



## Research Article

# Involvement of Exportin 1 Mediated c-Myc and Cullin 4A Signaling in Apoptotic Effect of Fisetin in Non-small Cell Lung Cancer Cells

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## Abstract

Though Fisetin was known to exhibit antitumor effect in prostate, colon, breast, stomach, and lung cancers, the underlying antitumor mechanism is not fully understood in non-small cell lung cancers (NSCLCs). Thus, the aim of the present work is to elucidate the antitumor mechanism of Fisetin in A549 and H460 NSCLC cells in association with ribosomal biogenesis and ubiquitin ligase proteins. Fisetin showed cytotoxic and antiproliferative effects in a concentration and time dependent manner in A549 and H460 cells. Also, Fisetin increased the number of Annexin V/PI positive apoptotic portion and sub G1 accumulation in A549 and H460 cells. Additionally, Fisetin cleaved Poly ADP-ribose polymerase (PARP) and caspase 3 and increased p53, attenuated the expression of G1 phase related proteins such as cyclin D1, cyclin E and CDK2 and ribosomal biogenesis related gene Exportin 1 (XPO1) in A549 and H460 cells. Of note, Fisetin abrogated the expression of driver oncogenes such as c-Myc, S-Phase Kinase Associated Protein 2 (SKP2) and Cullin 4A (CUL4A) in A549 and H460 cells. Conversely, depletion of XPO1 enhanced p53 and PARP cleavage, while depletion of CUL4A promoted PARP cleavage without p53 upregulation in H460 cells. Overall, these findings highlight evidence that XPO1 mediated c-Myc and CUL4A signaling is critically involved in Fisetin induced apoptotic effect in NSCLCs as a potent antitumor candidate

**Keywords:** Fisetin; Non-small Cell Lung Cancer; c-Myc; p53; XPO1; CUL4A

## Introduction

Non-small-cell lung cancer (NSCLC) is one of the most common malignant diseases in humans worldwide [1]. The three major types of NSCLC are adenocarcinoma (ADC), Squamous Cell Carcinoma (SCC) and Large Cell Carcinoma (LCC) [2,3]. However, current treatments have a drawback to cause a variety of side effects. For instance, cisplatin is known to induce adverse reactions such as anemia, joint pain, face swelling, convulsions

and trouble in walking [4]. Recently molecular target therapy is attractive for effective cancer therapy via regulation of specific molecules.

It is well documented that the Myc family gene consisting of c-Myc, n-Myc and L-Myc work as oncogenes in several cancers, while p53 acts as one of tumor suppressors [5]. Furthermore, the cullin 4 subfamily of genes including CUL4A and CUL4B ubiquitin ligases or Cullin RING ligase (CRL) complexes are often overexpressed in human malignancies [6,7]. Also, exportin1 (XPO1) or Chromosome region maintenance 1 (CRM1), is often overexpressed or mutated in several cancer by regulation of

nuclear export of proteins and RNAs, and ribosome biogenesis for cancer growth and survival [8,9]. Furthermore, in terms of lung cancer chemoprevention, some natural compounds are of interests to selectively induce apoptosis and growth arrest in lung cancer cells without severe cytotoxicity in normal cells [10]. For instance, sulforaphane [11], resveratrol [12], tanshinone IIA [13], shikonin [14] and quercetin [15].

In the same line, Fisetin, one of flavonol [16], was known to have anti-inflammatory [17], anti-aging [18], anti-angiogenic [19], anti-proliferative [20] and anti-tumor [21, 22] effects. In particular, Fisetin induced apoptosis in prostate [23], pancreatic [24], glioblastoma [25], gastric [26], breast [27], colon [28], ovarian [29] cancer cells. It also exerted antitumor effect in lung cancer cells via modulation of ERK1/2[30, 31] and AKT/PI3K/mTOR [32] signaling pathway. Nevertheless, the underlying antitumor mechanism of Fisetin is not fully understood in non-small cell lung cancer cells so far. Thus, in the present study, the apoptotic mechanism of Fisetin was explored in association with c-Myc/p53 signaling mediated by XPO1 ribosomal protein and ubiquitin ligase CUL4A in non-small cell lung cancer cells.

## Materials and Methods

### Cell culture and reagents

H460 and A549 cells (human epithelial non-small cell lung cancers; NSCLCs) were purchased from the American Type Culture Collection (ATCC, USA). The cells were cultured in RPMI containing 10% FBS (Fetal bovine serum) and 1% antibiotic (Welgene, South Korea) and incubated at 37 °C with 5% CO<sub>2</sub>. Fisetin ((3, 7, 3', 4'-tetrahydroxyflavone) was purchased from Sigma Chemical Co., (St. Louis, MO, USA) and dissolved in DMSO (dimethyl sulfoxide) as a 100 mM stock.

