

Wogonin induced apoptosis in human nasopharyngeal carcinoma cells by targeting GSK-3 β and Δ Np63

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Abstract

Purpose Wogonin, a plant flavonoid, has antitumor activity in various cancers. Dysregulation of GSK-3 β has been implicated in tumorigenesis and cancer progression. In this study, we investigated the antitumor activity and the mechanistic action of wogonin in human nasopharyngeal carcinoma (NPC) cells.

Methods The effects of wogonin on the cell survival and apoptosis in NPC cells were investigated by MTS assay, flow cytometry, and PARP cleavage assays. Pharmacological inhibitors (BIO, LiCl, and OA), or small interfering RNA (siRNA) were used to address the expression status of GSK-3 β and the anticancer effect of Δ Np63 in NPC cells.

Results Wogonin was shown to induce dose-dependent cell apoptosis due to the induction of sub-G1-phase cells, PARP cleavage, and downregulation of Δ Np63, a survival factor in NPC cells. Strikingly, the apoptotic effect of wogonin involved GSK-3 β inactivation via prominent inhibition of phosphorylation at Tyr216 and slightly increment of phosphorylation at Ser9, while there is no

change in total GSK-3 β proteins. Dysregulation of GSK-3 β caused cell apoptosis was confirmed by pharmacological inhibitors (lithium chloroid, LiCl, and 6-bro-moindirubin-3-oxime, BIO). Administration of okadaic acid (OA, a protein phosphatase inhibitor) that significantly inactivated GSK-3 β also induced Δ Np63 downregulation and apoptosis. Targeted silencing of Δ Np63 repressed the phosphorylation of GSK-3 β at Tyr216 and sensitized NPC cells to wogonin-induced apoptosis. Furthermore, GSK-3 β or PP2A inhibitors enhanced wogonin-induced apoptosis via activation of caspase 3/7.

Conclusion These results indicate that GSK-3 β , as well as Δ Np63, are novel targets for wogonin action and suggest that wogonin might provide a potential therapeutic option in NPC. Further in vitro and in vivo studies will help to clarify the therapeutic role of wogonin in NPC.

Keywords Wogonin · GSK-3 β · Δ Np63 · Apoptosis

Introduction

Nasopharyngeal carcinoma (NPC), a squamous cell carcinoma, is most commonly diagnosed cancer in southern Asia [1]. While environmental factors and genetic susceptibility play important roles in NPC pathogenesis, the Epstein–Barr virus in particular has been implicated in the molecular abnormalities leading to NPC [2]. Δ Np63 has been shown to be the predominant form of p63 expressed in squamous cell carcinomas, including NPC cells [3, 4]. Overexpression of a Δ Np63 isoform in Rat-1A cells that derived from spontaneously immortalized rat embryo fibroblast increases colony growth in soft agar and xenograft tumor formation in nude mice, suggesting an oncogenic role [5]. Protein phosphate 2A (PP2A) was shown to

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tether GSK-3 β and induce the ability of GSK-3 β to phosphorylate its molecular target, β -catenin [6]. It was shown that Δ Np63 associated with GSK-3 β and PP2A to form a protein complex in the nuclei and led to an increase in intracellular β -catenin, indicating that Δ Np63 would affect the GSK-3 β kinase activity [6]. Moreover, it was shown that inhibition of GSK-3 β might regulate Δ Np63 gene expression through β -catenin signaling pathway in a human embryonic kidney cell (HEK 293T) [7]. These studies suggested there is a closely regulated relationship between GSK-3 β and Δ Np63. In our previous studies, overexpression of Δ Np63 has shown to play an anti-apoptotic role in NPC cells [8, 9]. However, the role of GSK-3 β in NPC is still uninvestigated.

Glycogen synthase kinase 3 β (GSK-3 β) is a multifunctional serine/threonine kinase, which was first identified as a critical mediator in glycogen metabolism [10]. Dysregulation of GSK-3 β activity has been implicated in the regulation of cell fate, protein synthesis, cell mobility, proliferation, and apoptosis [11]. GSK-3 β inactivation can exert contrasting effects on cell survival depending on the cell type and the nature of the stimulus [12]. Although it has reported that a proapoptotic role for GSK-3 β activation in triterpenoids- and ceramide-induced cell death [12, 13], disruption of the murine GSK-3 β gene results in embryonic lethality, and mouse embryonic fibroblast derived from these embryos are more sensitive to cell apoptosis [14]. GSK-3 β suppression sensitizes prostate cancer cells to TRAIL-induced caspase-8-mediated apoptosis [15]. Consistent with this finding, GSK-3 β inhibition associates with the cell apoptosis in pancreatic cancer cells, gliomablastoma cells and colorectal cancer [16–20].

Phosphorylation has been shown to regulate the activity of GSK-3 β that positively regulated by phosphorylation at Tyr 216 (p-GSK-3 β (Y216)) and negatively regulated by phosphorylation at Ser 9 (p-GSK-3 β (S9)) [13]. P-GSK-3 β (S9) by kinases such as Akt inhibits its activity and by protein phosphatase such as PP2A activates its activity [12]. PP2A can revert the phosphorylation of P-GSK-3 β (S9) by its phosphatase action [21]. Thus, GSK-3 β has been shown to act downstream of PP2A [12]. Recently, it has been shown that higher expression levels of GSK-3 β and p-GSK-3 β (Y216) were frequently detected in glioblastoma, colorectal cancer cells [19, 20]. Inhibition of GSK-3 β induced the apoptosis and attenuated the cell survival that associated with increased expression of p53 and p21, inhibition of cyclin D1 or NF- κ B expression [19]. In addition to phosphorylation, protein complex formation also has important regulatory influence on GSK-3 β activity, such as Wnt signaling negatively regulates GSK-3 β activity by disrupting a protein complex that brings GSK-3 β into close proximity with its substrate, β -catenin [22].

