

Research Article

p53 Is a Key Regulator for Osthole-Triggered Cancer Pathogenesis

**Ssu-Ming Huang,^{1,2,3,4} Cheng-Fang Tsai,⁵ Dar-Ren Chen,⁶
Min-Ying Wang,¹ and Wei-Lan Yeh⁷**

¹ Graduate Institute of Biotechnology, National Chung Hsing University, Taichung 402, Taiwan

² Preventive Medicine Center, Department of Community Medicine, Taichung Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Taichung 427, Taiwan

³ Division of Colon and Rectal Surgery, Department of Surgery, Taichung Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Taichung 427, Taiwan

⁴ School of Medicine, Tzu Chi University, Hualien 970, Taiwan

⁵ Department of Biotechnology, Asia University, Taichung 413, Taiwan

⁶ Comprehensive Breast Cancer Center, Changhua Christian Hospital, Changhua 500, Taiwan

⁷ Department of Cell and Tissue Engineering, Changhua Christian Hospital, Changhua 500, Taiwan

Correspondence should be addressed to Wei-Lan Yeh; ibizayeh0816@hotmail.com

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Osthole has been reported to have antitumor activities via the induction of apoptosis and inhibition of cancer cell growth and metastasis. However, the detailed molecular mechanisms underlying the anticancer effects of osthole in human colon cancer remain unclear. In the present study, we have assessed osthole-induced cell death in two different human colon cancer cell lines, HCT116 and SW480. Our results also showed that osthole activated proapoptotic signaling pathways in human colon cancer cells. By using cell culture insert system, osthole reduced cell motility in both human colon cancer cell lines. This study also provides evidence supporting the potential of osthole in p53 activation. Expression of p53, an apoptotic protein, was remarkably upregulated in cells treated with osthole. Importantly, the levels of phosphorylation of p53 on Ser15 (p-p53) and acetylation of p53 on Lys³⁷⁹ (acetyl-p53) were increased under osthole treatment. Our results also demonstrated that p53 was activated followed by generation of reactive oxygen species (ROS) and activation of c-Jun N-terminal kinase (JNK). Our study provides novel insights of p53-mediated responses under osthole treatment. Taken together, we concluded that osthole induces cancer cell death and inhibits migratory activity in a controlled manner and is a promising candidate for antitumor drug development.

1. Introduction

Colon cancer is one of the leading causes of cancer-related deaths worldwide. Surgery can be applied in the early stage, while chemotherapy and/or radiation therapy are used to treat malignant tumors. The metastatic dissemination of primary tumors is linked directly to patients' survival and accounts for about 90% of all colon cancer deaths. It should therefore be obvious that tools and methodologies that allow early cancer detection will affect the survival time and rate of patients. The development of colon cancer is increasing

in recent years; however, the knowledge of treatments is still limited.

The p53 tumor suppressor gene is one of the most commonly mutated genes found in human malignancies [1]. More than 50% of human cancer cells are associated with missense mutations or deletions of p53 [2], which results in chemoresistance of those cancer cells [3]. p53 protein is important in the transcription of growth inhibiting genes, apoptosis, cell cycle arrest, and DNA repair [4]. p53 is also a sequence-specific transcription factor that transactivates p21, which is involved in cell growth regulation [5]. The MAP

kinase family comprises extracellular-signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK), and these signal pathways have been implicated in many physiologic processes, including cell growth and death through p53-dependent or p53-independent mechanisms [6]. Importantly, JNK signal transduction pathway is also activated by some anticancer drugs [7, 8]. JNK1 protein kinase is the major isoform in JNK family proteins that its activation requires phosphorylation on Thr¹⁸³ and Tyr¹⁸⁵ [9]. Dominant-negative mutation of JNK (DN-JNK) is a mutant at these sites (on Thr¹⁸³ and Tyr¹⁸⁵), which effectively suppresses paclitaxel-induced cell apoptosis in cancer cells [10].

Frequent consumption of natural fruits and vegetables has been considered to reduce risk of developing cancers and mortality [11, 12]. Osthole (7-methoxy-8-(3-methyl-2-butenyl)coumarin) is an active constituent isolated from *Cnidium monnieri* (L.) Cusson, which has been shown to exert a wide variety of biological effects such as contractility-based motility of different cells and tissues [13]. Osthole has also been shown to have anti-inflammatory [14], antiosteoporosis [15], and antiseizure [16] effects. In recent years, accumulating evidence also suggests that osthole has antitumor activities that are thought to occur via the induction of apoptosis and inhibition of cancer cell growth and metastasis [17–19].