### Cell viability assay

Cell viability was assessed by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, St. Louis, MO, USA) according to the manufacturer's instruction. After H460 and A549 cells were seeded onto 96-well microplate, the cells were treated by various concentrations (0, 10, 20, 40, 60, 80 μM) of Fisetin for 24 h. For time or concentration dependent study, the cells were exposed to Fisetin at 40 and 80 μM for 24h, 48 h, and 72 h. MTT solution (1 mg/ml) was added and formazan was dissolved with DMSO (dimethyl sulfoxide). To measure the optical density, microplate reader (TECAN, Austria) was used at 570 nm. Cell viability was determined as a percentage of viable cells in Fisetin treated group versus untreated control.

### Colony formation assay

H460 and A549 cells (1,000 cells) were seeded in 6-well plates RPMI 1640 medium containing 10% serum. The cells were treated with Fisetin (40, 80 μM) for 24 h and replaced by new

media. The cells were incubated at 37 °C with 5% CO<sub>2</sub> for 10 days, the colonies were fixed and stained with Diff-Quick solution (Sysmex, Japan). Colonies containing ≥50 cells were counted using ImageJ software.

### Annexin-V-FITC and propidium iodide staining for apoptotic portion analysis

H460 and A549 cells were treated with Fisetin (40, 80 μM) for 24 h. Then apoptosis was measured by Annexin V staining-based FACS analysis by using FITC Annexin V Apoptosis Detection Kit (BD Biosciences, Korea).

### Cell cycle analysis for sub G1 population

H460 and A549 cells were treated with Fisetin (40 or 80 μM) for 24h. The cells were washed using PBS and fixed with ethanol (75%), incubated with 1mg/ml RNase A in PBS at 37 °C for 30 min, and stained in PBS containing 50 μg/mL PI (propidium iodide). The DNA contents of the stained cells were analyzed by using Cell Quest Software (BD Biosciences, San Jose, CA, USA) with the FACS Calibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

### Western blotting

H460 and A549 cells were exposed to various concentrations of Fisetin for 24h. The cells were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, and 1% TritonX-100) containing protease inhibitors (Roche, Germany), and phosphatase inhibitors (Sigma, USA). Total cell lysates were separated in 8 to 15% SDS-PAGE (SDS-polyacrylamide gels), and electrophoretically transferred to nitrocellulose membranes. The antibodies against p53, cyclin D1, CDK4, CDK2, Bcl-2, XPO1 were obtained from Santa Cruz Biotechnology. Cyclin E, Poly (ADP-ribose) polymerase (PARP), and cleaved-PARP were purchased from Cell signaling (Beverly, MA, USA). β-actin antibody was from Sigma-Aldrich (St. Louis, MO, USA), and c-Myc was bought from Abcam (Cambridge, England). The antibodies were diluted in 3% BSA in PBS-Tween20 (1:500-1:2000), and the membranes were incubated overnight at 4 °C. Then the membranes were washed three times with PBS-Tween20, and incubated with HRP-conjugated secondary antibodies. The expression was visualized by using ECL Western blotting detection reagent (GE Healthcare, UK).

### Small interfering RNA (siRNA) transfection assay

H460 or A549 cells were transfected with scrambled siRNA, XPO1 siRNA or CUL4A siRNA (Bioneer, Korea) with Interferin<sup>TM</sup> transfection reagent (Polyplus-transfection Inc., New York, NY, USA). The mixtures of XPO1 siRNA with Interferin<sup>TM</sup> transfection reagent were incubated for 10 min at room temperature and the cells were incubated at 37 °C for 48 h before exposure to Fisetin 80 μM for 24 h.