Wogonin (5,7-dihydroxy-8-methoxyflavanone) is a flavonoid compound derived from the traditional Chinese medicine of Huang-Qin (*Scutellaria radix*) with various therapeutic potential including anti-inflammatory [23], anticancer activities [24–28], and its low toxicity to normal tissues [29, 30]. The anticancer activity has been reported in various tumors recognized as a new source of anticancer drugs and new chemotherapy adjuvant to enhance the efficacy of chemotherapy and to ameliorate the effects of cancer chemotherapies [29, 31]. In this study, the effects of wogonin on the cell survival and apoptosis in NPC cells were investigated. Wogonin, pharmacological inhibitors (BIO, LiCl, and OA) [32], or small interfering RNA (siRNA) were used to address the expression status of GSK-3 β and the anticancer effect of Δ Np63 in NPC cells. Our data demonstrated that GSK-3 β and Δ Np63 might be as a potentially attractive target for human NPC therapy.

Materials and methods

Cell culture and reagents

Human nasopharyngeal carcinoma cell lines NPC-TW076 and NPC-TW039 were isolated from keratinized nasopharyngeal squamous cell carcinoma [33]. The cells were maintained in basal medium (DMEM/F-12 at 1:1 v/v; Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum in a humidified incubator at 37°C under 5% CO₂/95% air. Wogonin (>99% pure) were obtained from Wako Chemical Co. (San Francisco, CA, USA). Most chemicals were obtained from Sigma (St. Louis, MO) unless otherwise indicated. 6-Bromoinirubin-3'-oxime (BIO) was purchased from Calbiochem (San Diego, CA). Antibodies to (ADP-ribose) polymerase-1 (PARP-1), GSK-3 β , and phosphorylated GSK-3 β (Ser9) were purchased from Cell Signaling (Beverly, MA). Antibody to Δ Np63 was purchased from Biolegend (San Diego, CA). Antibody to phosphorylated GSK-3 β (Tyr216) was purchased from GenScript (Piscataway, NJ).

Measurement of cell viability

Briefly, 2×10^4 cells per well were plated in 96-well plates and incubated overnight. The cells were then treated with wogonin or inhibitors at indicated concentrations and time course. Alternatively, cells were transiently transfected with appropriate siRNA and cultured at the indicated time points post-transfection. Cell viability was determined by the colorimetric MTS assay using the CellTiter 96[®] AQueous One Solution Proliferation Assay System from Promega (Madison, WI). After incubation for 2 h at 37°C, the absorbance, which is directly proportional to the

number of viable cells in cultures, was measured at 490 nm using a microplate reader.

The sub-G1 cells distribution analysis

PI staining was used to analyze the DNA content. Cells were plated in 35-mm dishes at concentrations determined to yield 60–70% confluence within 24 h. Cells were then treated with wogonin (50 μ M) for 24 h. Both the adherent and floating cells were harvested, and the cells were resuspended in PBS, fixed with 70% ethanol, labeled with propidium iodide (PI, 0.05 mg/ml) and incubated at room temperature in the dark for 30 min. DNA content was then analyzed using a FACScan instrument equipped with FACStation running cell Quest software (Becton–Dickinson).

Apoptosis assay

Cell apoptosis was assayed by annexin V–Cy5 and PI staining (BioVision Inc., Mountain View, CA) followed by FACS analysis. The cells were treated with wogonin at the indicated concentrations for 24 h. The cells were pelleted and resuspended in annexin V binding buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.4) containing annexin V–Cy5 (1:1,000) and 1 μ g/ml PI. After incubation at room temperature for 5 min, the cells were analyzed with a FACSCalibur flow cytometer (Becton–Dickinson, USA). The percentage of total apoptotic events was defined as the sum of the cells in the early stage (annexin-V-Cy5 positive/PI negative) or late stage (annexin-V-Cy5 positive/PI positive) of apoptosis as previously described [9].

Preparation of cell lysates and Western blot analysis

The cells were seeded at a 1×10^6 per 100-mm culture dish. The cells were incubated for 24 h and were treated with wogonin or GSK 3 β inhibitors at the desired concentrations. Twenty-four hours after treatment, the cells were washed with ice-cold phosphate-buffered saline and lysed in Mammalian Protein Extraction Reagent (M-PER; Pierce Chemical Co., Rockford, IL). Protein samples (20 μ g per lane) were separated on a 10% SDS–polyacrylamide gel and blotted onto polyvinylidene difluoride membranes (Immobilon(TM)-P, Millipore, Bedford, MA), blocked in TTBS and probed with primary antibodies overnight at 4°C. The membranes were then incubated with the appropriate horseradish peroxidase–conjugated secondary antibodies (1:2,000). The immunoreactive protein bands were developed by Enhanced Chemiluminescence (ECL) (Amersham Pharmacia Biotech, Freiburg, Germany).

siRNA transfection

To explore the function of Δ Np63 or GSK-3 β in NPC cells, small interfering RNA (siRNA) was used to silence their expressions. The siRNA targeting Δ Np63 mRNA was designed and synthesized by Dharmacon Research Inc. (Lafayette, CO, USA). The siRNA sequence for Δ Np63 targeting was 5'ACAAUGCCCAGACUCAUU3'. The GSK-3 β siRNA and non-targeting siRNA was purchased from Cell Signaling Technology (Beverly, MA, USA; 5'-CUACUCCUGAAAACAACGTT). The transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The effectiveness of siRNA silencing was assayed by Western blot analysis using anti- Δ Np63 or anti-GSK-3 β antibody.

Luminescence-based caspase-3/7 activity assay

Active caspase 3 was assayed with the caspase 3/7-GLO assay (Promega, Madison, WI) according to the manufacturer's instructions. Cells were plated in triplicate at 2×10^3 per well in white-walled 96-well plates (Becton–Dickinson). Cells were pretreated with inhibitor as described above. Caspase-3/7 activity was measured at 24-h treatment with wogonin. Caspase-Glo 3/7 assay uses a caspase-3/7 tetrapeptide DEVD substrate that produces a luminescent signal on cleavage. Relative light units were measured on an Lmax Microplate Luminometer (Molecular Devices). Experiments were performed in triplicate. Means and standard deviation for 3 independent experiments were shown. For comparison of caspase activity, we designated the level of caspase 3 activity in negative control cells as 1 and expressed the level of caspase 3 activity in the experimental sample relative to the negative control.

