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# Grifolin, a potent antitumour natural product upregulates death-associated protein kinase 1 DAPK1 via p53 in nasopharyngeal carcinoma cells

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ABSTRACT

Grifolin, a secondary metabolite isolated from the fresh fruiting bodies of the mushroom *Albatrellus confluens*, has been shown to inhibit the growth of some cancer cell lines *in vitro* by induction of apoptosis in previous studies of our group. However, the mechanisms of action are not completely understood. An apoptosis-related gene expression profiling analysis provided a clue that death-associated protein kinase 1 (*dapk1*) gene was upregulated at least twofold in response to grifolin treatment in nasopharyngeal carcinoma cell CNE1. Here, we further investigated the role of DAPK1 in apoptotic effect induced by grifolin. We observed that protein as well as mRNA level of DAPK1 was induced by grifolin in a dose-dependent manner in nasopharyngeal carcinoma cell CNE1. We found that grifolin increased both Ser392 and Ser20 phosphorylation levels of transcription factor p53 protein, which could promote its transcriptional activity. Moreover, induced by grifolin, the recruitment of p53 to *dapk1* gene promoter was confirmed to enhance markedly using EMSA and ChIP assays analysis. The involvement of DAPK1 in grifolin-induced apoptosis was supported by the studies that introducing siRNA targeting DAPK1 to CNE1 cells remarkably interfered grifolin-caused apoptotic effect as well as the activation of caspase-3. Grifolin induced upregulation of DAPK1 via p53 was also observed in tumour cells derived from human breast cancer and human colon cancer. The findings suggest that upregulation of DAPK1 via p53–DAPK1 pathway is an important mechanism of grifolin contributing to its ability to induce apoptotic effect. Since growing evidence found a significant loss of DAPK1 expression in a large variety of tumour types, grifolin may represent a promising candidate in the intervention of cancer via targeting DAPK1.



## 1. Introduction

Natural products and their derivatives have historically been invaluable as sources of chemopreventive and therapeutic agents.[1] Besides the polysaccharides and polysaccharide–protein complexes, another source for substances of therapeutic interest originated from mushroom is the pool of low-molecular-weight secondary metabolites.[2] In more recent years,

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secondary metabolites from mushrooms have been found to possess significant antitumour properties *in vitro* and *in vivo* via targeting processes such as signal transduction cascades, cell cycle regulation and apoptosis. Grifolin, a secondary metabolite isolated from the fresh fruiting bodies of the mushroom *Albatrellus confluens*, has shown various pharmacological and microbiological effects.[3–5] Grifolin was reported to exhibit inhibitory activity against nitric oxide (NO) production in RAW 264.7 cells.[6] Grifolin and its derivatives presented novel partial agonists for free fatty acid receptors GPR120.[7] The anticancer activities of grifolin were first reported by our group. Our previous studies have shown that grifolin was able to inhibit the growth of some cancer cell lines by induction of apoptosis, meanwhile, the normal cells were less sensitive to grifolin, which suggests that grifolin has some selective antitumour effect.[8] Our group further identified grifolin induced cell-cycle arrest in G1 phase via the ERK1/2 pathway.[9] Some other studies showed grifolin induced apoptosis in human osteosarcoma cells.[10] Although it represents encouraging antitumour activities, the central mechanism responsible for grifolin efficacy is not fully known.

To better understand which key regulatory genes are involved in the apoptotic effect induced by grifolin in nasopharyngeal carcinoma (NPC) cell, a 205 apoptosis-related genes expression profiling analysis was carried out in our earlier study. It provided a clue that death-associated protein kinase 1 (*dapk1*) gene was upregulated significantly in response to grifolin treatment.[9] DAPK1 belongs to a newly classified family of $Ca^{2+}$/calmodulin (CaM)-regulated Ser/Thr kinase, whose members not only possess significant homology in their catalytic domains, but also share cell death-associated functions. Increased DAPK1 activity, due to overexpression of the kinase, leads to pronounced death-associated cellular changes such as membrane blebbing, cell rounding and detachment from extracellular matrix.[11–18] DAPK1 gene expression increases in response to stimuli that activate p53, such as DNA-damaging agents and oncogene expression.[15,19] Documents showed that the DAPK1 promoter contain a p53-binding element.[19] Under certain circumstances, particularly in cells with minimal DAPK1 protein expression, transcriptional or translational mechanisms may be not only important means of activation of the kinase but also rescue its pro-apoptotic function repressed by promoter methylation.

In order to evaluate the therapeutic potential of grifolin, in the present study, we developed the molecular mechanisms of its pro-apoptotic effect employing human nasopharyngeal carcinoma cells CNE1. Our results provided first evidence for the upregulation of DAPK1 via p53–DAPK1 pathway as an important mechanism of grifolin-induced apoptosis in NPC cells.

## 2. Materials and methods

### 2.1. Cell lines and culture

Human nasopharyngeal carcinoma cell line CNE1, human cervical cancer cell line HeLa (ATCC CCL-2), human breast cancer cell line MCF7 (ATCC HTB-22), human colon cancer cell line SW480 (ATCC CCL-228), human lung cancer cell line A549 (ATCC CCL-185) and human non-small cell lung cancer cell line H1299 (ATCC CRL-5803) were grown in RPMI 1640 supplemented with 10% v/v heat-inactivated foetal bovine serum, 1% w/v glutamine and 1% w/v antibiotics. All were cultured at 37 °C in a humidified incubator containing 5%$CO_2$.

### 2.2. Reagents and plasmids

The following antibodies were used for Western blot: β-actin (sc-8432, Santa Cruz), anti-DAPK1 (D1319, Sigma), anti-p53 (sc-126, Santa Cruz), anti-p-p53(ser15) (9284s, Cell signalling), anti-p-p53(Ser392) (sc-7997, Santa Cruz), anti-p-p53(ser20) (sc-18079, Santa Cruz), anti-p-p53(Thr81) (2676s, Cell signalling) and horseradish peroxidase-conjugated goat anti-mouse (sc-2005, Santa Cruz), goat anti-rabbit (sc-2004, Santa Cruz) or donkey anti-goat (sc-2020) secondary antibodies.