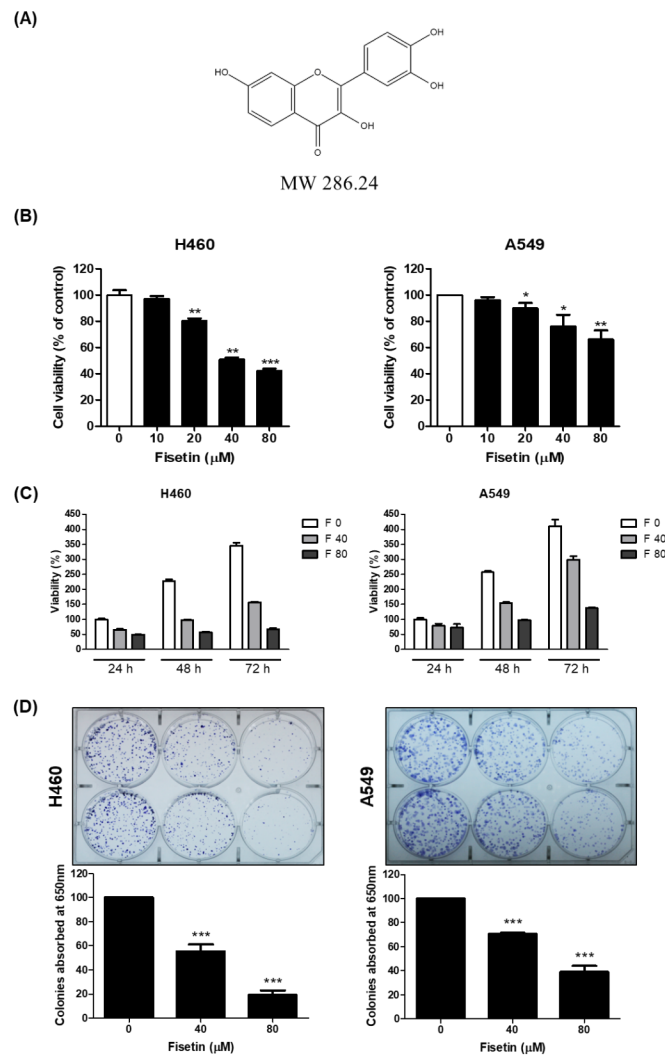
## Statistical analysis

Data were presented as the means  $\pm$  standard deviation. Statistical significance of the differences was determined by Student's t-test using Sigma Plot Software (Systat Software Inc., USA).

## Results

### Fisetin exerted cytotoxic and antiproliferative effect in H460 and A549 non-small cell lung cancer cells

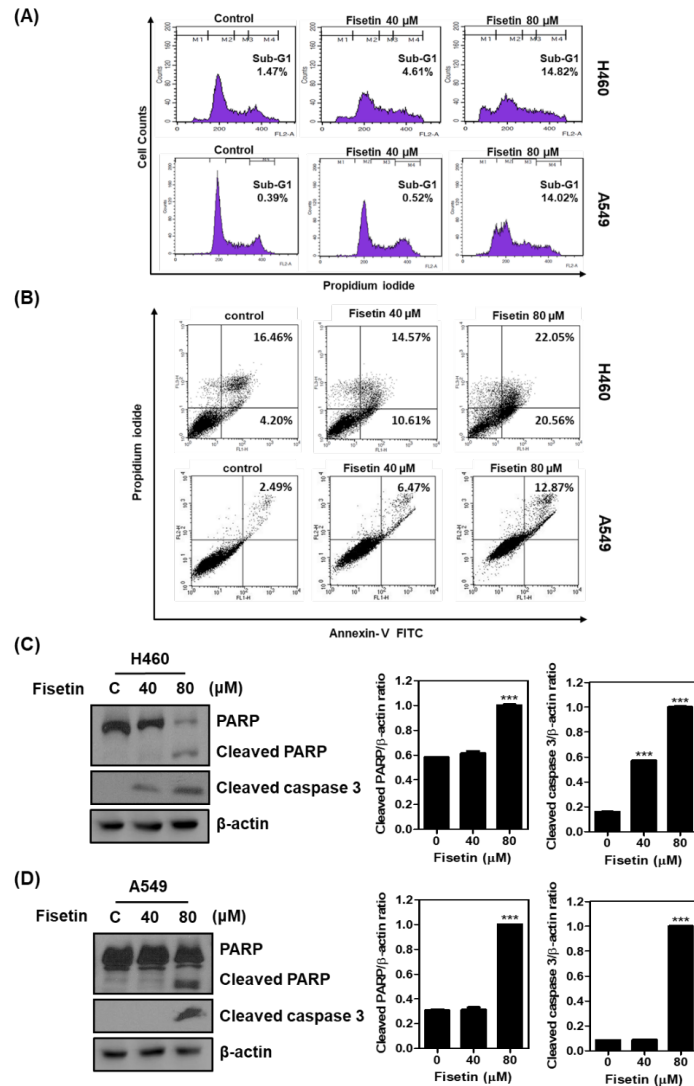
To evaluate the cytotoxic and antiproliferative effect of Fisetin (Figure 1A), MTT assay and colony formation assay were performed in H460 and A549 cells. Here Fisetin significantly reduced the viability of H460 and A549 cells in a concentration and time dependent fashion (Figure 1B and 1C). Also, colony formation assay showed that the sizes and number of colonies were reduced in Fisetin treated group compared to untreated control (Figure 1D).



**Figure 1:** Effect of Fisetin on cytotoxicity and colony formation in H460 and A549 cells. (A) Chemical structure of Fisetin (B). Concentration dependent cytotoxicity of Fisetin in H460 and A549 cells. The cell viability by Fisetin was assessed by MTT assay after exposure to Fisetin for 24 h. \*,  $p < 0.05$ , \*\*,  $p < 0.01$  vs untreated control. (C) Time dependent cytotoxicity of Fisetin in H460 and A549 cells by MTT assay after exposure to Fisetin for 24 h. (D) Effect of Fisetin on colony formation in H460 and A549 cells. Ten days after exposure to Fisetin (40, and 80  $\mu$ M) for 24 h, the colonies were stained with Diff Quick Solution to visualize, and then the colonies were counted. \*\*\*,  $p < 0.001$  vs untreated control.