Statistical analyses

Data are presented as means \pm SEM. The statistical differences were determined using Student–Newman–Keuls Test and Dunn's Test (Sigma Stat Software Program, Jandel Scientific, San Rafael, CA). A *P* value of 0.05 or less was considered as significant.

Results

Wogonin inhibits cell growth and blocks cell cycle progression

To examine the possible effect of wogonin on the cell viability in human NPC cells, including NPC-TW076 and

NPC-TW039 cells, with colorimetric MTS assay. Cell viability was dose-dependently reduced in both NPC-TW076 and NPC-TW039 cells after wogonin treatment as indicated concentrations for 24 h (Fig. 1a). An IC₅₀ value of NPC-TW076 and NPC-TW039 cells were 34.6 and 30.5 μ M, respectively. Next, to determine whether this reduction of cell viability by wogonin was related to the cell apoptosis, the percentage of sub-diploid cells was detected by flow cytometry. Exposure to wogonin (50 μ M) for 24 h induced 21.5 and 26.6% of the sub-G1 fraction in NPC-TW076 and NPC-TW039 cells (Fig. 1b). The data indicate that wogonin treatment induced apoptosis of both NPC cell lines.

Wogonin induces the downregulation of Δ Np63 and cell apoptosis

Δ Np63 overexpression promotes the survival and enhances oncogenic growth of NPC cells [8, 9]. To verify whether downregulation of Δ Np63 was responsible for wogonin induction of NPC cell apoptosis, Δ Np63 expression, and poly(ADP-ribose) polymerase (PARP) cleavage were detected in wogonin-treated cell lysates by Western blot. As shown in Fig. 2a, wogonin induced the reduction of Δ Np63 as well as the induction of PARP cleavage was in a dose-dependent manner. Increased cell apoptosis was further confirmed by PI and annexin V staining by analysis of flow cytometry. Figure 2b shows the percentage of annexin V-cy5 binding both NPC cell lines markedly increased

after treatment with wogonin at concentrations of 25 and 50 μ M for 24 h in both cell lines. These data suggest that an association between Δ Np63 downregulation and apoptotic induction.

Wogonin inhibits the GSK-3 β activation

GSK-3 β is considered as an active regulator of oncogenic pathways in human cancers as a tumor promoter or a tumor suppressor [20]. To determine whether GSK-3 β activation was involved in wogonin-inhibited Δ Np63 expression, the effects of wogonin on the protein level, and phosphorylation status of GSK-3 β were examined. GSK-3 β activation was measured after NPC cells treated with wogonin (25 and 50 μ M) for 24 h. Upon wogonin treatment, the total protein of GSK-3 β was unchanged, while its phosphorylation status was obviously affected. Wogonin not only increased p-GSK-3 β (S9) but also dramatically decreased p-GSK-3 β (Y216) (Fig. 3a). Next, to confirm that the activity of GSK-3 β was affected, the Ser37/41 phosphorylation level of β -catenin, a marker of GSK-3 β activity, was checked. Figure 3b shows that wogonin caused a dose-dependent dephosphorylation of β -catenin, suggesting a functional blockage of GSK-3 β .

GSK-3 β inhibitors induce cell apoptosis

NPC-TW076 and NPC-TW039 cells are isolated from keratinized nasopharyngeal squamous cell carcinoma from

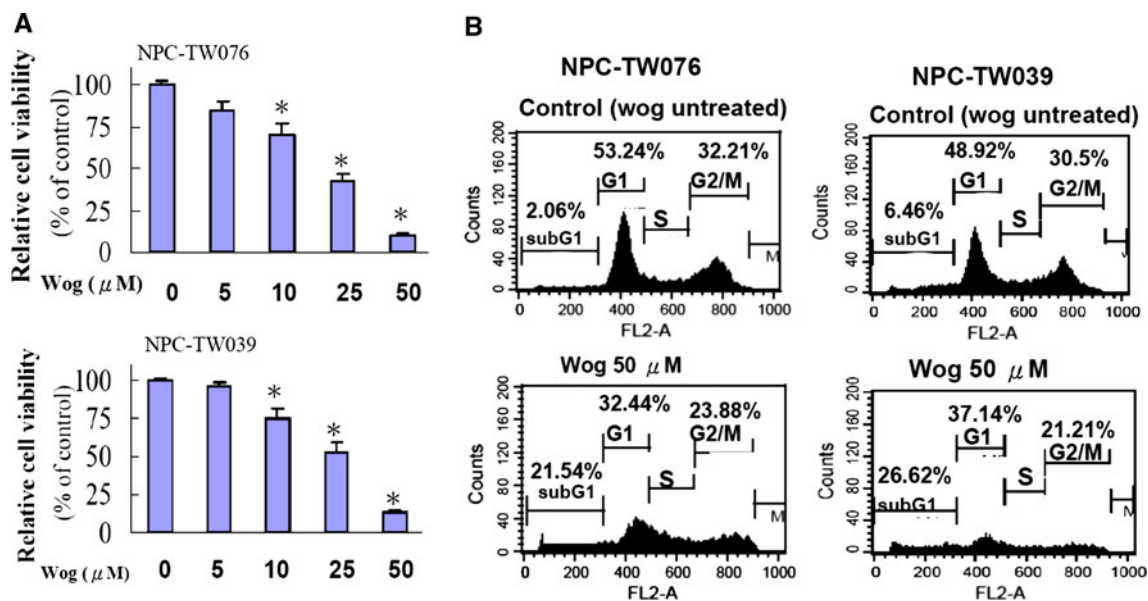
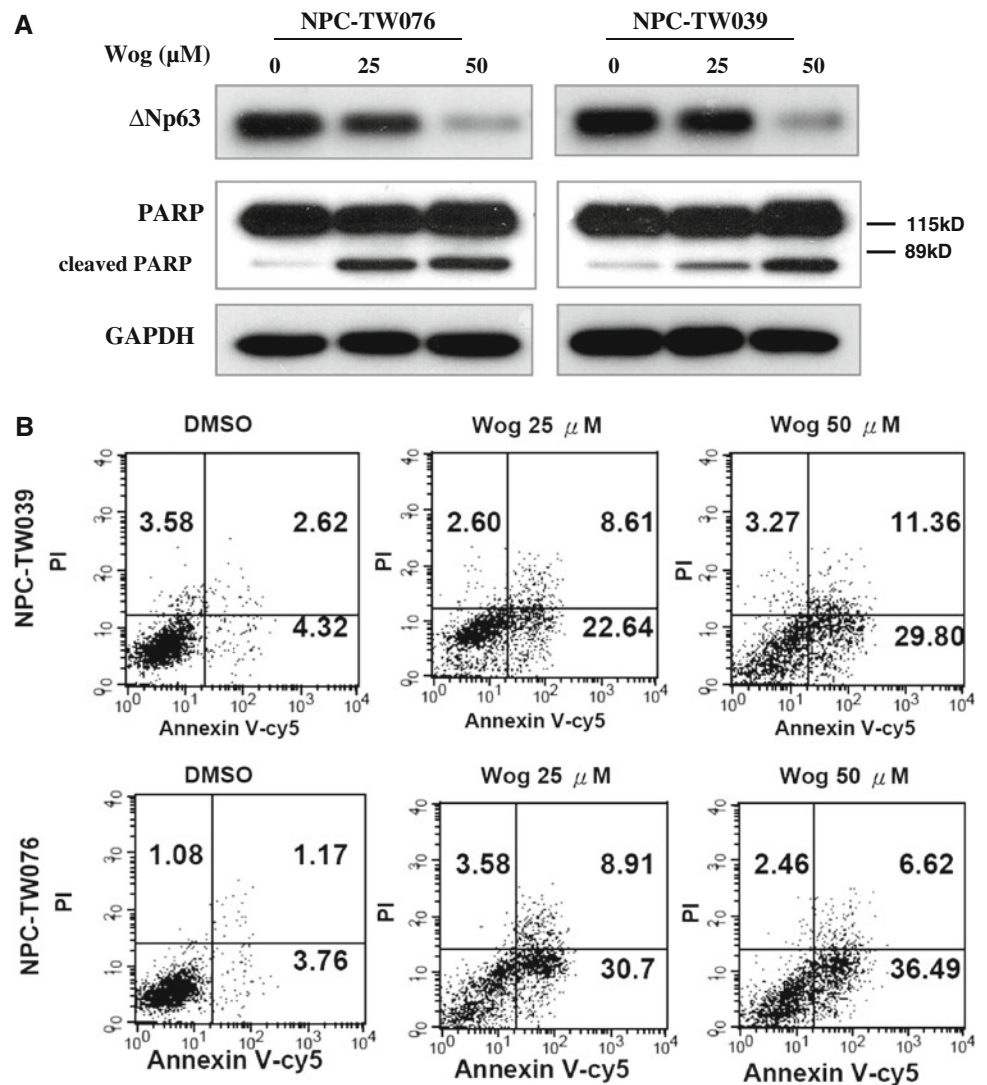