Grifolin (2-trans, trans-farnesyl-5-methylresorcinol) was provided by Kunming Institute of Botany, the Chinese Academy of Sciences (purity > 99%, HPLC analysis). Dimethyl sulphoxide (DMSO, Sigma) was used to dissolve grifolin. The final concentration of DMSO in the culture media was kept less than 0.1% v/v which had no significant effect on the cell growth.pSUPER-p53 siRNA vector and pEGFP-p53 expression vector were kindly provided by Professor Wu Qiao (Xiamen University, China). pSIREN-dapk1 siRNA vector was generated by targeting the coding region of *dapk1* gene. pEGFP-DAPK1 expression vector was constructed by cloning the whole DAPK1 coding fragment into EcoRI/SalI sites of the pEGFP-N1 vector.

### 2.3. Western blot analysis

Cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS), and then lysed in the lysis buffer 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 2% SDS w/v, 5 mM dithiothreitol, 10 mM phenylmethyl sulphonylfluoride, 1 mM $Na_3VO_4$, 1 mM NaF, 10% v/v glycerol, protease inhibitor cocktail tablet (Roche). Equal amounts of the total proteins from cell preparations and PageRuler™ molecular weight markers (Fermentas life sciences) were resolved by SDS–polyacrylamide gel electrophoresis and then electrotransferred onto the nitrocellulose membrane. The membranes were blocked and then incubated with specific primary antibodies according to the manufacturer's recommendations. The primary antibody complexes were then stained with horseradish peroxidase-conjugated secondary antibody and developed with the enhanced chemiluminescence detection kit (ECL; Pierce).

### 2.4. Preparation of nuclear extract and electrophoretic mobility shift assay (EMSA)

Cells were processed using the kit (NE-PER®, Thermo Scientific). Cells were washed in cold PBS, resuspended in 100 μl of ice-cold CER I, vortexed and incubated on ice for 10 min, then added with 5.5 μl ice-cold CER II, incubated for a further 1 min followed by centrifugation. The cell pellet was re-suspended in 50 μl of ice-cold NER with alternative vortex and incubation for a total of 40 min, and then centrifuged at 16,000*g* for 10 min at 4 °C. The nuclear extract was aliquoted and frozen at –70 °C until further use. Oligonucleotide containing the p53-binding site of dapk1 promoter as well as its complementary sequence was commercially synthesised (Takara) and labelled with 5′-biotin. The oligonucleotides

used for EMSA were WT oligo 5′-GAACTTGTTCTAATGG-CATGTCT-3′, WT oligo mutant 5′-GAACTTGTTCTAATGGTA-GATCT-3′. The EMSAs were performed using 10 μg of nuclear protein extract in a 20 μl of binding buffer at room temperature for 20 min prior to the addition of the appropriate biotin-labelled double-stranded oligo probe. After incubating for 20 min, samples were resolved under non-denaturing conditions on a 6% polyacrylamide gel using 0.5×TBE (45 mM Tris, pH 7.5, 45 mM boric acid, 2 mM EDTA) as a running buffer and the results were recorded by Chemiluminescent Nucleic Acid Detection Module Kit (Pierce). To determine the binding specificity of the DNA–protein complexes formation, nuclear extracts were incubated for 10 min with a 200-fold excess of unlabelled p53 probe prior to the addition of labelled double-stranded oligos. For demonstrating the presence of a specific protein in the complexes, 1 μg of p53 antibody was added to the nuclear extracts and the mixture was incubated at room temperature for an additional 30 min. DNA–protein complexes were detected as described above.

### 2.5. Chromatin immunoprecipitation (ChIP) assay

Cells were grown to 70–80% confluency in 10-cm plates, and were then processed for ChIP assays using the kit (EZ-ChIP, Upstate). DNA was sheared to a size of 200–1000 base pairs (bp) prior to performing the immunoprecipitation (IP). Sheared chromatin was precleared by incubating with protein G beads for 1 h at 4 °C to reduce non-specific background. Precleared chromatin was then incubated with various antibodies (2 μg) overnight at 4 °C. The kit's negative control IgG and positive control anti-RNA Polymerase II were also used in parallel reactions. Protein G bead incubation, washing of ChIP reactions, DNA elution from protein G, cross-link reversal, RNA removal and purification of eluted DNA were performed following the kit's protocol. Isolated DNA was subjected to RT-PCR and analysed by running agarose gels, stained with ethidium bromide, followed by UV visualisation.

### 2.6. Apoptosis and flow cytometric analysis

Cells were treated with grifolin for 48 h and resuspended in binding buffer containing annexin V-fluorescein isothiocyanate (FITC) and propidium iodide according to the supplier's instructions (BD Biosciences), and assessed by flow cytometry using a BD FACS Calibur cytometer. Each sample was run in twice.

### 2.7. Statistical analysis

Data are expressed as the mean S.E. Statistical significance was determined using *t* test and accepted at $p < 0.05$. All assays were performed in two independent samples, and each experiment was repeated at least two times.

## 3. Results

### 3.1. Grifolin promotes DAPK1 expression

In previous studies, we screened and found *dapk1* gene was upregulated in response to grifolin treatment by cDNA array analysis. We further set up experiments to determine the mRNA and protein expressions of DAPK1 in CNE1 cells.[9] Human cervical cancer cell line Hela was used as positive control.[20] As DAPK1 positive control, Hela cells expressed DAPK1 at very high level. We observed induction of *dapk1* mRNA in response to different concentrations of grifolin treatment monitored by RT-PCR. Treatment with grifolin induced a marked increase in *dapk1* mRNA in a dose-dependent manner (Fig. 1A). Induction of DAPK1 expression was further confirmed at the protein level by Western blot. DAPK1 protein