### Fisetin induced apoptosis and increased the accumulation of sub-G1 population in H460 and A549 cells

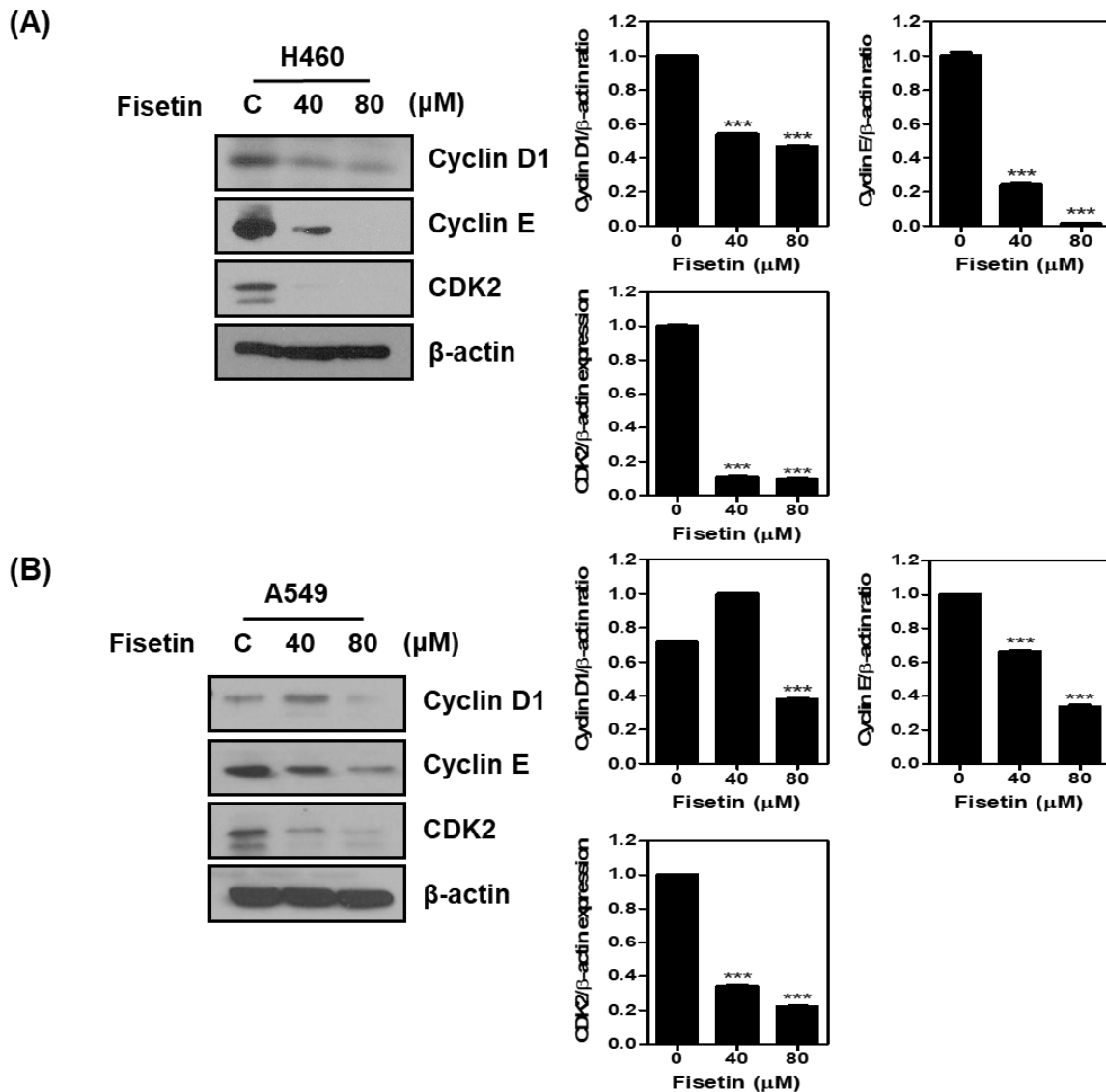
To examine the apoptotic effect of Fisetin, cell cycle analysis and Western blotting were carried out in Fisetin treated H460 and A549 cells. Apoptotic assay by using Annexin V/propidium iodide staining reveals that Fisetin increased apoptotic portion to 14.82% and 14.02% at 80  $\mu$ M compared to untreated control in H460 and A549 cells, respectively (Figure 2A). Consistently, Fisetin increased sub G1 population to 22.5 and 12.87% cells compared to untreated control in H460 and A549 cells, respectively (Figure 2B). Also, to confirm above apoptotic effect of Fisetin, Western blotting was conducted. As expected, Fisetin effectively cleaved PARP and caspase 3 in H460 and A549 cells (Figure 2C, 2D).