Fig. 1 Wogonin inhibited cell survival and increased the sub-G1 cell distribution. NPC cells were treated with or without wogonin (as indicated concentrations) for 24 h. **a** Cell viability was assessed by MTS assay. Data from each experimental group were compared to the control (drug-untreated) group by analysis of variance. * $P < 0.05$.

b Cell cycle analysis of wogonin-treated cells using FACScan flow cytometer. The x and y axes indicate fluorescent intensity and count, respectively. The data are a representative of three independent experiments

Fig. 2 Wogonin induced cell apoptosis. Cells were treated with the indicated concentrations of wogonin for 24 h, and the apoptotic status was assayed. **a** The apoptotic cells were assessed by Western blot. Cell lysates were subjected to immunoblotting for Δ Np63 and PARP/cleaved PARP. Immunoblots for GAPDH were shown as loading controls. The blots shown are representative of three independent experiments. **b** The apoptotic cells were assessed by annexin V–Cy5/PI double-staining. The data, shown as % of total cells, are a representative of three independent experiments



different patients [33]. It shows no detectable Epstein–Barr virus DNA sequence and the doubling time is 10.5 and 10.8 h, respectively. Since the similar effects of wogonin on inducing cell apoptosis and GSK-3 β inactivation in both cell lines, NPC-TW076 cell was used to examine the following molecular events. To determine whether GSK-3 β inactivation was responsible for Δ Np63 downregulation and cellular apoptosis, the well-known GSK-3 β pharmacological inhibitors, lithium chloride (LiCl), and synthetic, cell-permeable derivative 6-bromoindirubin-3-oxime (BIO), were applied. NPC-TW076 cell was treated with LiCl or BIO for 2 h prior to treatment with or without wogonin for 24 h, and subsequently, the cell viability and apoptotic effect were measured. Importantly, LiCl and BIO induced viability suppression (Fig. 4a) and cell apoptosis (Fig. 4b). The effects of BIO and LiCl on the phosphorylation status of GSK-3 β were further investigated by Western blotting (Fig. 4c). Treatment with LiCl (30 mM) increased p-GSK-3 β (S9), with no significant effect on the

relative phosphorylation level of GSK-3 β at Tyr216. Conversely, BIO (2 μ M) significantly reduced the phosphorylation level of GSK-3 β at Tyr216 and was no obvious increase in p-GSK-3 β (S9) (Fig. 4c). Co-treatment with wogonin did not change the GSK-3 β phosphorylation status of BIO treatment, but decreased the relative phosphorylation level of GSK-3 β at Tyr216, which was unchanged by LiCl treatment alone. The apoptotic effect of BIO and LiCl were further investigated by Western blotting (Fig. 4d). BIO (2 and 5 μ M) as well as LiCl (10 and 30 mM) had a dose-dependent reduction in Δ Np63 expression and increment in PARP cleavage. These data suggest that inactivation of GSK-3 β was involved in cell apoptosis.