ROS plays a key role in regulation of biological functions including differentiation and immune responses [20]. ROS is generated through a number of environmental stimuli, and excessive production of ROS causes oxidative stress leading to adverse events like cell death [21, 22]. Previous reports showed that the protective effects of osthole were revealed by reducing ROS production in ischemia-reperfusion injury models [23, 24]. However, the effect of osthole in generation of ROS in cancer cells is still unknown, and the detailed mechanisms underlying the anticancer effects of osthole in human colon cancer remain unclear. This study investigates the effects and underlying mechanisms of osthole-induced cell death and migration in human colon cancer. Our study reports that osthole induces cell death and reduces cell migration through the induction of ROS production, JNK activation, and p53 activation.

2. Experimental Section

2.1. Reagents and Antibodies. Osthole was purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), OPTI-MEM, and Lipofectamine 2000 (LF2000) were purchased from Gibco BRL (Invitrogen Life Technologies, Carlsbad, CA, USA). Primary antibodies against Bax, Bcl2, procaspase-3, PARP, JNK, and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies against p-JNK Thr¹⁸³/Tyr¹⁸⁵, p-p53 Ser¹⁵, and acetyl-p53 Lys³⁷⁹ were purchased from Cell Signaling and Neuroscience (Danvers, MA). The primary antibody against p53 was purchased from BD Pharmingen Transduction Laboratories (San Diego, CA). The dominant-negative (DN) mutant of JNK plasmid was a gift from Dr. W.-M. Fu (National Taiwan University, Taipei, Taiwan).

2.2. Cell Culture. Human colon carcinoma cell lines HCT116 and SW480 were obtained from the American Type Culture Collection (Manassas, VA, USA). The procedures of cell culturing were conducted according to previous reports [25, 26].

2.3. Reactive Oxygen Species (ROS) Assay. The procedures of ROS assay were conducted according to previous reports [27, 28]. ROS production was determined by the oxidation of specific probe, H₂DCFDA (2',7'-dichlorodihydrofluorescein diacetate), by using flow cytometry. Cells were incubated with H₂DCFDA (10 μ M) at 37°C for 30 min and then stimulated with osthole or hydrogen peroxide. Fluorescence intensities were determined with an excitation filter of 488 and 525 nm emission wavelengths.

2.4. Western Blot Analysis. Cells were lysed in a homogenization buffer on ice, and equal protein amounts of samples were loaded in an SDS-PAGE (polyacrylamide gel electrophoresis) [29]. The membrane was blocked with 5% nonfat milk and then probed with primary antibody. After several PBST washes, the membrane was incubated with a peroxidase-conjugated secondary antibody. The blots were visualized by enhanced chemiluminescence using Fuji medical X-ray film (Fujifilm, Tokyo, Japan). The blots were then stripped by incubation in stripping buffer [25] and reprobed a loading control. Quantitative data were obtained using a densitometer and Image J software (National Institute of Health, Bethesda, MA).

2.5. Migratory Activity Assay. *In vitro* migration assay was performed using Costar Transwell inserts (pore size, 8 μ m) (Corning, Albany, NY) as described previously [30–32]. Cells in 200 μ L of media were seeded in the upper chamber, and 300 μ L of media was placed in the lower chamber (incubated at 37°C in 5% CO₂). After seeding cells in the upper chamber, cells were treated with osthole for 2 h. After a 24 h migration period, cells were stained with 0.05% crystal violet in 2% methanol. Nonmigratory cells on the upper surface of the filters were removed by wiping with a cotton swab. Cell number was counted in five random fields per well under a microscope at 200x magnification. Images of migratory cells were acquired using a digital camera and light microscope.

2.6. Reverse Transcriptase-PCR (RT-PCR). Total RNA was extracted from cells using TRIzol kit (MDBio Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μ g of total RNA, which was reverse transcribed into cDNA using the oligo(dT) primer and then amplified using oligonucleotide primers:

p53: 5'-AATTTGCGTGTGGAGTATTT-3' and 5'-GTGGAGTCTTCCAGTGTGAT-3';
p21: 5'-AGGCACCGAGGCACTCAGAG-3' and 5'-AGTGA CAGGTCCACATGGTCTTCC-3';
GAPDH: 5'-TGGGCTACACTGAGCACCAG-3' and 5'-GGGTGTCGCTGTTGAAGTCA-3'.