**Figure 2:** Effect of Fisetin on Annexin V/PI stained apoptotic portion and sub G1 population in H460 and A549 cells. (A) Apoptotic effect of Fisetin on Sub G1 population in H460 and A549 cells. Sub G1 population was evaluated in Fisetin treated H460 and A549 cells by FACS analysis. The cells were pretreated with 40 and 80  $\mu$ M of Fisetin for 24 h and stained with PI. Then the data were analyzed by using Cell Quest Software. (B) Apoptotic effect of Fisetin in H460 and A549 cells by flow cytometric analysis using Annexin V/PI. After H460 and A549 cells were treated by Fisetin at 40 and 80  $\mu$ M for 24 h, the apoptotic portion was evaluated in H460 and A549 by FACS analysis. (C) Effect of Fisetin on apoptosis related proteins in H460 cells. The cells were treated with Fisetin for 24 h and were subjected to Western blotting. \*\*\*,  $p < 0.001$  vs untreated control. (D) Effect of Fisetin on apoptosis related proteins in A549 cells. The cells were treated with Fisetin for 24 h and were subjected to Western blotting. \*\*\*,  $p < 0.001$  vs untreated control.

### Fisetin down-regulated cell cycle related proteins in H460 and A549 cells.

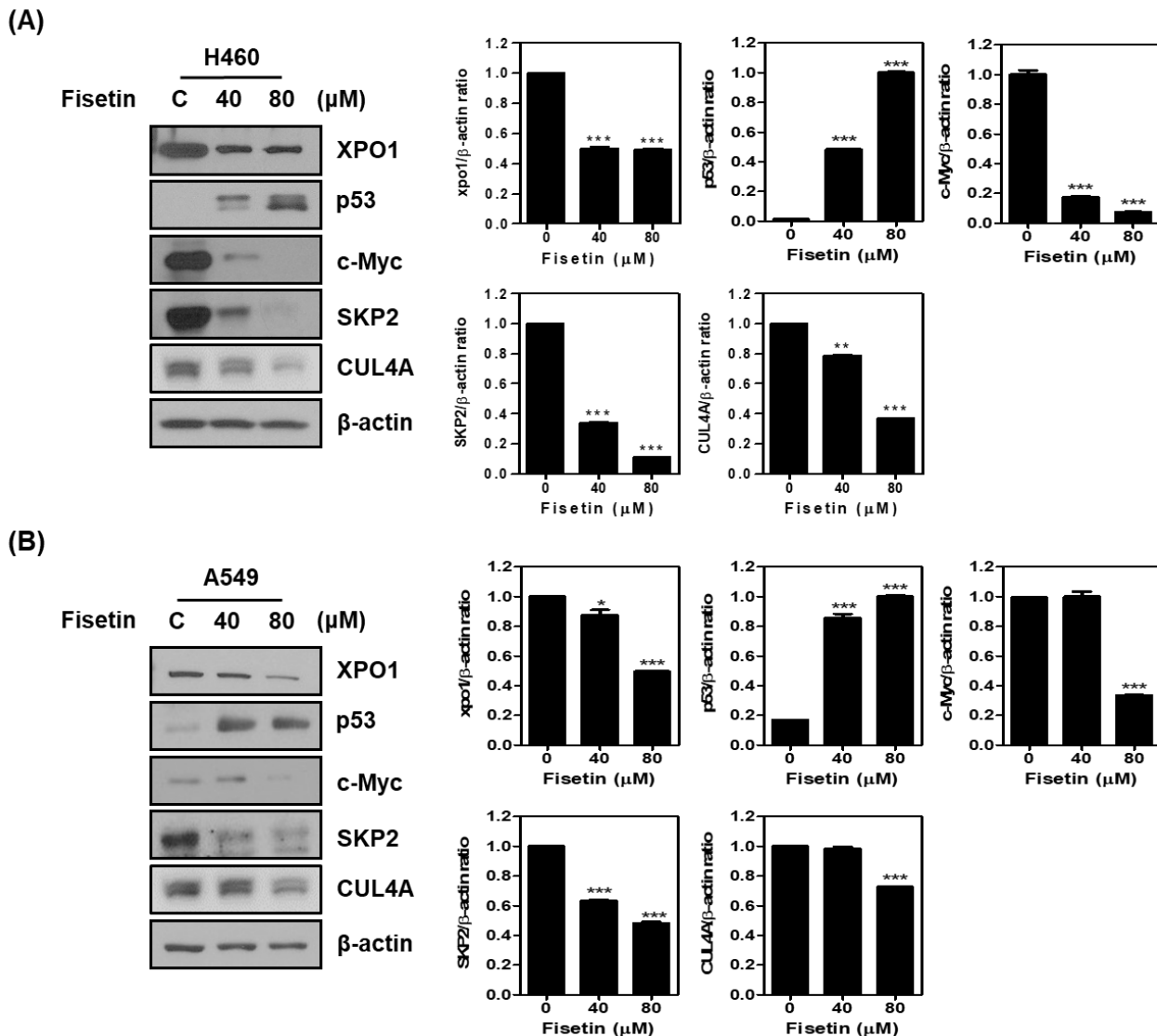
To confirm whether Fisetin affects cell cycle related proteins, Western blotting was performed. As shown in Figure 3A, Fisetin activated p53 and p21, and attenuated the expression of cyclin D1, cyclin E, CDK2 compared to untreated control in H460 and A549 cells (Figure 3B).