Inactivated GSK-3 β by OA induces cell apoptosis

GSK-3 β is shown to act a downstream molecule of PP2A [12]. PP2A induces Ser9 dephosphorylation of GSK-3 β and activates it [21]. To examine whether wogonin-induced

Fig. 3 Wogonin changed the phosphorylation status of GSK-3 β . NPC cells were treated with wogonin at the 25 and 50 μ M for 24 h. **a** The cell viability was assessed by MTS assay. **b** The cell lysates were separated by 10% SDS–polyacrylamide gel electrophoresis and the level of Δ Np63 protein, the fractions of p-GSK-3 β (S9), p-GSK-3 β (Y216), and total GSK-3 β were detected by Western blot. **b** GSK-3 β inactivation was detected by the expression of p- β -catenin and β -catenin. The blots shown are representative of three independent experiments. GAPDH was shown as a loading control

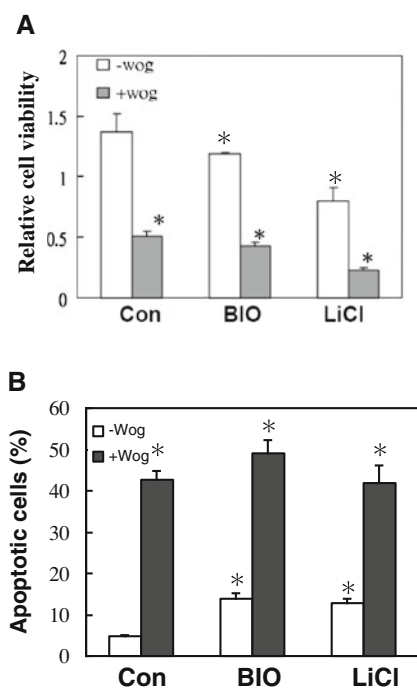
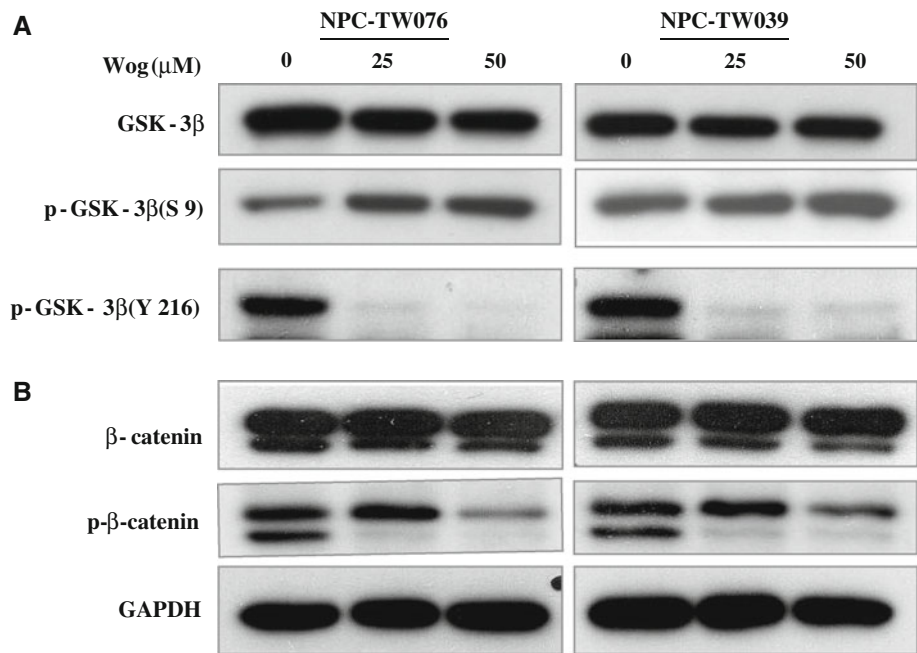
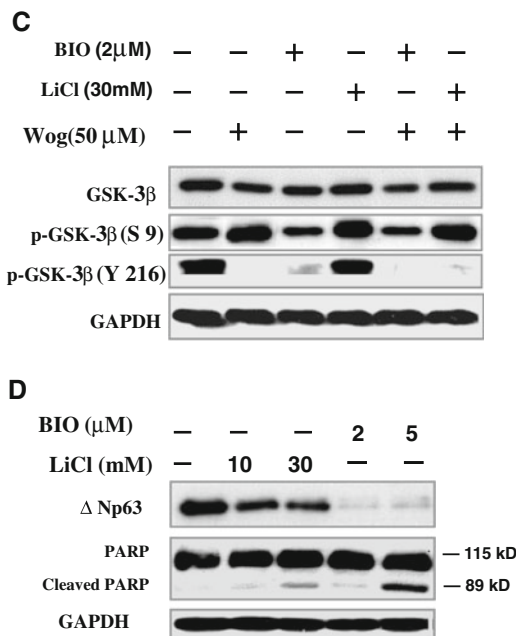


Fig. 4 Pharmacological inhibitors of GSK-3 β -induced cell apoptosis. **a** NPC-TW076 cell was treated with LiCl (30 mM) or BIO (2 μ M) for 2 h prior to treatment with or without wogonin (50 μ M) for 24 h. **a** Cell viability was assessed by MTS assay. Data from each experimental group were compared to the wogonin-untreated control group by analysis of variance. $*P < 0.05$. **b** NPC cells were harvested and double stained with annexin V–Cy5/PI and analyzed by flow cytometry. The percentage of the apoptotic cells is bar graphed as

inhibition of GSK-3 β is via PP signaling, okadaic acid (OA, 5 and 10 nM), a protein phosphatases inhibitor, was used to estimate on the basis of presence of the



mean \pm SEM ($n = 3$). Data from each experimental group were compared to the wogonin-untreated control group by analysis of variance. $*P < 0.05$. **c** The expression level of p-GSK-3 β (S9), p-GSK-3 β (Y216), and total GSK-3 β was detected by Western blot. **d** NPC-TW076 cell was treated with LiCl (10 and 30 mM) or BIO (2 and 5 μ M) for 24 h. The cell lysates were prepared for detected the apoptotic event that was measured by Δ Np63, PARP/cleaved PARP expression. The blots shown are representative of three independent experiments

phosphorylated forms of GSK-3 β in NPC-TW076 cells. As shown in Fig. 5, OA dose dependently reduced Δ Np63 expression and GSK-3 β inactivation via significantly