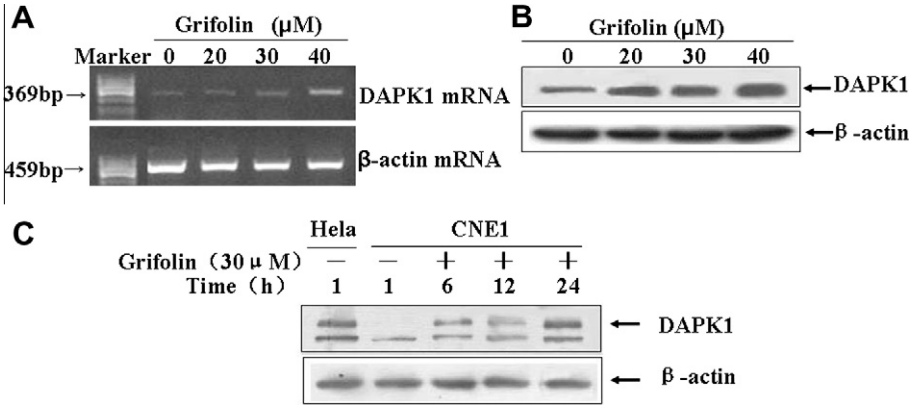


**Fig. 1 – Effect of grifolin on the induction of DAPK1 expression in CNE1 cell line. (A) Induction of *dapk1* mRNA levels by grifolin. CNE1 cells were incubated with medium containing the indicated concentration of grifolin for 24 h. Total RNA was isolated from cells and subjected to RT-PCR, using specific primers designed to amplify *dapk1* and actin mRNAs. Actin was used as a loading control. (B) Induction of DAPK1 protein expression by grifolin. CNE1 cells were cultured and treated with various concentrations of grifolin for 24 h and detected by Western blot. β-Actin served here as a normalisation control. (C) CNE1 cells were cultured and treated with 30 μM of grifolin from 1 to 24 h as indicated in the figure. At the end of treatments, total cell lysates were prepared and subjected to SDS–PAGE followed by Western blot. Membranes were probed with antibody against DAPK1. HeLa cells were used as a positive control.**

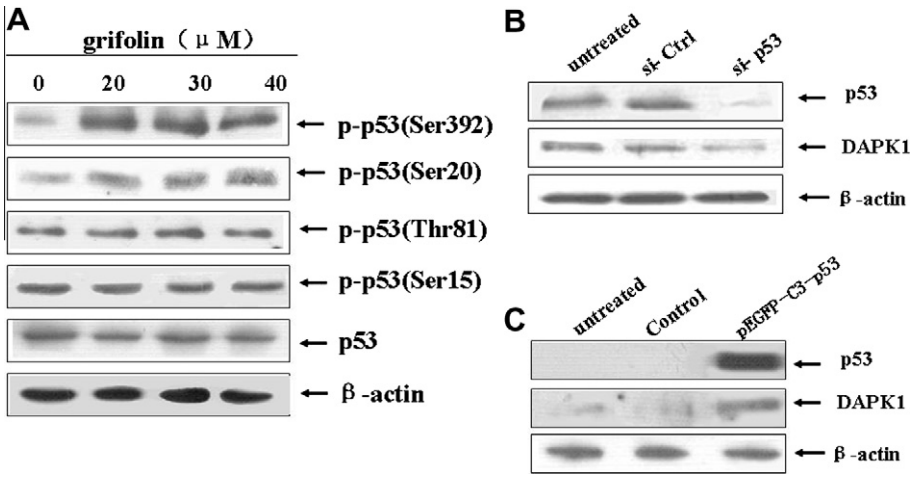


**Fig. 2 – Upregulation of DAPK1 via activating p53 induced by grifolin. (A) Effect of grifolin on phosphorylation of p53 protein. CNE1 cells were cultured and treated with various concentrations of grifolin for 24 h. Cell lysates were prepared and examined by Western blot analysis with antibodies against p-p53(Ser392), p-p53(ser20), p-p53(Thr81), p-p53(ser15) and p53, respectively. (B) Downregulation of DAPK1 expression in CNE1 cells as a result of p53 inhibition by siRNA. CNE1 cells were transfected with p53 siRNA(si-p53) or control siRNA (si-Ctrl) for 72 h, then p53 and DAPK1 expression were examined by Western blot analysis. (C) DAPK1 was upregulated as the result of re-expression of p53. P – which is a p53-deficient cell line was transfected with p53 expression vector pEGFP-C3-p53 or mock vector for 72 h, then p53 and DAPK1 were detected by Western blot analysis.**

in CNE1 cells treated with grifolin was significantly elevated in a dose-dependent manner (Fig. 1B). In addition, as shown in Fig. 1C, DAPK1 expression increased from 1 to 24 h induced by grifolin (Fig. 1C). Collectively, these observations indicate that grifolin increases the production of DAPK1 in CNE1 cells at both mRNA and protein levels.

### 3.2. *Grifolin induces phosphorylation of p53*

Transcription factor binding-site prediction suggests that p53 protein might bind the promoter of *dapk1* gene. More than 36 different amino acids within p53 protein have been shown to be modified in various biochemical and cell culture studies.[21]