**Figure 3:** Effect of Fisetin on cell cycle related proteins in H460 and A549 cells. (A) Effect of Fisetin on cell cycle related proteins in H460 cells. After exposure to Fisetin at 40 and 80 μM for 24 h, cell cycle related proteins were evaluated in H460 cells by Western blotting. \*\*\*, p<0.001 vs untreated control. (B) Effect of Fisetin on cell cycle related proteins in A549 cells. The expression of cell cycle and ribosomal biogenesis related proteins was assessed in Fisetin treated A549 cells by Western blotting. \*\*\*, p<0.001 vs untreated control.

### Fisetin attenuated the ubiquitination related proteins in H460 and A549 cells

Exportin-1 (XPO1) controls nuclear-cytoplasmic transport of protein, ribosomal RNA and certain mRNAs for ribosomal biogenesis, which is closely associated with ubiquitination [5,33]. The *c-Myc* is known an unstable *protein* susceptible to ubiquitination [34], while *SKP2* (S-phase kinase-associated protein 2) acts as the F-box protein of the E3 *ubiquitin ligase* complex [35] and Cullin 4A (CUL4A), an E3 ubiquitin ligase, is overexpressed in several cancers [36]. Hence, Western blotting was performed to assess the effect of Fisetin on ubiquitination related proteins in A549 and H460 cells. Here Fisetin attenuated the expression of XPO1, c-Myc, SKP2 and CUL4A compared to untreated control in A549 and H460 cells (Figure 4A and 4B). However, H460 cells were more susceptible to Fisetin than A549 cells.