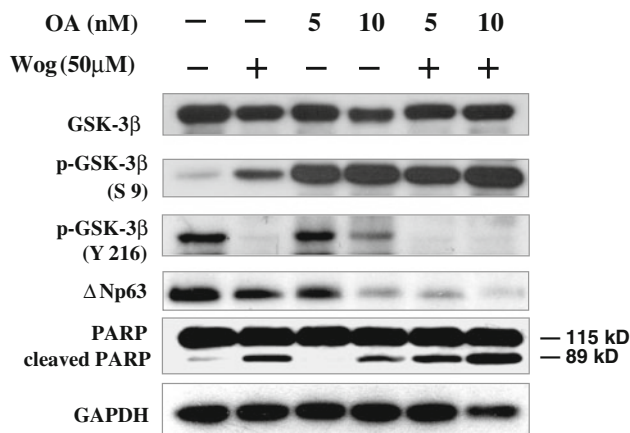


Fig. 5 OA inactivated GSK-3β led to ΔNp63-mediated cell apoptosis. NPC-TW076 cell was treated with OA (5 and 10 nM) for 2 h prior to incubation with or without wogonin (50 μM) for 24 h. Cell lysates were prepared, and the fractions of p-GSK-3β(S9), p-GSK-3β(Y216), and total GSK-3β were detected by Western blot. The apoptotic event was measured by ΔNp63 and PARP/cleaved PARP expression. The blots shown are representative of three independent experiments

phosphorylation at Ser9 and slightly dephosphorylation at Tyr216. The phosphorylation status of GSK-3β induced by OA was different from the state by wogonin. However,

OA-inhibited PP resulted in GSK-3β inactivation and led to cell apoptosis shown by PARP cleavage and ΔNp63 down-regulation. OA was also shown to enhance the wogonin-induced apoptotic events. These data clearly indicate that wogonin-inactivated GSK-3β was PP-independent and suggest that inactivation of GSK-3β might have partially contributed to induction of cell apoptosis.

GSK-3β activation is regulated by the level of ΔNp63 expression

To verify the regulation of GSK-3β associated with ΔNp63 in NPC cells, we determined the ΔNp63 expression in GSK-3β siRNA-transfected cells or the GSK-3β phosphorylation status in ΔNp63 siRNA-transfected cells. To our surprise, knockdown of GSK-3β with specific siRNA did not interfere with the expression of ΔNp63 (Fig. 6a), while ΔNp63 siRNA treatment reduced endogenous ΔNp63 expression that resulted in reduction of p-GSK-3β(Y216) (Fig. 6b). Further, ΔNp63 siRNA transfection enhanced the extent of wogonin-inhibited p-GSK-3β(Y216) (Fig. 6c). These data indicate that ΔNp63 expression level might regulate GSK-3β in NPC cells.

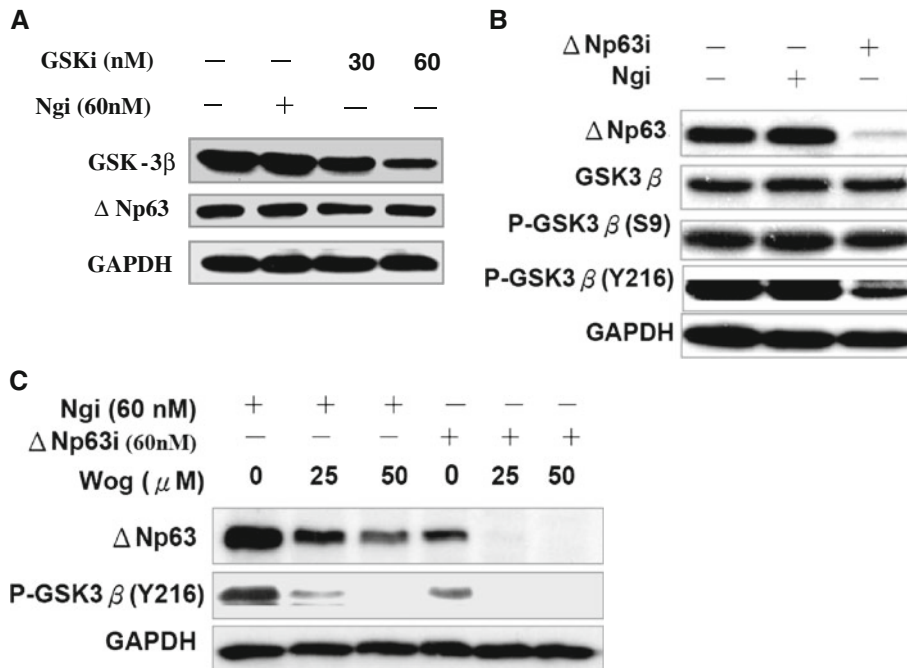


Fig. 6 ΔNp63 regulated the phosphorylation status of GSK-3β. **a** NPC-TW076 cell was transfected with control siRNA or GSK-3β siRNA (30 and 60 nM) for 48 h, the cell lysates were prepared, and the expression level of GSK-3β and ΔNp63 was investigated by Western blot. **b** ΔNp63 protein level regulated the phosphorylation status of GSK-3β. NPC-TW076 cell was transfected with control siRNA or ΔNp63 siRNA for 48 h. Cell lysates were prepared, and the expression level of ΔNp63, p-GSK-3β(S9), p-GSK-3β(Y216), and

total GSK-3β protein was detected by Western blot. **c** Target inhibition of ΔNp63 enhanced the effect of wogonin on GSK-3β. NPC-TW076 cell was transfected with control siRNA or ΔNp63 siRNA for 48 h and incubated with or without wogonin (25 and 50 μM) for 24 h. The expression level of ΔNp63 and p-GSK-3(Y216) was detected by Western blot. GAPDH was shown as a loading control. The blots shown are representative of a pattern of three independent

Silencing of Δ Np63 with siRNA sensitized NPC cells to wogonin

To further validate the biological significance of the Δ Np63 downregulation involved in wogonin-induced cell apoptosis, NPC cells were transfected with Δ Np63 siRNA for 48 h, and then Δ Np63 expression and cell apoptosis were analyzed. As shown in Fig 7a, targeted inhibition of Δ Np63 with siRNA effectively reduced the expression of Δ Np63, and this effect was enhanced by co-treatment with wogonin. Additionally, silence of Δ Np63 also enhanced wogonin-induced apoptosis evaluated by flow cytometry of annexin-V-stained cells (Fig. 7b), implying that administration of Δ Np63 siRNA facilitated NPC cells to wogonin induction of apoptosis. The data demonstrate that wogonin-induced Δ Np63 downregulation resulted in cell apoptosis.