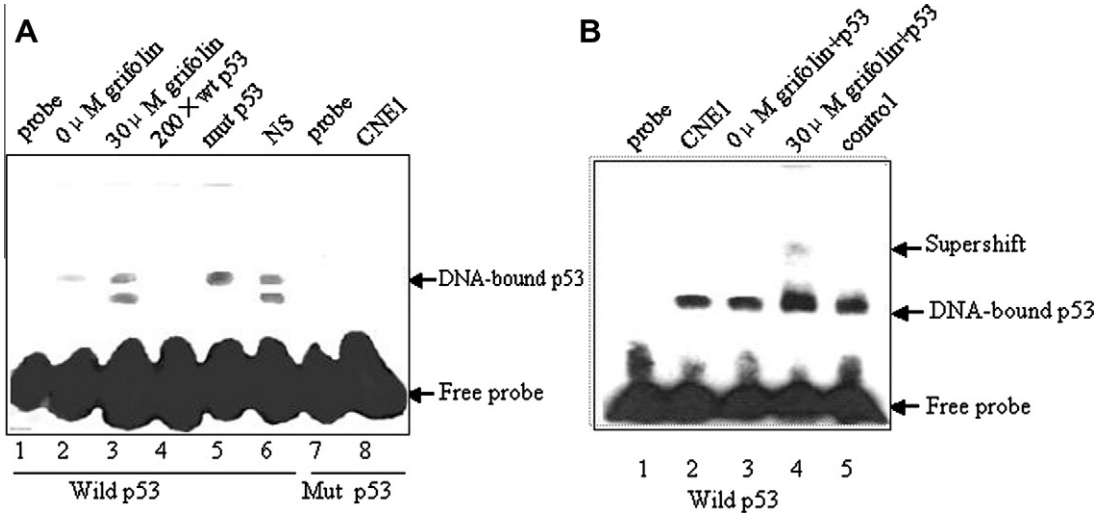


**Fig. 3 – Induction of p53 binding with *dapk1* DNA by grifolin. (A) Biotin-labelled wild type p53 oligonucleotide probe was incubated with nuclear extracts of CNE1 in the presence of grifolin treatment at a concentration of 30 μM for 24 h (lane 3), a 200-fold excess of unlabelled wildtype p53 (lane 4), a 200-fold excess of unlabelled oligonucleotide containing a 3 bp mutation of the p53 sequence (designated mut p53, lane 5) or a 200-fold excess of non-competitive unlabelled oligonucleotide (NS, lane6), and then p53 DNA binding activities were examined by EMSA. Biotin-labelled mutant p53 oligonucleotide probe only (lane 7) or probe was incubated with nuclear extracts of CNE1 cell line (lane 8), and then p53 DNA binding activities were detected by EMSA. (B) Ten micrograms of CNE1 nuclear extracts were preincubated with biotin-labelled p53 oligonucleotide probe in the absence (lane 2) or presence of antibody against p53 (lanes 3 and 4) or control antibody (lane 5) and then supershift assays were performed. CNE1 nuclear extracts were in the absence (lane 3) or presence (lane 4) of grifolin treatment.**

As a transcriptional activator, phosphorylation of p53 is classically regarded as the crucial step of p53 activation. N-terminal Ser 15 and Ser20 of p53 are phosphorylated after DNA damage and other types of stress by ATM, ATR, DNA-PK Chk1 and Chk2.[22] The C-terminal region of p53 was thought to act as a negative modulator that had to be modified to allow sequence-specific DNA binding.[22,23] Damage-stimulated phosphorylation of p53 at the C-terminal CK2 site (Ser392) forms a paradigm whereby sequence-specific DNA binding is stimulated by allosteric mechanisms.[24] Li and colleagues[25] reported that p53 was accumulated and activated by phosphorylation at Ser15, Ser20, Thr81 and Ser392 in NPC cells. To determine whether grifolin could affect p53 phosphorylation status, we treated CNE1 cells with grifolin at different concentrations and detected the phosphorylation level of Ser15, Ser20, Thr81 and Ser392 of p53, respectively by Western blot.

As shown in Fig. 2A, grifolin can increase Ser392 and Ser20 phosphorylation levels of p53, which could promote its transcription activity. However, there was no detectable

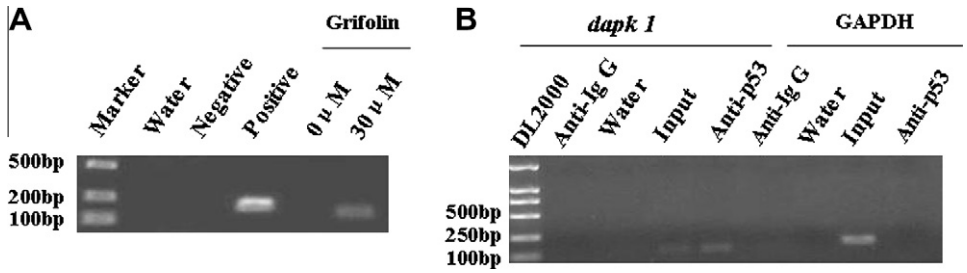


**Fig. 4 – Induction of p53 DNA binding activity by grifolin *in vivo* using ChIP assays. (A) ChIP assays of p53 DNA-binding activity in CNE1 cells in the presence of 30 μM of grifolin or not were performed as described in Section 2. A mouse monoclonal antibody against p53(sc-126) or negative control mouse IgG or positive control anti-RNA Polymerase II was used. PCR analysis using dapk1 primers 5′-TACC AGGAAGGGGTGACGGAA-3′, 5′-CTGTGCACAGTAACTGCAC TGT-3′ was shown using DNA after ChIP. (B) Specificity binding of transcription factor p53 to *dapk*1 promotor region. PCR analysis using dapk1 primers or GAPDH primers were shown using input DNA (1/10 of ChIP) or DNA after ChIP, followed by gel electrophoresis detection.**