Each PCR cycle was carried out for 30 sec at 95°C, 30 sec at 55°C, and 1 min at 72°C. PCR products were then separated

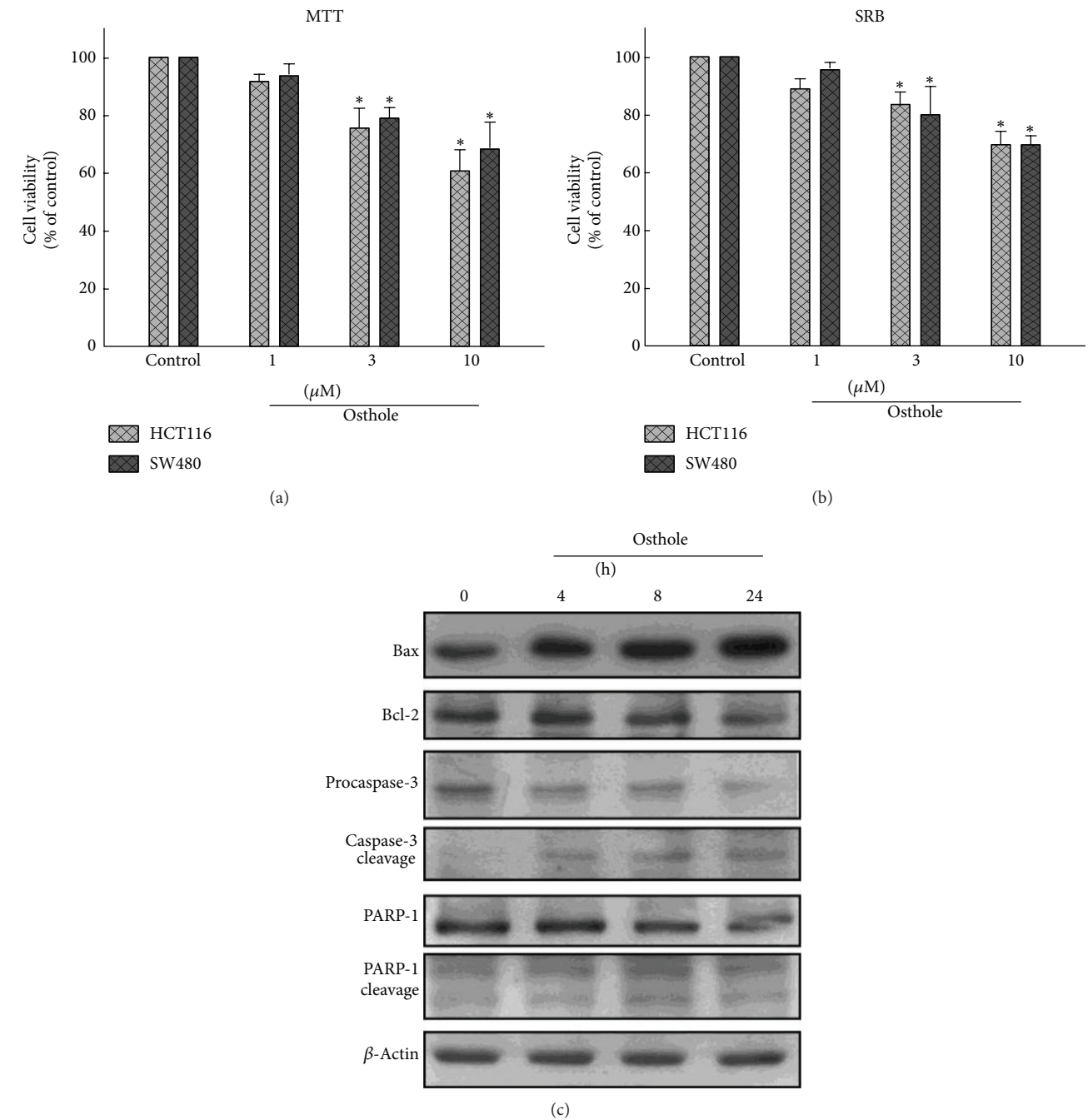


FIGURE 1: Osteohole induces cell death in human colon cancer. Osteohole-induced cell death of HCT116 and SW480 human colon cancer cells was shown. Cells were incubated with various concentrations of osteohole (1, 3, or 10 μ M) for 24 h, and the cell viability was examined by MTT (a) or SRB (b) assays. Results are expressed as the means \pm S.E.M. from three independent experiments. * $P < 0.05$, compared with the vehicle treatment group. (c) SW480 cells were incubated with osteohole (10 μ M) for indicated time periods, and the protein expressions of Bax, Bcl-2, procaspase-3, cleaved caspase-3, and PARP-1 were examined by western blot analysis. Results are the representative of three independent experiments.

electrophoretically in a 2% agarose gel and stained with Novel Juice (GeneDireX, Las Vegas, Nevada).