Wogonin induces the activation of caspase 3/7

To examine whether pharmacological inhibitors enhanced wogonin-induced apoptosis via activation of caspase 3/7, NPC-TW076 cell was treated with BIO, LiCl, or OA for 2 h prior to treatment with or without wogonin (50 μ M) for 24 h, then cell was subjected to analyze the caspase 3/7 activity using the caspase 3/7 Glo assay. As shown in Fig. 8, wogonin and GSK-3 β inhibitors treatment alone

increased caspase 3/7 activity about 0.2 ~ 0.8-fold compared with control group (wogonin-untreated group). Pre-treatment cells with the inhibitors (LiCl, BIO, and OA) for 2 h significantly enhanced the wogonin-induced caspase 3/7 activity (Fig. 8). These inhibitors were shown to enhance the wogonin-induced cell apoptosis in Figs. 4b and 5. Taken together, treatment with wogonin or GSK-3 β inhibitors induced cell apoptosis via activation of caspase 3.

Discussion

Wogonin is an effective drug with antiproliferative and apoptotic activity in human cancer cells, including lung epithelial cancer cells [26], colon cancer cells [27], leukemia cells [24, 28, 34], malignant lymphocytes [30], and breast cancer cells [25]. However, its healing mechanisms are still largely unknown. This study presented molecular mechanisms of wogonin responsible for the apoptotic effects, by inducing Δ Np63 downregulation and GSK-3 β inactivation in NPC cells. We found inactivation of GSK-3 β by its inhibitors (LiCl, BIO, and OA), and wogonin was associated with Δ Np63 downregulation that led to cell apoptosis. Depletion of Δ Np63 protein by siRNA inactivated GSK-3 β via inhibition of phosphorylation at Tyr216

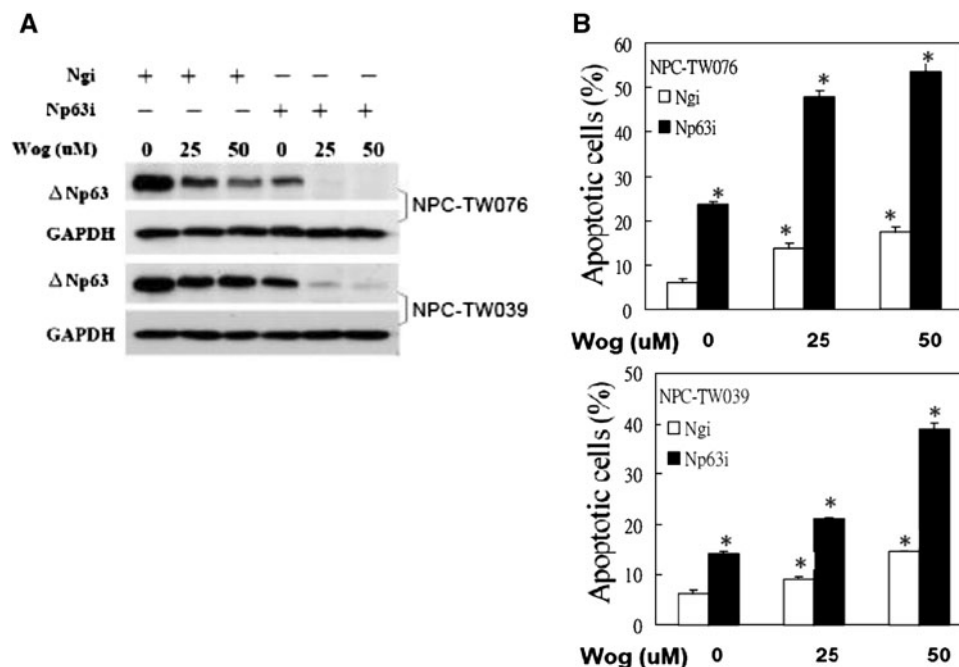


Fig. 7 Silencing of Δ Np63 expression by siRNA enhanced the apoptotic effect of wogonin. NPC-TW076 cell was transfected with control siRNA or Δ Np63 siRNA for 48 h and incubated with or without wogonin (25 and 50 μ M) for 24 h. **a** The cell lysates were prepared, and the expression level of Δ Np63 was shown the efficiency of protein silencing by siRNA. The blots shown are representative of

three independent experiments. GAPDH was shown as a loading control. **b** NPC-TW076 cell was harvested and stained with annexin V-Cy5/PI and analyzed by flow cytometry. The percentage of the apoptotic cells are bar graphed as mean \pm SEM ($n = 3$). Data from each experimental group were compared to the control group by analysis of variance. * $P < 0.05$

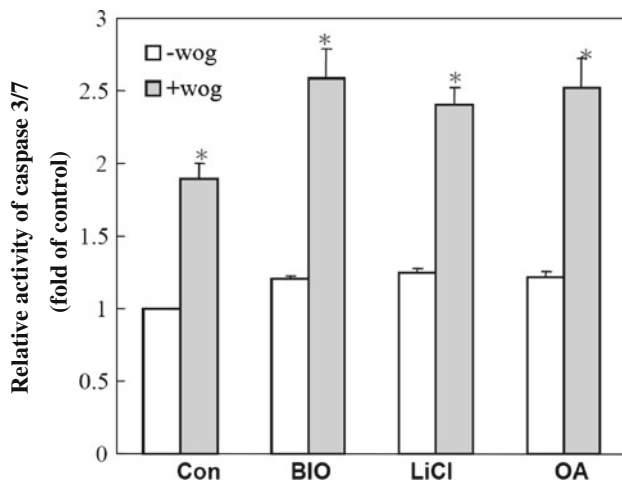


Fig. 8 Wogonin-induced caspase 3/7 activation caused cell apoptosis. NPC-TW076 cell was treated with BIO (2 μ M), LiCl (30 mM), or OA (5 nM) for 2 h prior to treatment with or without wogonin (50 μ M). Caspase 3/7-GLO activity assays were performed at 24 h after treatment. Caspase 3/7 activity of wogonin-untreated control group of each cell line was set as 1. Activities of other inhibitors treatment were compared to that of wogonin-untreated control group. The data are bar graphed as mean \pm SEM ($n = 3$)

and enhanced wogonin-induced cell apoptosis. These data indicate GSK-3 β and Δ Np63 are the molecular targets of wogonin and suggest that wogonin acts as a promising therapeutic option for NPC.

