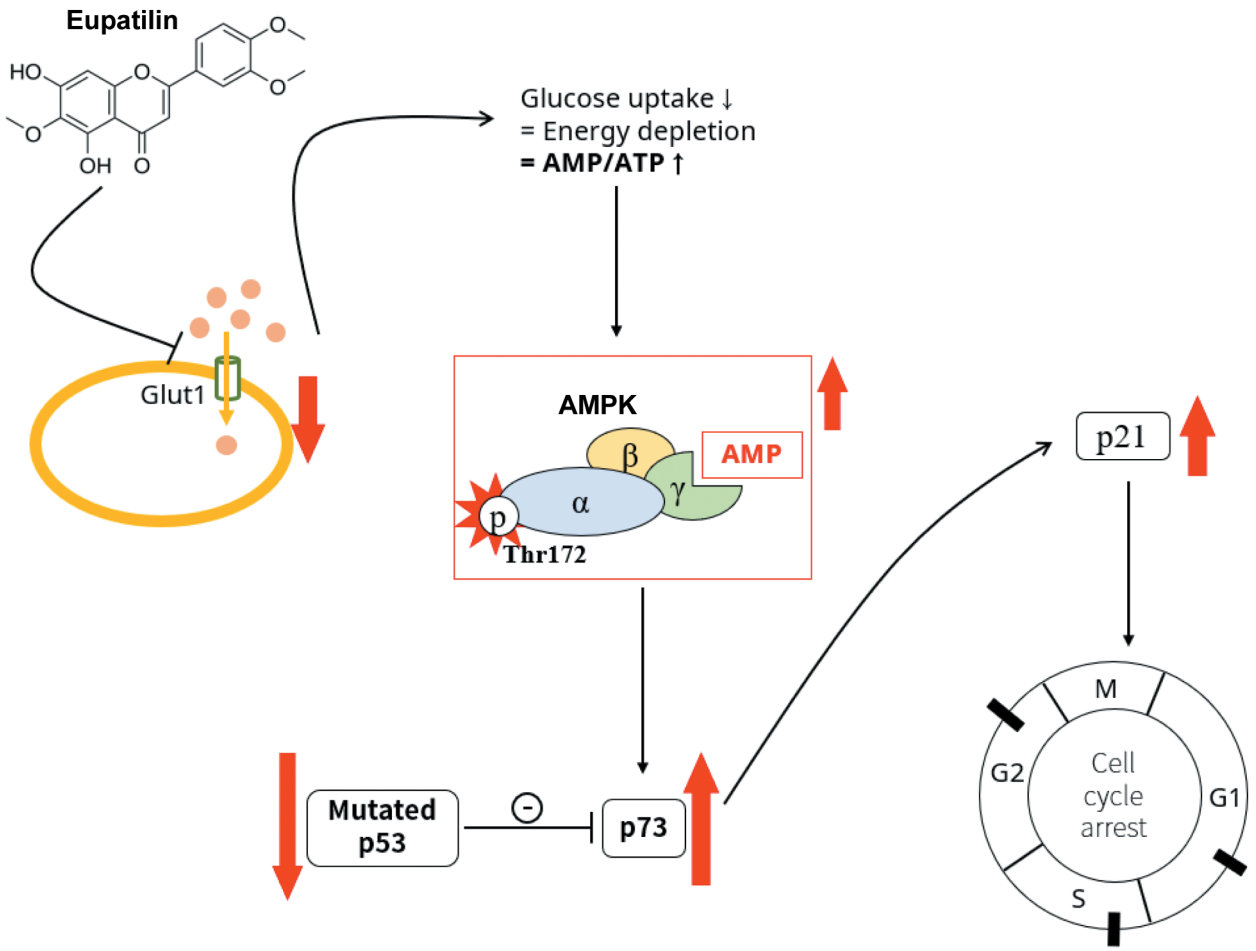


Figure 6. *The inhibitory mechanism of eupatilin on pancreatic cancer cells. Eupatilin induced energy depletion of MIA-PaCa2 cells by suppressing Glut1, which provides glucose as a primary energy source. Increased AMP/ATP ratio activated AMPK. AMPK activation induced cell cycle arrest via up-regulation of p21. Pro-oncogenic mutated p53 protein was down-regulated and TAp73 expression was increased, possibly substituting p53 protein in AMPK-p53-p21 signaling pathway of cell cycle arrest.*

the distribution of cells in each phase of the cell cycle was analyzed by flow cytometry. The results showed expansion of G2 and sub-G1, which indicates increased apoptosis and G2/M cell cycle arrest of MIA-PaCa2 cells (Figure 4A). p21 is one the most important proteins in cell cycle arrest. Treatment with eupatilin increased p21 levels in a time- and concentration-dependent manner (Figure 4B and C). Theis result indicates that eupatilin suppresses cell cycle progression in MIA-PaCa2 cells.