2.7. MTT and SRB (Sulforhodamine B) Assays. The procedures of MTT and SRB assays determining cell viability were

conducted according to previous reports [33–35]. After treatment with various concentrations of osteohole for 24 h, cell culture media were aspirated. In MTT assay, MTT (0.5 mg/mL) was added to each culture well and incubated for 2 h at 37°C. The MTT reagent was then removed and washed with PBS for several times. DMSO (200 μ L per well)

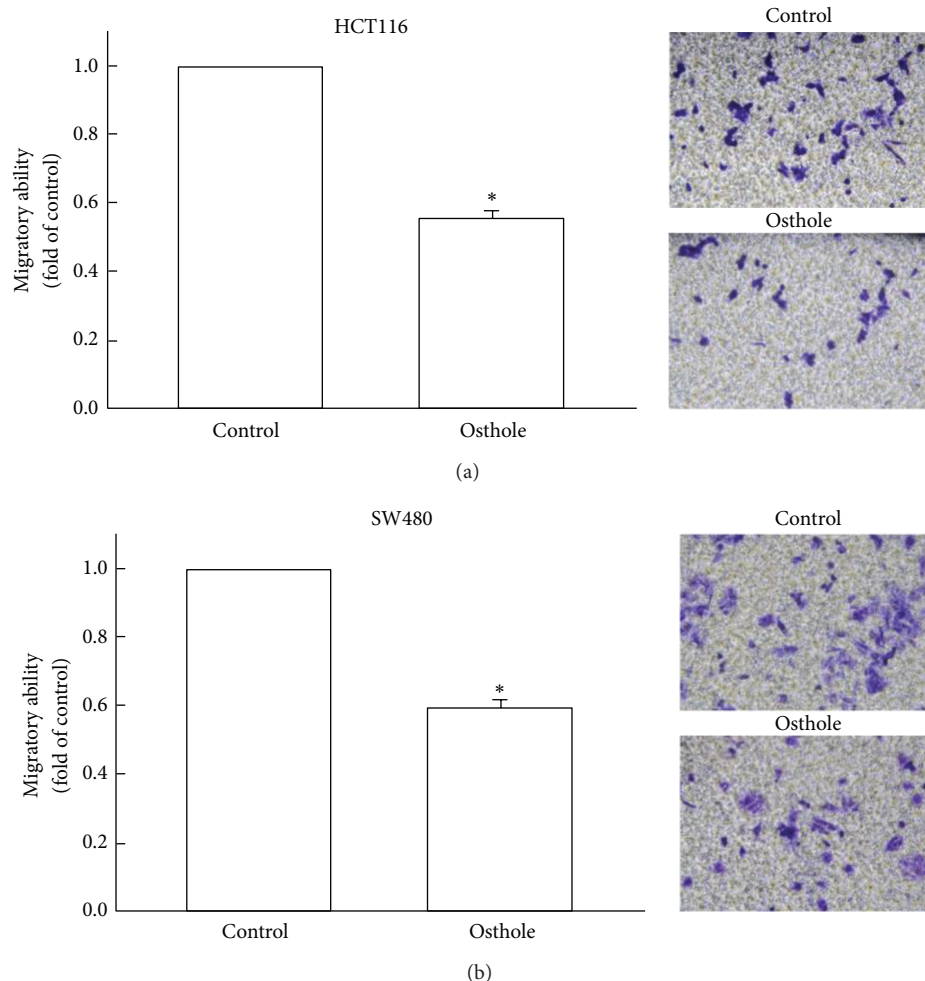


FIGURE 2: Osthole inhibits migration activity of human colon cancer. By using cell culture insert system, migratory activities of human colon cancer cells were examined. After incubating cells with osthole (10 μ M) or vehicle control for 24 h, we found that osthole inhibited migration activity in both HCT116 (a) and SW480 (b) cells. Migrated cells were visualized by phase-contrast imaging (right panels). Results are expressed as means \pm S.E.M. from three independent experiments. * $P < 0.05$, compared with control group.

was added to dissolve formazan crystals. In SRB assay, cells were fixed *in situ* by gentle addition of 50 μ L per well of 10% TCA, and culture plates were incubated for 1 h at 4°C. After discarding supernatant, cells were then washed with PBS and 50 μ L of SRB solution was then added to each well for 10 min at room temperature. After staining, cells were washed with 1% acetic acid and dissolved in tris-base. The absorbance was determined at 550 nm (for MTT) or 515 nm (for SRB) using a microplate reader (Thermo Scientific, Vantaa, Finland).

2.8. Statistical Analyses. The values are reported as mean \pm S.E.M. Statistical analyses between two groups were performed using Student's *t*-test. The difference was determined to be significant if the *P* value was <0.05 .

3. Results

3.1. Osthole Induces Cell Death and Apoptosis in Human Colon Cancer. In order to investigate whether osthole affects

cell viability, HCT116 and SW480 human colon cancer cells were incubated with various concentrations of osthole (1, 10, or 30 μ M) for 24 h, and cell viability of both cell lines was determined by MTT and SRB assay. We observed that osthole induced cell death of human colon cancer in a concentration-dependent manner (Figures 1(a) and 1(b)). Treatment of SW480 cells with osthole also increased Bax protein expression and the cleavage of caspase-3 and PARP-1. Meanwhile, osthole decreased the protein expression of Bcl-2, procaspase-3, and PARP-1 as well (Figure 1(c)).