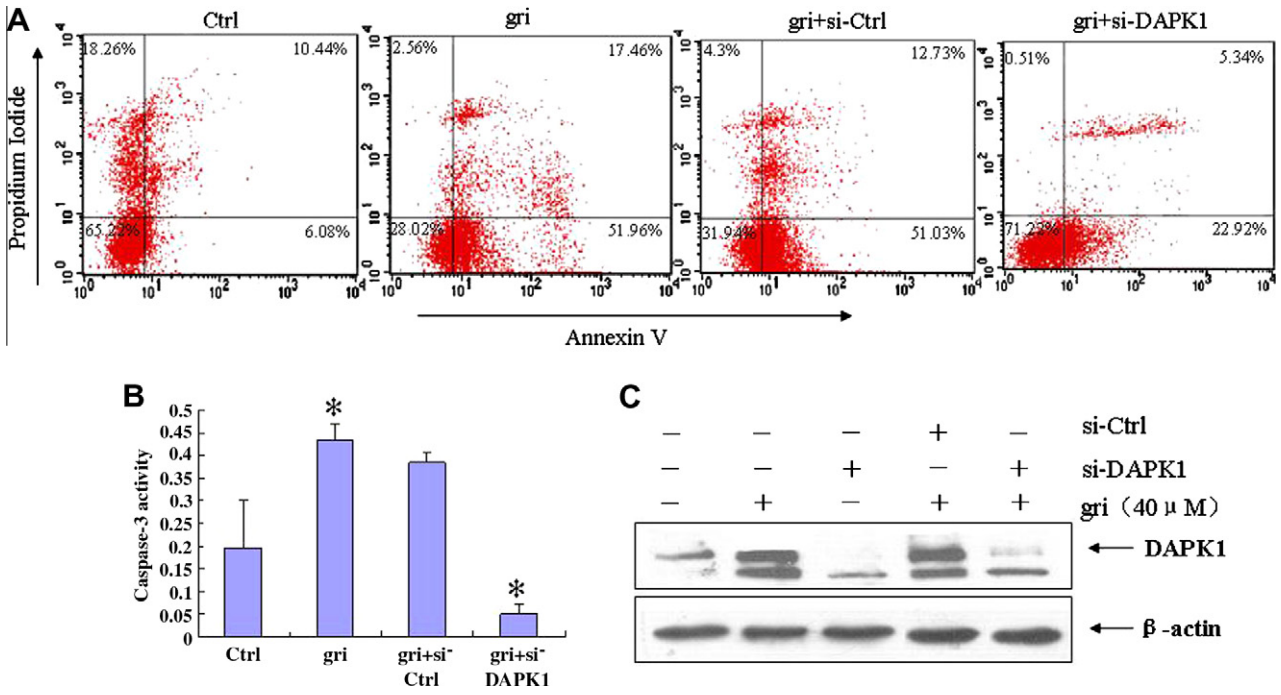


**Fig. 5 – Grifolin-induced apoptosis in CNE1 cells is DAPK1 dependent. (A) Percent live cells were measured in CNE1 cells transfected with scrambled(si-Ctrl) or DAPK1 siRNA(si-DAPK1), treated with 40 μM of grifolin as indicated in the figure. After 48 h, cells were harvested and suspended in binding buffer with annexin V-FITC and propidium iodide, followed by flow cytometry to assess cell apoptosis. (B) DAPK1 mediated the activation of caspase-3 induced by grifolin. CNE1 cells were transfected with DAPK siRNA for 72 h followed by incubation with 40 μM of grifolin for 6 h before the caspase-3 substrate DEVD-pNA (50 mM) was added. Mixtures were incubated for 1 h at 37 °C. Activation of caspase-3 was measured by ELISA Micro-plate Reader (Bio-tek) at 405 nm. Results are presented as means ± S.D. (three independent experiments). (C) Total cell lysates were prepared and subjected to SDS–PAGE followed by Western blot for DAPK1 detection. Ctrl, control; gri, grifolin.**

change on the total levels of p53 protein or its Ser15 or Thr81 phosphorylation level in the absence or presence of grifolin.

### 3.3. *Dapk1 is transcription target of p53*

p53 activates the expression of numerous genes, including genes encoding for proteins that have a purported activity in triggering apoptosis, such as the mitochondrial proteins Bax, BID and proteins that play a role in death receptor-mediated apoptosis, such as Fas and PIDD.[26] The *dapk1* gene expression increases in response to stimuli that activate p53, such as DNA-damaging agents and oncogene expression.[19] To further identify *dapk1* expression is p53 inducible, we transfected CNE1 cells with p53 siRNA for 72 h. Western blot showed successful p53 depletion following transfection, whilst control siRNA did not affect p53 expression. Meanwhile, DAPK1 expression decreased significantly, which correlated with p53 depletion (Fig. 2B). Furthermore, we transfected p53 expression vector to H1299 cells, which is a p53-deficient cell line. As shown in Fig. 2C, with p53 re-expression, DAPK1 was upregulated in transfected H1299 cells compared with control or mock-transfected cells. From the above experiments, we identify that *dapk1* expression is p53 inducible in CNE1 cells.

### 3.4. *Grifolin upregulates the binding activity of p53 to dapk1*

To explore whether grifolin promoted p53 binding to the response elements in the human *dapk1* gene, an electrophoretic mobility shift assay (EMSA) was performed with the p53 oligo. Using nuclear extracts from CNE1 cells, shift bands were observed (Fig. 3A). Competition assays with unlabelled oligo confirmed specificity (lane 4). The specificity of binding was further illustrated by mutating three bases in core sequence within the oligos. Incubation of nuclear extracts with a 200-fold excess of the indicated unlabelled mutant oligos did not block the formation of complex (lane 5). In supershift assays, the p53 antibody induced a clear shift (Fig. 3B). We could conclude from the experiments that human p53 DNA-binding sites in *dapk1* gene were transcriptionally regulated by p53 *in vitro* and the recruitment of p53 to *dapk1* gene promoters was enhanced markedly by grifolin.

Furthermore, to confirm that grifolin promoted the ability of p53 to bind *dapk1 in vivo*, chromatin immunoprecipitation (ChIP) followed by PCR analysis was performed. CNE1 cells were grown for 24 h in the presence of grifolin or not. Chromatin was immunoprecipitated using p53-specific polyclonal antibody, normal Mouse IgG as negative control and anti-RNA Polymerase II as positive control, then *dapk1* promoter region containing the p53-binding sites was amplified by PCR. Results revealed an increased recruitment of p53 to *dapk1* after grifolin treatment compared with the control, thereby proving that grifolin promoted p53 binding to *dapk1 in vivo* (Fig. 4A). As controls, ChIP assays with GAPDH promoters revealed specificity of p53 protein binding to *dapk1* gene, in that p53 was not associated with the GAPDH gene promoters (Fig. 4B).

### 3.5. *DAPK1 mediates the ability of grifolin to induce apoptosis*

In our previous studies, the result showed that treatment at different concentrations of grifolin resulted in a dramatic increase of apoptosis.[8] Next we tested whether DAPK1 mediated the ability of grifolin to induce apoptosis. We observed 51.96% of the cells to be apoptotic when treated with grifolin for 48 h. Introduction of siRNA against DAPK1 into CNE1 cells resulted in a ~2.3-fold reduction in grifolin-induced apoptosis (22.92%) as compared with the siRNA control (51.03%) (Fig. 5A).

We have demonstrated that the catalytic activity of caspase-3 was rapidly elevated after grifolin treatment in previous studies.[8] To further identify whether DAPK1 mediated the activation of caspase induced by grifolin, we transfected DAPK1 siRNA to CNE1 cells in the presence of 40 μM grifolin and observed that the caspase activity was reversed by more than 70% (Fig. 5B). These findings allowed us to conclude the involvement of DAPK1 in grifolin-induced caspase activation in CNE1 cells.