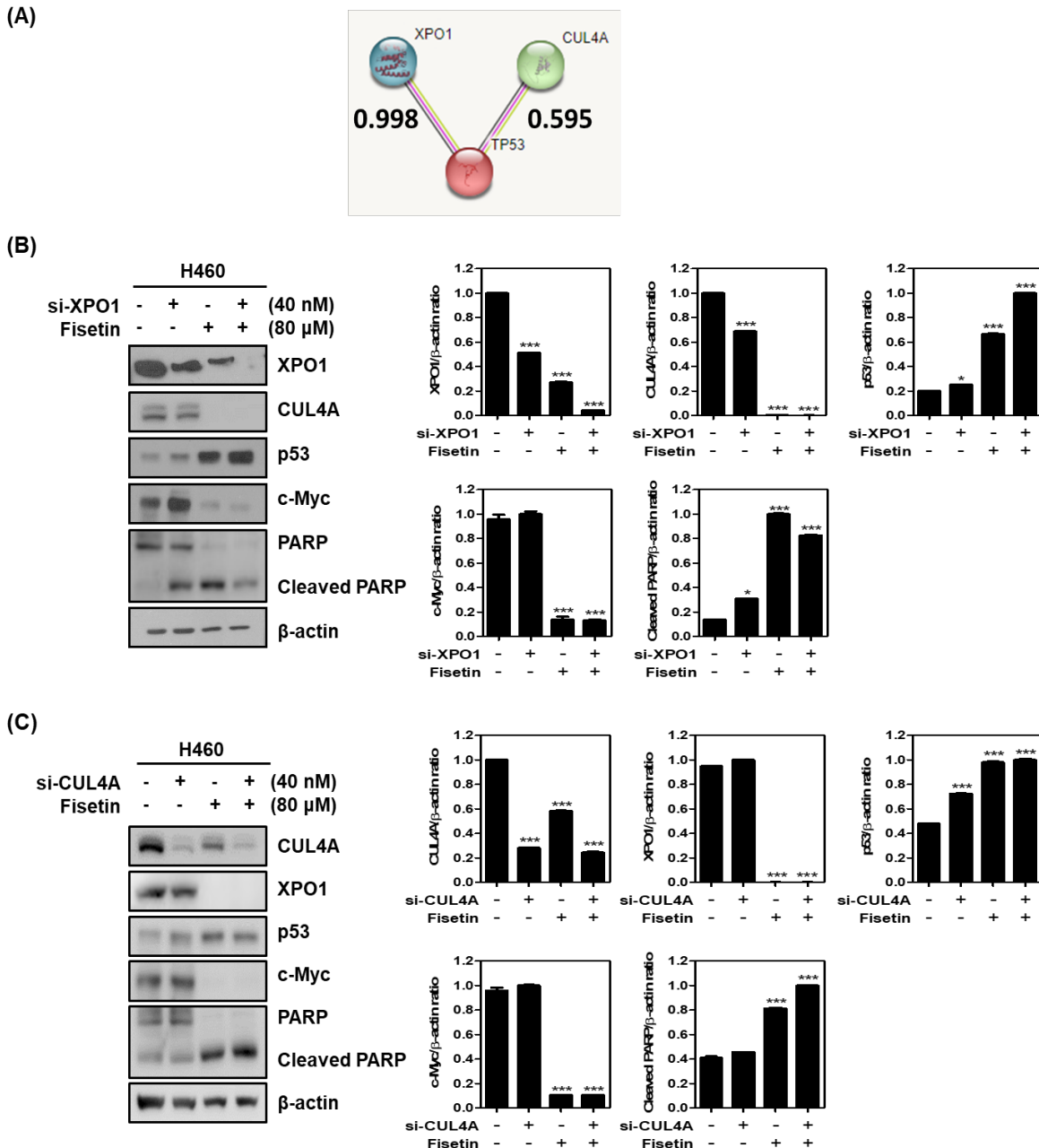


**Figure 4:** Effect of Fisetin on ubiquitination related proteins in H460 and A549 cells. (A) Effect of Fisetin on ubiquitination related proteins in H460 cells. After exposure to Fisetin at 40 and 80 μM for 24 h, ubiquitination related proteins were evaluated in H460 cells by Western blotting. \*\*, p<0.01, \*\*\*, p<0.001 vs untreated control. (B) Effect of Fisetin on ubiquitination related proteins in A549 cells. \*, p<0.05, \*\*\*, p<0.001 vs untreated control.

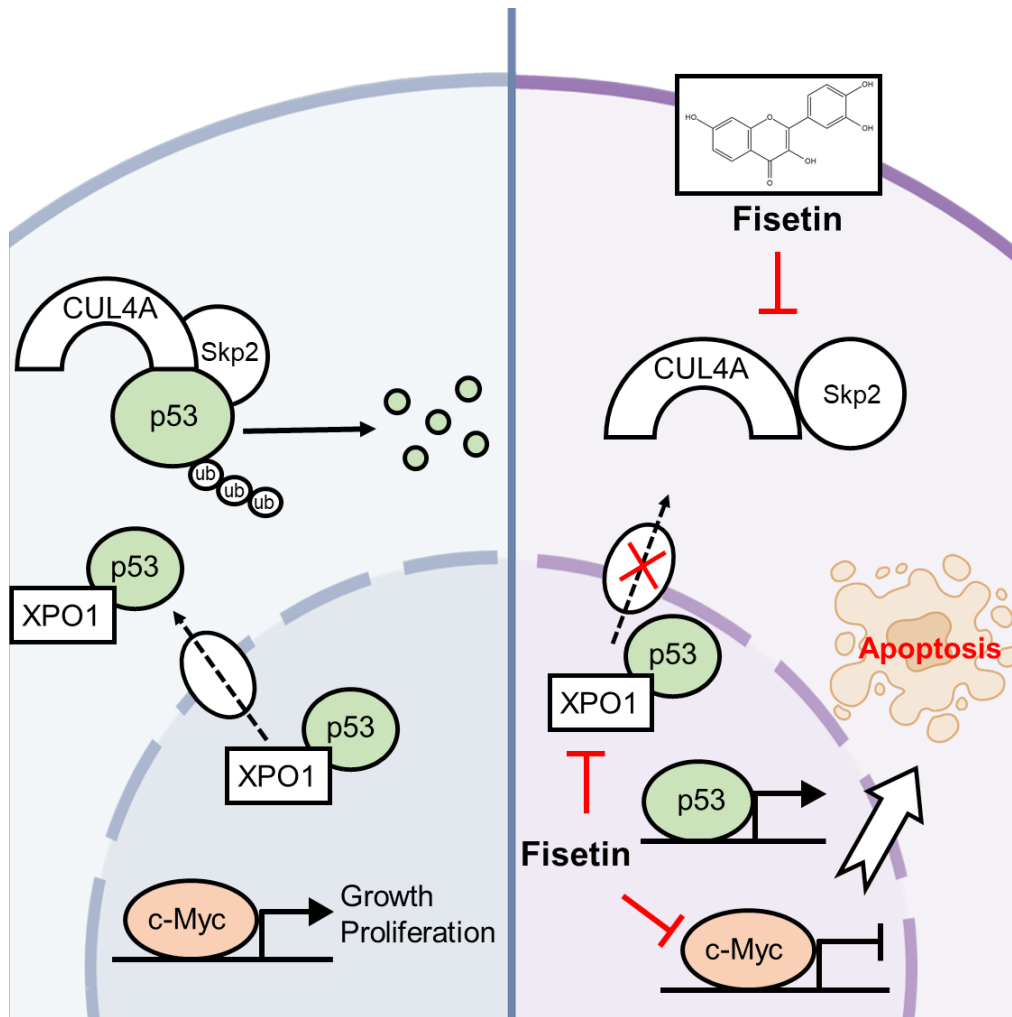


### XPO1 or CUL4A depletion reduced the capacity of Fisetin to cleave PARP and induce p53 in H460 cells

To confirm the important role of XPO1 and IPO7 in Fisetin-induced apoptosis in H460 cells, Western blotting was performed in H460 cells transfected with siRNA vector, XPO1 siRNA plasmids. Herein XPO1 depletion enhanced p53 activation, while CUL4A depletion enhanced PARP cleavage in Fisetin treated H460 cells (Figure 5A and 5B).