3.2. Osthole Inhibits Migratory Activity of Human Colon Cancer. Osthole-regulated human colon cancer migration was examined by using cell culture insert system. As shown in Figures 2(a) and 2(b), human colon cancer cells (HCT116 and SW480 cells) migrated from the upper to the lower chamber, and images of migrated cells were shown in right panels. Our results indicated that osthole effectively reduced human colon cancer migration in a concentration-dependent manner.

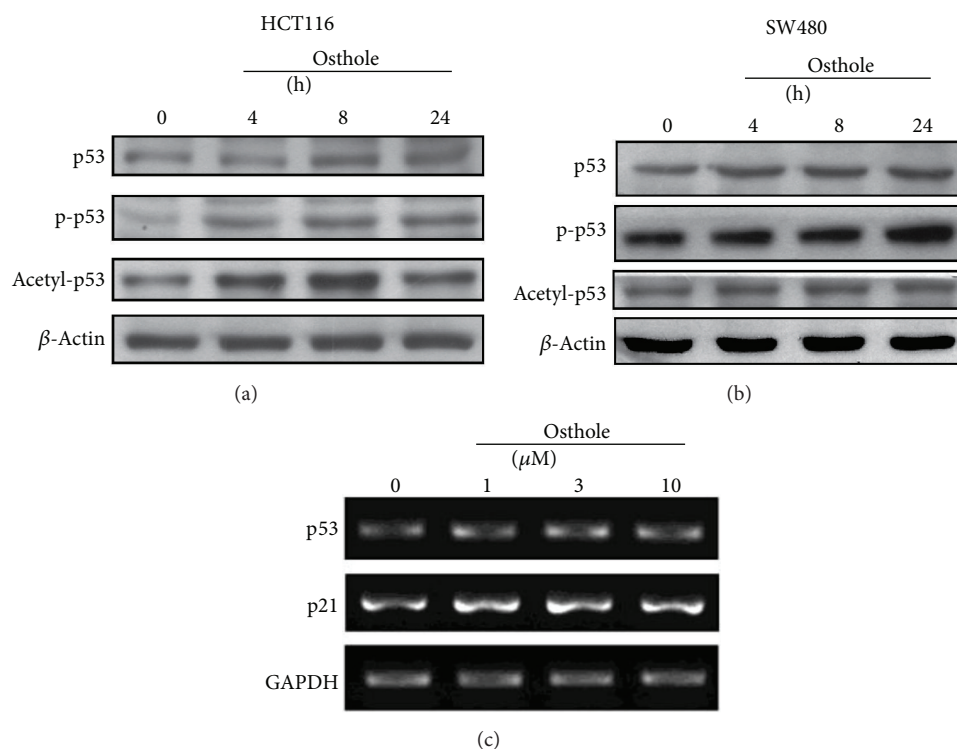


FIGURE 3: Osthole increases p53 protein activation in human colon cancer. HCT116 (a) and SW480 (b) were incubated with osthole (10 μ M) for indicated time periods. p53, phosphorylated p53 (p-p53), and acetylated p53 (acetyl-p53) expressions were examined by western blot analysis. (c) SW480 cells were stimulated by osthole for 6 h, and p53 and p21 expressions were examined by RT-PCR analysis. Results are the representative of three independent experiments.

3.3. Osthole Induces p53 Protein Activation in Human Colon Cancer. As shown in Figures 3(a) and 3(b), HCT116 and SW480 cells were incubated with osthole before cell lysate extracts were collected. p53 protein levels, phosphorylation of p53 on Ser15 (p-p53), and acetylation of p53 on Lys³⁷⁹ (acetyl-p53) were all increased after osthole stimulation. The effects of osthole on gene expression of p53 and p21 were estimated in SW480 cells using RT-PCR analysis. As shown in Figure 3(c), p53 and p21 gene expression levels were mildly increased in osthole-treated group.

3.4. JNK Activation in Osthole Involves Protein p53-Induced Activation. After incubating the SW480 cells with various concentrations of osthole (1, 3, or 10 μ M) for 24 h, we found that osthole increased the level of phosphorylated JNK (Figure 4(a)). Moreover, transfection with DN-JNK for 24 h reduced JNK phosphorylation (Figure 4(a)). Treatment with JNK inhibitor SP600125 reduced osthole-induced p53 protein activation (Figure 4(b)). Furthermore, transfection with DN-JNK also attenuated the effects of osthole-induced human colon cancer cell death (Figures 4(c) and 4(d)) and cell migratory effect (Figure 4(e)). Incubation with JNK inhibitor SP600125 also reversed osthole-inhibited cell migratory effect in SW480 human colon cancer cells (Figure 4(f)).