Under the same conditions, cell lysates were analysed for DAPK1. DAPK1 siRNA transfection abolished basal as well as the grifolin induced upregulation of DAPK1, consistent with the observed reversal in grifolin-induced apoptosis and caspase-3 activity. Cells transfected with scrambled siRNA did

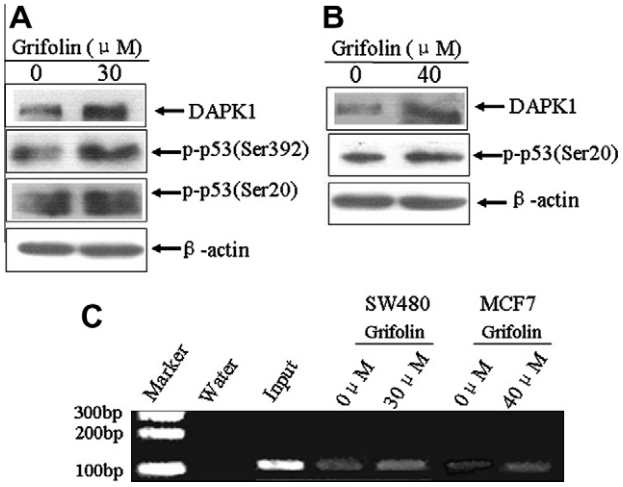


**Fig. 6 – Activation of p53-DAPK1 pathway by grifolin in SW480 (A) and MCF7 (B) cell line. Cells were cultured and treated in the presence of grifolin or not for 24 h as indicated in the figure. At the end of treatments, total cell lysates were prepared and subjected to SDS–PAGE followed by Western blot. Membranes were probed with antibody against DAPK1, p-p53(Ser392), p-p53(ser20), respectively. β-Actin served as a normalisation control. (C) Induction of p53 DNA binding activity by grifolin *in vivo* using ChIP assays in SW480 and MCF7 cell line. ChIP assays of p53 DNA-binding activity in the presence of grifolin or not were performed as described in materials and methods. A mouse monoclonal antibody against p53(sc-126) was used. PCR analysis using dapk1 primers 5′-TACC AGGAAGGGGTGACGGAA-3′, 5′-CTGTGCACAGTAACTGCAC TGT-3′ was shown using DNA after ChIP.**

not affect apoptotic effect, caspase-3 activity or DAPK1 level induced by grifolin (Fig. 5C). Taken together, these findings clearly suggest that upregulation of DAPK1 be an essential event for grifolin-induced apoptosis in CNE1 cells.

### 3.6. *Grifolin upregulates DAPK1 to induce apoptotic effect in tumour cells derived from human breast cancer and human colon cancer*

As we observed in the above results that grifolin upregulated DAPK1 via p53 in CNE1 cells, additional studies were also performed to evaluate whether observed effects of grifolin are applicable for other tumour types.

In our previous studies, we found grifolin induced apoptotic effect also in human breast cancer cell MCF7 and human colon cancer cell SW480.[8] Treatment of SW480 cells for 24 h with grifolin at 30 μM dose resulted in a marked increase both on DAPK1 protein and the Ser392 and Ser20 phosphorylation level of p53 as compared with untreated control (Fig. 6A). In MCF7 cells, DAPK1 protein expression as well as Ser20 phosphorylation level of p53 was induced by grifolin at 40 μM dose compared with untreated control (Fig. 6B). ChIP assays were further used to assess whether grifolin promoted the binding ability of p53 to DAPK1 promoter region. The results showed an increased recruitment of p53 to dapk1 promoter region after grifolin treatment compared with the control both in SW480 and MCF7 cells (Fig. 6C). So we confirmed that similar to CNE1 cells, grifolin up-regulates DAPK1 via p53 pathway in SW480 and MCF7 cells. To evaluate whether DAPK1 mediates the apoptotic effect induced by grifolin, we then introduced DAPK1 siRNA to SW480 and MCF7 cells for 24 h followed by an additional 48 h treatment with grifolin at 40 μM dose, then detected by FACS, respectively. We observed that in SW480 cells 37.79% of the cells were apoptotic when treated with grifolin for 48 h. Introduction of siRNA against DAPK1 resulted in a reduction in grifolin-induced apoptosis (22.51%) as compared with the siRNA control (47.93%) (Fig. 7A). In MCF7 cells, introduction of DAPK1 siRNA resulted in reduction in grifolin-induced apoptosis (14.79%), as compared with the siRNA control (63.72%) (Fig. 7B).

### 3.7. *Grifolin-induced cellular apoptosis is mediated by p53–DAPK1 pathway*

To further determine functions for p53–DAPK1 pathway in grifolin induced apoptosis, p53-null H1299 lung cancer cell line was used. We first examined the p53 and DAPK1 protein levels in H1299p53$^{-/-}$ cells and the results demonstrated that p53 protein was lost and DAPK1 expression was too weak to be detectable. p53-wildtype A549 cells were taken as positive control (Fig. 8A). Then p53 or DAPK1 expression was re-introduced into H1299p53$^{-/-}$ cells. By flow cytometric analysis we observed that grifolin only induced 4.67% population of cells to be apoptotic in H1299p53$^{-/-}$ cells when compared to the control (4.54%). Re-introduction of p53 or DAPK1 resulted in 4.67% and 5.57% of the cells to be apoptotic, respectively. Re-introduction of p53, DAPK1 or co-transfection of p53 and DAPK1 expression vectors and subsequent treatment with grifolin for 48 h showed enhancement of apoptosis by 1.8-, 3.9- and 5.5-folds, respectively, when compared to grifolin-induced apoptosis