Eupatilin overcomes the p53 mutation by up-regulating Tap73 in pancreatic cancer cells. The AMPK-p53-p21 pathway is well known to arrest cell cycle. AMPK phosphorylates p53, and p-p53 up-regulates p21. To clarify signals between AMPK and p21, we analyzed p53 and p-p53 (Ser15) levels by western blotting. Surprisingly, p-p53 and p53 level protein levels decreased (Figure 5A). In fact, MIA-PaCa2 cells carry mutations in p53 gene, which has pro-oncogenic potential, while promoting self-accumulation and down-regulation of wild-type p53 family members (32, 33). Therefore, the cancerous potential of mutated p53 was decreased by eupatilin. Then, we examined another candidate, Tap73, which is a member of the p53 family and has a function similar to wild type p53. We found that expression of Tap73 increased by eupatilin (Figure 5B). This result suggests that Tap73 can replace the normal role of p53.

Discussion

Pancreatic cancer is notorious for its low survival and extremely aggressive behavior. Due to the disappointing treatment results, many chemicals other than of traditional

chemotherapy regimens are being studied. The main objective of our study was to examine whether and how eupatilin suppresses cancer. The three main components of 'drugability' of a chemical are its potency, safety, and cost. Eupatilin, a flavonoid from *Artemisia Asiatica*, shows acceptable safety and cost, and is already used widely for gastric ulcer. Exploring the anticancer effect of natural products always has been a primary method for identifying drug candidate. Eupatilin was recently found to suppress several cancer types (15-20). In this research we found eupatilin shows anticancer effect against additional cancer types (pancreatic cancer, neuroblastoma, and breast adenocarcinoma), which has not been previously reported.
Additionally, we elucidated the mechanism through which eupatilin suppresses the growth of pancreatic cancer. Eupatilin inhibits glucose uptake into pancreatic cancer cells. The depleted energy status is shown as elevated AMP:ATP ratio which activates AMPK. Notably, treatment with 30 μM eupatilin for 1 h caused a 18.46% decrease in glucose uptake compared control. This result suggests that eupatilin can rapidly and powerfully decrease glucose uptake by MIA-PaCa2 cells and significantly affect cancer cells, since they use glucose as a primary energy source (Warburg effect). It is known that higher blood glucose levels are associated with shorter overall and disease-free survival, and higher risk of distant metastasis (34). Based on these facts, the idea of glucose restriction during chemotherapy was proposed (35). Eupatilin can play a significant role in the starvation of cancer cells, since it halts glucose uptake from MIA-PaCa2 cells.

Eupatilin can bind and activate PPARα selectively (13). In this study we showed inhibition of PPARα partially reversed decreased glucose uptake, but the effect was not statistically significant. Furthermore, up-regulation of PPARα inhibits GLUT1 transcription (31). Therefore, we inferred that expression of GLUT1 will mediate the decrease in glucose uptake, but this was not the case. Considering the rapid effect of eupatilin, there is the possibility that decreased glucose uptake may result from GLUT1 translocation inhibition. The ambiguous results related to PPARα and GLUT1 suggest that additional studies are required to further elucidation the mechanism of glucose inhibition by eupatilin.

AMPK up-regulates p21 and induces cell cycle arrest of MIA-PaCa2 cells. P53, known as the 'guardian of the genome', is the bridge 'signal' between AMPK and p21. However, in MIA-PaCa2 cells p53 is mutated and shows pro-oncogenic potential. We found that level of mutated p53 decrease and that of Tap73, which is a member of p53 family, increase. P53 mutation is common in cancer cell and prognosis is worse in p53-mutated cancer. Furthermore, a large proportion of patients with pancreatic cancer carry a p53 mutation or deletion (36). Eupatilin, by decreasing mutated p53 and increasing Tap73, has the potential to overcome a p53 mutation, while Tap73 substitutes mutated p53. The anticancer effect of eupatilin is illustrated in Figure 6 collectively.

In recent years, AMPK is widely studied as a tumor suppressor. Metformin, known as AMPK activator, is the drug of choice for type 2 diabetes mellitus (DM). DM is strongly associated with pancreatic cancer and can induce pancreatic cancer and end-stage of pancreatic cancer leads to DM. Metformin is studied as a drug of cancer treatment and prevention and can also suppress pancreatic cancer. The link between AMPK, DM and pancreatic cancer makes eupatilin a more attractive drug since it can activate AMPK powerfully. The link between metformin and eupatilin and their putative synergistic effects upon co-treatment will be further studied.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study.

Authors' Contributions

THP and HSK conceived and designed the experiments. THP performed the experiments and wrote the paper. THP and HSK reviewed and revised the manuscript. All Authors read and approved the final manuscript.

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