3.5. Osthole Induces p53 Protein Activation through Reactive Oxygen Species Production in SW480 Human Colon Cancer.

As determined by probe H₂DCF-DA which was analyzed by flow cytometry assay, osthole was found to increase intracellular ROS levels. Treatment with an ROS scavenger NAC (n-acetylcysteine) reduced hydrogen peroxide (H₂O₂) induced ROS production (Figures 5(a) and 5(b)). Moreover, incubation with osthole also dramatically increased ROS generation in human colon cancer cells (Figure 5(c)). Additionally, treatment with NAC antagonized osthole-induced ROS production in SW480 cells as well (Figure 5(d)). As shown in Figure 5(e), treatment with NAC along with osthole in human colon cancer also reduced osthole-enhanced p53 protein expression, p-p53, and acetyl-p53 activation.

4. Discussion

Several clinical uses of drugs were derived or modified from plant extracts and have been successfully applied to treat a variety of human cancers [36]. We have reported some natural products and chemical compounds exerting anticancer effects in human glioblastoma [27, 28] and colon cancer [25]. Recently, we also reported that osthole induces cell death and attenuates cell migration in brain tumor [37]. However, the detailed mechanisms underlying the anticancer effects of osthole remain unclear. Our present study reported that osthole increased phosphorylation of p53 on Ser¹⁵ (p-p53) and acetylation of p53 on Lys³⁷⁹ (acetyl-p53), which were regulated by ROS generation and JNK activation.