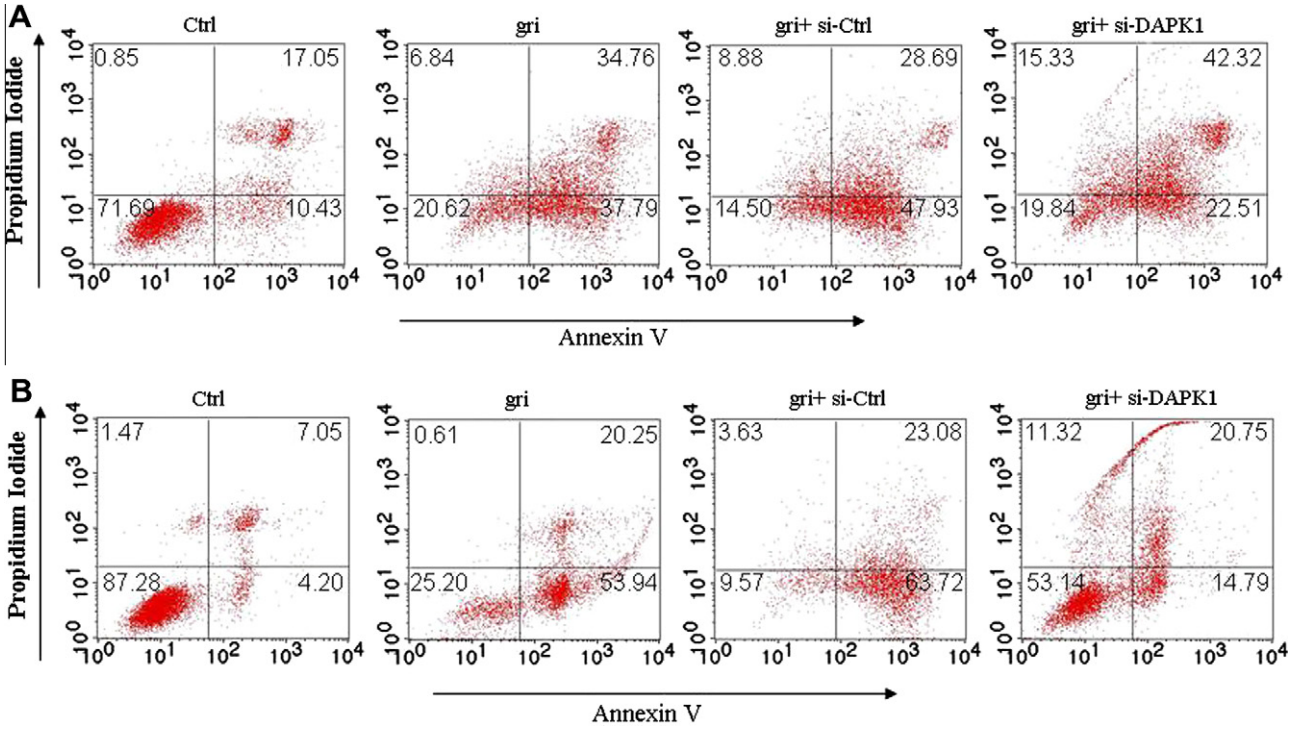


**Fig. 7 – Grifolin-induced apoptosis in SW480 and MCF7 cells are DAPK1 dependent. Percent live cells were measured in SW480 (A) and MCF7 (B) cells transfected with scrambled(si-Ctrl) or DAPK1 siRNA(si-DAPK1), treated with 40 μM of grifolin as indicated in the figure. After 48 h, cells were harvested and suspended in binding buffer with annexin V-FITC and propidium iodide, followed by flow cytometry to assess cell apoptosis.**