Downregulation of Δ Np63 by siRNA induces NPC cell apoptosis via activation of caspase 3 [9]. The present study indicates that wogonin acts like GSK-3 β inhibitors that induced cell apoptosis. The involvement pathway is shown to induce the GSK-3 β inactivation, Δ Np63 downregulation, caspase 3 activation, and the increment of PARP cleavage. These effects of wogonin might contribute to the increment of the sub-G1 cells and annexin-V-staining cells. Especially, this study also indicates silence of Δ Np63 expression induced the Tyr 216 dephosphorylation of GSK-3 β . The role of GSK-3 β in the regulation of apoptosis is controversial [35]. Recent reports on other cancers, including pancreatic cancer [16], medullary thyroid cancer [36], and glioblastoma cells [19], have shown to inhibit the GSK-3 β kinase activity by inhibitors or genetic depletion of its protein by siRNA that lead to decrease in cancer cell proliferation and survival. Consistent with these studies, this study shows wogonin might exert its apoptotic effect through inhibition of GSK-3 β / Δ Np63 pathway in NPC cells.

GSK-3 β is a dual specificity kinase differentially regulated by Tyr and Ser phosphorylation and correlated with a net inactivation of the enzyme. Subsequent dephosphorylation at Ser 9 residue restores activity, whereas dephosphorylation at Tyr216 residue leads to further inactivation [37]. This study shows wogonin-inactivated GSK-3 β with

phosphorylation or dephosphorylation of GSK-3 β at different residues resulted in cell apoptosis that was evidenced by pharmacological inhibitors (LiCl and BIO). LiCl inhibits GSK-3 β by acting as a competitive inhibitor of Mg^{2+} inducing Ser9 autophosphorylation [38]. In addition, PKA is shown to function as a GSK-3 kinase that, in parallel with PKB, controls the activity of the multifunctional enzyme GSK-3 [39]. Increasing evidence suggests that GSK-3 β activity is increased by Tyr216 phosphorylation [18, 20]. Phosphorylation of GSK-3 β is an intramolecular autophosphorylation event in the cells [40] or by Pyk2, a tyrosine kinases [41]. Dephosphorylation at Tyr216 diminishes its activity [41]. BIO is a potent pharmacological inhibitor of GSK-3 β that inhibits its phosphorylation at Tyr216, a GSK-3 β activation site [42]. With BIO treatment, the apoptosis was evaluated to be associated with dephosphorylation of GSK-3 β at Tyr216 in NPC cells. Thus, wogonin-induced apoptosis like BIO's effect that concomitantly related with significantly dephosphorylation of GSK-3 β at Tyr216. Comparing with LiCl, BIO, OA, and wogonin, this study indicates GSK-3 β inactivation that the absence of phosphorylation at Tyr216 and/or the presence of phosphorylation at Ser9 would play an important role for induction of cancer-specific apoptosis. Thus, wogonin acts like LiCl and BIO displayed remarkable selective inhibition of GSK-3 β [42].

Dephosphorylation Tyr216 or phosphorylation Ser9 of GSK-3 β is critical for the specification of cell fate [11]. Wogonin induced deleterious signals against cancer cell viability and survival that was evidenced by downregulation of Δ Np63. Δ Np63 is frequently overexpressed in NPC cells, and its downregulation exhibits proapoptotic function via increasing the caspase 3 activity and PARP cleavage [4, 8, 9]. NPC cell that treated with LiCl or BIO induced GSK-3 β inactivation, Δ Np63 downregulation, caspase 3/7 activation, and apoptosis. However, this event seems due to the effect of GSK-3 β inactivation (phosphorylation at Ser9 or dephosphorylation at Tyr216) and is not related with the level of GSK-3 β protein. GSK-3 β is shown to regulate Δ Np63 gene expression through β -catenin signaling pathway in HEK 293T cells [7]. However, there was no change on the expression level of Δ Np63 while NPC cell treated with GSK-3 β siRNA. Wogonin treatment also did not cause significantly change on the total protein of GSK-3 β . In addition, the effect of GSK-3 β inactivation induced by OA also responses to the downregulation of Δ Np63. These findings indicate the level of GSK-3 β protein might not involve in the regulation of Δ Np63 gene expression, but a change in the status of phosphorylation might associate with the regulation of Δ Np63 in NPC cells.

An alternative mechanism that could lead to disruption of GSK-3 β activity is a substrate trap mechanism by a complex associated with the PP2A, Δ Np63, and GSK-3 β

[6]. Δ protein is shown to bind to the N terminus of B56 α , a catalytic unit of PP2A and forms a complex with GSK-3 β , leading to the proposal that the expression of Δ Np63 reduces the PP2A-dependent activation of GSK-3 β [6]. However, in this study, we find that target silence of Δ Np63 induced GSK-3 β inactivation via inhibition of Tyr216 phosphorylation in NPC-TW076 cells. Further, knockdown of Δ Np63 in the presence of wogonin was shown to account for the enhancing effect of GSK-3 β inhibition. These findings suggest that Δ Np63 protein expression might regulate the GSK-3 β activation. Δ Np63 expression as well as GSK-3 β activation might play a role in the pathogenesis of NPC cells.

In the present study, the proapoptotic role of wogonin in NPC cells is explored. The signaling mechanisms of wogonin are shown by GSK-3 β inactivation and Δ Np63 downregulation that lead to cell apoptosis. This study also shows there is a closely regulation of GSK-3 β activation associated with the Δ Np63 expression. Thus, wogonin is suggested to have potential therapeutic application in the treatment of NPC cancer.

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