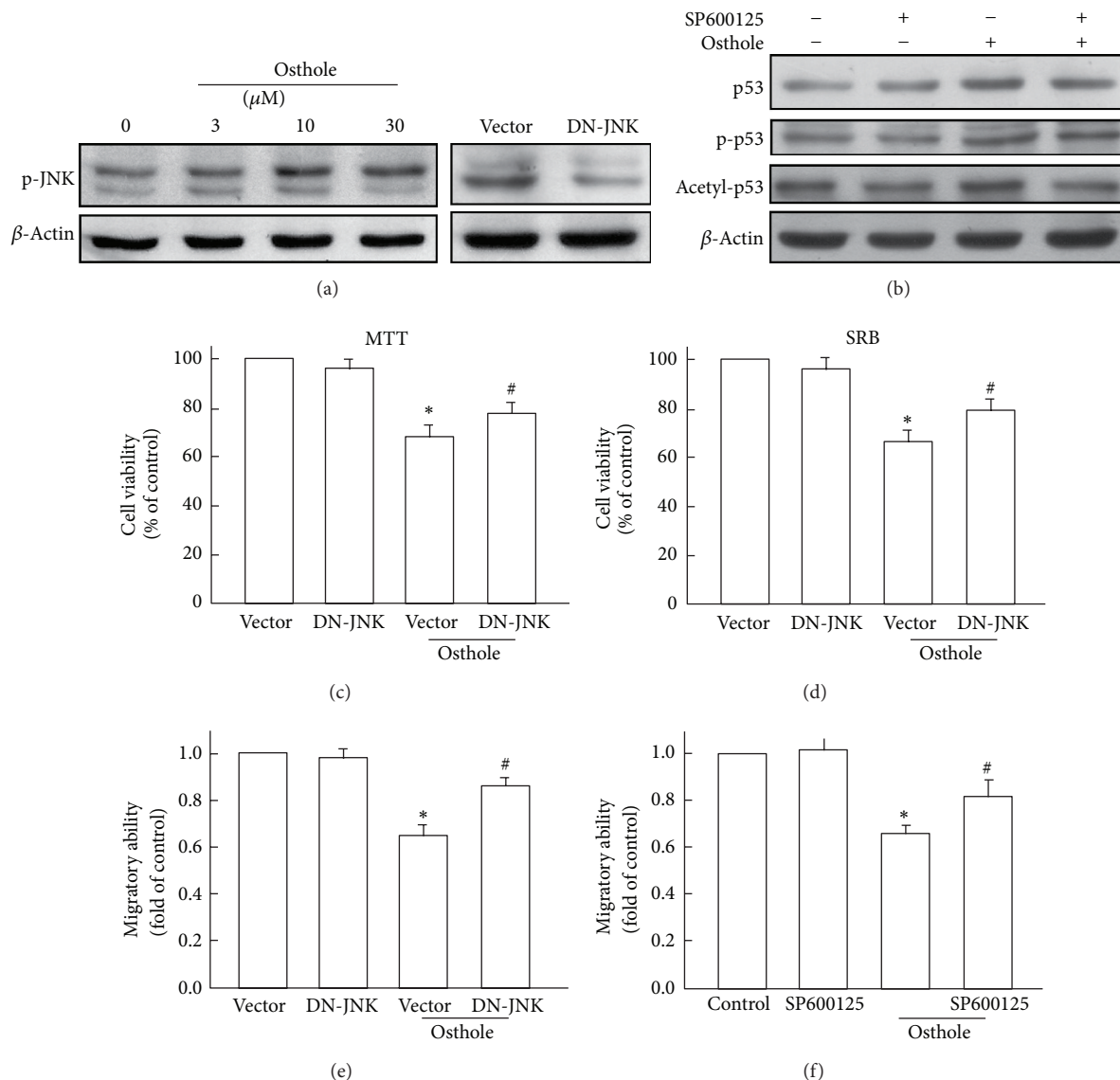


FIGURE 4: JNK activation contributes to osthole-induced p53 activation in SW480 human colon cancer. (a) Cells were incubated with various concentrations of osthole for 24 h (left panel). Cells were transfected with DN-JNK or empty vector for 24 h (right panel) before cell lysates were collected. Levels of phosphorylated JNK were determined by western blot. (b) Cells were pretreated with or without SP600125 (10 μ M) followed by stimulation with osthole for another 24 h; the protein expressions of p53, p-p53, and acetyl-p53 were examined by western blot analysis. Results are the representative of three independent experiments. (c) Cells were transfected with DN-JNK for 24 h followed by stimulation with osthole for another 24 h, and cell viability was examined by MTT (c) or SRB (d) assays. Osthole-inhibited migratory activities were examined by cell culture insert system after transfecting cells with DN-JNK or empty vector for 24 h (e). (f) Cells were pretreated with SP600125 (10 μ M) followed by stimulation with osthole for another 24 h, and migratory activities were also examined.

To our knowledge, this is the first report that shows the role of p53 in osthole-induced anticancer effects and identifies the molecular mechanisms through which ROS and JNK modulate p53 protein activation in human colon cancer cells.

Targeting p53, a tumor suppressor, is one of the promising strategies for anticancer therapy; therefore several compounds targeting p53 are currently being tested in clinical studies [38]. Numerous reports have supported this idea of pharmacological restoration of p53 activity for anticancer [39, 40]. Induction of p53 activation leads to cell growth

arrest or cell death, but investigation of the detailed molecular mechanism regulating the cancer pathogenesis by p53 remains a key challenge in p53 biology of cancer cells [41]. Importantly, p53-mediated cell fate decisions also prevent cancer cells to be killed by chemotherapeutic drugs, thus leading to poor clinical outcomes. It is imperative to understand the mechanism of p53-mediated cell fate decisions for the efficient clinical application of drugs activating p53 [42]. Our current study reported the crucial role of ROS and JNK in p53-related proapoptotic function in human