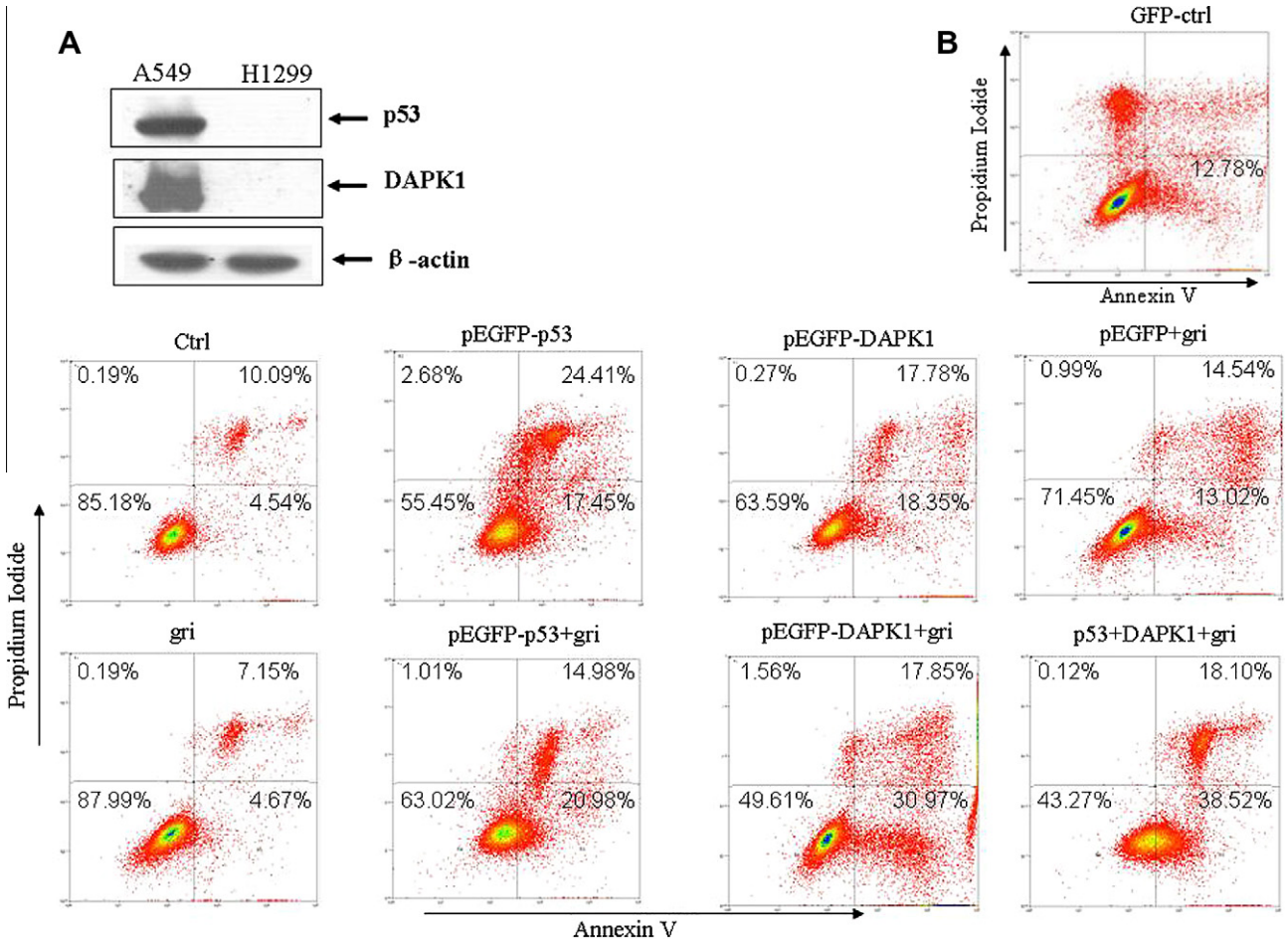


**Fig. 8 – Function of p53-DAPK1 pathway in grifolin- induced apoptosis. (A) Total cell lysates of H1299p53$^{-/-}$ cells were prepared and subjected to SDS–PAGE followed by Western blots analysis with antibodies against p53 and DAPK1, respectively. A549p53$^{+/+}$ cells were taken as positive control. (B) Percent live cells were measured in H1299p53$^{-/-}$ cells transfected with p53 or DAPK1 expression vector, treated with 40 μM of grifolin as indicated in the figure. After 48 h, cells were harvested and suspended in binding buffer with annexin V-FITC and propidium iodide, followed by flow cytometry to assess cell apoptosis. GFP-ctrl, cells transfected with pEGFP vector labelled with GFP, only suspended with propidium iodide without adding FITC are taken as green fluorescence background to be subtracted in FACS analysis. Ctrl, control; gri, cells were only treated with 40 μM of grifolin; pEGFP-p53, cells were only transfected with pEGFP-p53 expression vector; pEGFP-DAPK1, cells were only transfected with pEGFP-DAPK1 expression vector; pEGFP-p53+gri, cells were transfected with pEGFP-p53 expression vector, followed by grifolin treatment for additional 48 h; pEGFP-DAPK1+gri, cells were transfected with pEGFP-DAPK1 expression vector, followed by grifolin treatment for additional 48 h; pEGFP+gri, cells were transfected with pEGFP vector, followed by grifolin treatment for an additional 48 h; p53+DAPK1+gri, cells were co-transfected with p53 and DAPK1 expression vector, followed by grifolin treatment for an additional 48 h.**

(4.67%) in H1299p53$^{-/-}$ cells (Fig. 8B). The above results further confirmed the important role of p53–DAPK1 pathway in grifolin induced apoptosis in tumour cells.

## 4. Discussion

The elucidation of molecular and cellular targets critical in cancer development and prevention is an area of intensive research and is driving the development of novel small-molecule compounds, which may prevent carcinogenesis, curtail its progression, or even cure the disease.[27] DAPK1 is an apoptotic positive-mediator. DAPK1 acts as a tumour suppressor largely because of its ability to sensitise cells to many of the apoptotic signals including those generated by death receptors, cytokines, matrix detachment and oncogene-induced hyperproliferation that are encountered as a cell undergoes tumourigenesis.[28] Extensive data in human primary tumours are accumulating showing a significant loss of DAPK1 expression in a large variety of tumour types.[29,30] A recent study also showed loss of DAPK1 expression in NPC, which could be associated with its promoter region methylation.[31] Grifolin isolated from the mushroom *Albatrellus confluens*, has displayed various pharmacological and microbiological effects. Recent evidence was provided that grifolin also possess antioxidative and antitumour activity.[6–10]

Our results confirmed that grifolin upregulated DAPK1 mRNA as well as protein expression in a dose-dependent manner in NPC cell, which suggested that grifolin promoted

DAPK1 expression on transcriptional level in NPC. DAPK1 mRNA levels were demonstrated to increase in a p53-dependent manner in various cellular settings.[19,32] Transcription factor binding-site prediction also suggested that p53 might bind the promoter of *dapk1* gene. Although there are over 12 phosphorylation sites on p53 that alter transactivation, nuclear shuttling and degradation, only two known biochemical activities of p53 are directly affected: sequence-specific DNA binding and p300 co-activator stability.[24] General consensus remains that the phosphorylation of N-terminal regulatory sites of p53 occurs rapidly in response to various stress stimuli to activate p53.Reduced levels of Ser20 phosphorylation could attenuate p53 activity as a transcription factor.[24,32] It is currently believed that both the core DNA-binding domain and the C-terminal domain of p53 possess DNA-binding activities, in that the former primarily provides sequence specificity whereas the latter recognises structural features of target DNA. We observed that grifolin promoted the phosphorylation of p53 at Ser392 and Ser20, while it had little effect on the phosphorylation of Ser15 or Thr81 as well as the total p53 protein level. Using EMSA, we found grifolin significantly increased the binding activity of p53 to *dapk1* gene *in vitro*. ChIP assays of the p53–*dapk1* promoter complex confirmed that endogenous p53 interacts with this region *in vivo*.

In previous studies, we found that caspase-3 activities were rapidly elevated after the grifolin treatment. Here, we introduced siRNA targeting DAPK1 and scrambled siRNA as a control into CNE1. Depletion of DAPK1 reduced the apoptotic effect as well as the activation of caspase-3 induced by grifolin. Grifolin induced upregulation of DAPK1 was also observed in tumour cells derived from human breast cancer and human colon cancer. In SW480 and MCF7 cells, grifolin also upregulated DAPK1 via p53 pathway, and DAPK1 mediates grifolin-induced apoptotic effect. Reintroduction of p53 and DAPK1 into p53 null H1299 cells enhanced the apoptotic rate markedly, which further confirmed the function of p53–DAPK1 pathway in grifolin-induced apoptosis. Together, upregulation of DAPK1 expression by grifolin may be an important mechanism contributing to its ability to induce apoptotic effect in tumour cells. Since high-frequency loss of DAPK1 expression in a large variety of tumour types, grifolin may rescue pro-apoptotic function of DAPK1 via p53 pathway.

Grifolin, a secondary metabolite from mushroom *Albatrellus confluens*, may represent a promising candidate in the intervention of cancer via targeting DAPK1, and it may also be useful as a reagent to increase understanding of DAPK1 biological function.

## Conflict of interest statement

None declared.

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