**Figure 5:** The pivotal role of XPO1 or CUL4A in Fisetin induced apoptosis in H460 cells. (A) PPI binding score between XPO1 or CUL4A and p53 by String database (B) Effect of XPO1 depletion on apoptosis related proteins in Fisetin treated H460 cells. \*,  $p < 0.05$ , \*\*\*,  $p < 0.001$  vs untreated control. (C) Effect of CUL4A depletion on apoptosis related proteins in Fisetin treated H460 cells \*\*\*,  $p < 0.001$  vs untreated control.



**Figure 6:** Apoptotic mechanism of Fisetin via modulation of XPO1 and CUL4A in non-small lung cancer cells.

## Discussion

In the current study, the underlying mechanisms of Fisetin were explored in H460 and A549 cells in association with ribosomal protein XPO1 and CUL4A signaling axis. Herein we found that Fisetin significantly suppressed the cell proliferation A549 and H460 lung cancer cells by MTT assay and colony formation assay, implying antitumor potential of Fisetin in non-small cell lung cancer cells. Also, Fisetin significantly increased the Annexin V/PI positive apoptotic portion and sub G1 portion in H460 and A549 cells, indicating the cytotoxicity of Fisetin is mediated by apoptosis in H460 and A549 cells. Consistently, several papers reported the capacity of Fisetin to induce cell cycle arrest in several cancer cells [5,37]. Additionally, Fisetin attenuated the expression of G1 related proteins such as cyclin D1, cyclin E, CDK2 and activated p53 and p21, implying G1 arrest leading to cell death by Fisetin in H460 and A549 cells.

Ribosome biogenesis is known the course of producing a variety of ribosomes in the cytoplasm and nucleus to organize the production and processing of ribosomal RNAs (rRNAs) to sustain cell homeostasis and cell survival [38]. Also, the important role of ribosomal proteins was reported as the regulators of cell cycle and proliferation [38], though ribosomal proteins require the assistance of nuclear import factors during the active nuclear import process [9]. To date, exportin 1 (XPO1) is known to mediate the nuclear export of various proteins including p53 and RPL5, RPL4, RPL6 and RPL23A [39], since ribosomal proteins increase the cell proliferation of colon cancer cells through c-Myc [40,41] and p53 [39,42]. Our data demonstrate that Fisetin effectively suppressed the expression of XPO1 and c-Myc and activated the expression of p53 in A549 and H460 cells.



It is well documented that the ubiquitin-dependent proteolysis system (UPS) for protein degradation is involved in the regulation of cell proliferation, differentiation and apoptosis via the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin-protein ligase (E3) [43,44]. S-phase kinase-associated protein 2 (Skp2) for a Skp2-SCF E3 ligase complex induces proteasome mediated proteolysis through conjugating K63-linked polyubiquitin chains to targeting Akt, LKB1 and Aurora B [45] CUL4A ubiquitin ligase that forms a complex with DNA damage binding protein 1 (DDB1) and ring of cullin 1(ROC1) is known as a promising target for cancer treatment [46]. Here Fisetin attenuated the expression of cMyc, SKP2, CUL4A in A549 and H460 cells. Of note, XPO1 depletion enhanced p53 activation, while CUL4A depletion enhanced PARP cleavage in Fisetin treated H460 cells, indicating the pivotal roles of XPO1 and CUL4A. However, considering that interaction scores were 0.998 between XPO1 and p53, 0.595 between CUL4A and p53 by String database, it can be assumed that XPO1 binds to p53, while CUL4A as E3 ubiquitine ligase enhances ubiquitination in H460 cells.

## Conclusions

Fisetin increased cytotoxic and apoptotic effect by increasing the number of Annexin V/PI positive apoptotic portion and sub G1 accumulation, and the cleavages of PARP and caspase 3, activated p53/p21 and attenuating cyclin D1, cyclin E, CDK2, XPO1, cMyc, SKP2 and Cullin 4A in A549 and H460 cells. Conversely, depletion of XPO1 enhanced p53 and PARP cleavage, while depletion of UL4A promoted PARP cleavage without p53 upregulation in H460 cells. Taken together, our work strongly demonstrates the pivotal role of XPO1 mediated c-Myc and CUL4A signaling in Fisetin induced apoptotic effect in NSCLCs as a potent antitumor candidate (Figure 6). Overall, our findings suggest that Fisetin remarkably suppressed c-Myc and increased p53 in a concentration-dependent manner, along with decreased IPO7 and XPO1, suggesting the involvement of IPO7 and XPO1 linked c-Myc and p53 signaling in Fisetin-induced antiproliferative effect in NSCLCs.

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