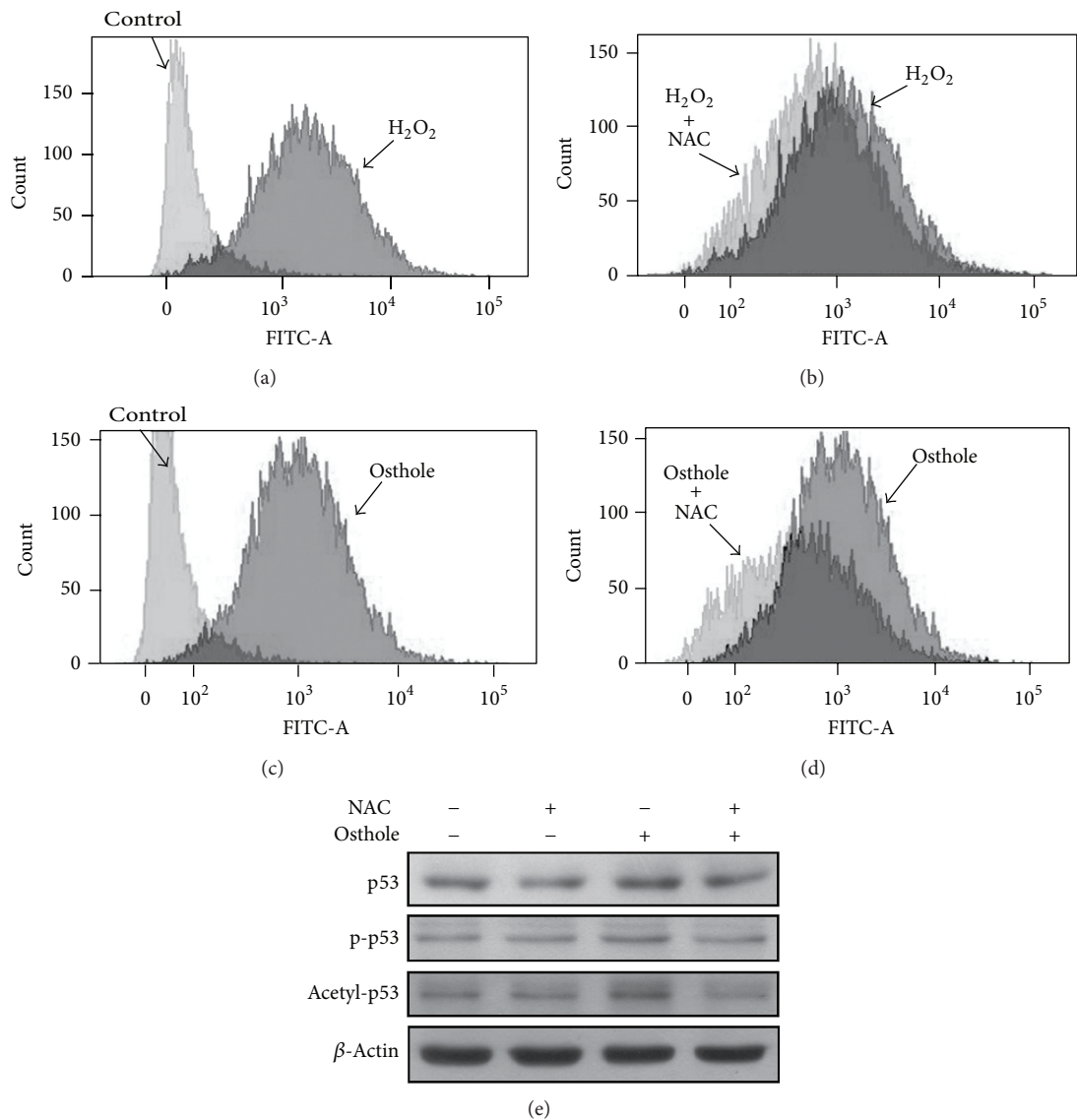


FIGURE 5: Reactive oxygen species involved in osthole-induced p53 activation. SW480 cells were incubated with H₂O₂ (200 μM; (a)), H₂O₂ plus N-acetyl-L-cysteine (H₂O₂ + NAC; (b)), osthole (10 μM; (c)), or osthole plus N-acetyl-L-cysteine (osthole + NAC; (d)) for 120 min. ROS generations were determined using flow cytometry with the fluorescence probe H₂DCFH-DA. (e) Cells were treated with NAC plus osthole for 24 h and the protein expressions of p53, p-p53, and acetyl-p53 were examined by western blot analysis. Results are the representative of three independent experiments.

colon cancer through activation of p53 by a natural product, osthole. Furthermore, our results also demonstrated that JNK-mediated mechanism played a key role in cell death and migratory ability. Interestingly, osthole induced significant cell death and inhibited migratory ability even in the p53-mutated colon cancer cell line SW480 cells.

Several lines of evidence demonstrate that the accumulation of ROS is correlated with the apoptotic response induced by several chemotherapeutic drugs [43]. ROS generation appears to be triggered by the activation of the mitochondrial-dependent cell death pathway through the proapoptotic Bcl-2 proteins Bax and is further transformed sequentially into more toxic ROS, like hydrogen peroxide, which, consequently, induces cell death [44]. Our study

showed that osthole elevated the intracellular ROS levels in human colon cancer cells. Furthermore, we observed that blocking ROS production with antioxidant NAC resulted in decreased intracellular ROS levels as well as osthole-induced p53 protein activation. These results indicated that ROS accumulation contributed to osthole-induced cell apoptosis in human colon cancer. Our results also reported that osthole-enhanced proapoptotic protein Bax expression and Bcl-2 degradation. Furthermore, treatment with osthole also increased caspase-3 activation and promoted PARP-1 cleavage. In conclusion, osthole-induced human colon cancer cell death may mediate by ROS generation, which subsequently induces Bax/Bcl-2 turnover and promotes caspase-3 activation and PARP-1 cleavage, resulting in cell apoptosis.

Moreover, our results in colon cancer are also in accordance with previous report that osthole effectively attenuates migratory ability in human cancer cells.

5. Conclusions

In the present study, osthole-induced p53 activation results from ROS generation and JNK activation, revealing a promising potential in the treatment of human colon cancer. The current study on a molecular basis provides valuable knowledge of osthole in effective antitumor therapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

All authors were involved in drafting this paper or revising it critically for important intellectual content, and all authors approved the final version to be published. Ssu-Ming Huang, Cheng-Fang Tsai, and Wei-Lan Yeh conceived and designed experiments. The acquisition, analysis, and interpretation of data were done by Ssu-Ming Huang and Wei-Lan Yeh. Ssu-Ming Huang, Min-Ying Wang, Dar-Ren Chen, and Wei-Lan Yeh drafted and revised the paper.

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