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Fisetin induces G2/M phase cell cycle arrest by inactivating cdc25C-cdc2 via ATM-Chk1/2 activation in human endometrial cancer cells

Zhan-Ying Wang¹, Wen-Qiong Liu², Si’e Wang¹ and Zeng-Tao Wei¹

¹Department of Obstetrics and Gynecology, Jinan Central Hospital Affiliated to Shandong University, Jinan 250013, China; ²Department of Obstetrics and Gynecology, The Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan 250011, China.

Introduction

Endometrial cancer is one of the most prevailing gynecological malignancies with increasing incidence (Leslie et al., 2012) and with limited therapeutic options available for advanced and recurrent cancers.

Dysregulation of the cell cycle is the most frequent alterations in tumor development (Collins et al., 1997; Hajduch et al., 1999; Buolamwini, 2000). Among the cell cycle checkpoints, the G2/M checkpoint maintains chromosomal integrity by allowing cells to repair the DNA damage prior to mitosis and thus a target for development and identification of novel effective drugs.

DNA damage sensor, ataxia telangiectasia mutated (ATM) controls cell cycle arrest at G1 and G2 and blocks DNA synthesis that is in progress. The functions of ATM are mediated partly by checkpoint effector kinases, Chk1 and Chk2 (Zhou and Elledge, 2000; Kastan and Lim 2000; Abraham, 2001). ATM phosphorylates Chk2 on threonine 68 (Thr 68) and Chk1 on serine 317 and 345 (Ser 317 and Ser 345), causing their activation (Matsuoka et al., 1998; Melchionna et al., 2000). In response to DNA damage and DNA replication stress, Chk1 and Chk2 phosphorylate Cdc25C phosphatase, an activator of cyclin dependent kinase, Cdc2 (Bulavin et al., 2003).

Studies have reported the effect of various phytochemicals on carcinogenesis. Resveratrol (Aggarwal et al., 2004; Tyagi et al., 2006) and jaceosidin caused G2/M cell cycle arrests (Lee et al., 2013). Recent investigations suggest that fisetin attenuated metastasis (Chou et al., 2013) and...
exerted anti-proliferative effects on various cancer cells (Chen et al., 2002; Haddad et al., 2006). Mechanisms by which fisetin acts on cancer cells though not completely understood, studies have reported that it reduces the cyclin D1 and E levels, increases p53 expression and activates caspase-3 (Chen et al., 2002; Lee et al., 2002, 2005; Lu et al., 2005; Haddad et al., 2006). This study is an attempt to explore the effect of fisetin on human endometrial cancer cells.

Materials and Methods

Antibodies and reagents

Fisetin and propidium iodide was purchased from Sigma (St. Louis, MO, USA). Antibodies for cyclin B1, phospho-Cdc2 (Tyr 15), Cdc2, phospho-ATM, p21, p27, and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for phospho-Cdc25C (Ser 216), Cdc25C, phospho-H2AX (Ser 139), phospho-Chk1 and phospho-Chk2 were from Cell Signaling (Beverly, MA, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified.

Cell lines

The endometrial cancer cell lines KLE and Hec1 A were obtained from American Type Culture Collection. Cells were cultured in DMEM media supplemented with 5% fetal bovine serum (FBS), penicillin (100 µg/mL) and streptomycin sulfate (100 µg/mL).

Cell viability assay

Cytotoxicity was assessed using MTT assay. Briefly, the cells (5 x 10^4) were seeded in DMEM media (50 µL/well) in a 96-well plate and incubated for 24 h, followed by treatment with various concentrations of fisetin (20 - 100 µM). After incubation for 48 hours, 25 µL of MTT (5 mg/mL stock solution) was added and the plates were further incubated for 4 hours. The blue colour formazan crystals formed after discarding the media, were dissolved in 50 µL DMSO. Optical density was measured at 540 nm using a spectrophotometer (SpectraMax, Molecular Devices, Sunnyvale, CA, USA).

Cell cycle analysis

Endometrial cells (Hec1 A and KLE) were treated with fisetin (20-100 µM). The cells were harvested following 6 and 12 hours of incubation and washed twice with ice cold PBS. The cells were fixed and permeabilised with 70% ice-cold ethanol at 4°C for 60 min. The cells were washed once with PBS and re-suspended in a staining solution containing propidium iodide (50 µL/mL) and RNase A (250 µg/mL). The cell suspensions were incubated for 30 min at room temperature followed by fluorescence-activated cell sorting (FACS) cater-plus flow cytometry (Becton Dickin-son Co., Germany) using 10,000 cells per treatment group.

Western blot analysis

Cells were washed with cold PBS and lysed using cell lysis buffer (25 mM Tris HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) for 30 min on ice. Total protein content was determined using protein assay kit of Invitrogen™, Life technologies. Clear protein lysates (30 - 80 µg) were then fractionated by SDS-PAGE (8-12%) and electro-transferred to nitrocellulose membranes. The mem-branes were blocked with 5% non fat milk in PBST buffer (PBS, pH = 7.3 and 0.05% Tween-20) and incubi-tated overnight at 4° C with primary antibodies, followed by hybridization with horseradish peroxidise conjuga-ted secondary antibodies. The bands were detected by enhanced chemiluminescence (GE Healthcare). The band signals of proteins were normalized to those of β-actin (internal control).

Statistical analysis

The data are expressed as mean ± standard deviation (SD). The significance of the differ-ence between different groups was determined using one-way analysis of variance (one-way ANOVA) using SPSS software (version 17.0). The differences were considered significant at p<0.05.

Figure 1: Fisetin inhibits the growth of endometrial cancer cells - KLE and Hec1 A

Fisetin effectively reduced the viability of KLE and Hec1 A cells. Values are represented as mean ± S.D from 3 individual experiments.
**Results**

The anti-proliferative effect of fisetin on KLE and Hec1A cells were assessed by MTT assay (Figure 1). Following treatment with 20 µM fisetin, the viability was 90.3% in Hec1A cells and 92.8% in KLE cells. However, with increasing concentrations of fisetin, the viable cell count sharply decreased thus exerting a dose-dependent effect in the viability of cancer cells. At 100 µM fisetin exhibited a multi-fold decrease in the viable cell number compared to the lower concentrations (20 and 40 µM). While the anti-proliferative effect was evident from the observed results, fisetin caused a more marked decline in the viability of Hec1 A than KLE cells.

![Figure 2: Effect of fisetin on cell cycle in KLE cells](image)

Fisetin at (20-100 µM) induces G2/M cell cycle arrest in KLE cells in a dose and time-dependent manner. The increase in G2/M cells following fisetin exposure at 12 hours (A) were higher than at 6 hours exposure (B).

To determine whether the growth inhibitory effect is associated with cell cycle arrest, the distribution of cells in each phase of the cell cycle was analyzed using flow cytometry. As depicted in Figures 2 and Figure 3, exposure of KLE and Hec1 A cells to growth suppressive concentrations of fisetin (20-100 µM) resulted in a significant increase in G2/M fraction. After treatment with 100 µM fisetin for 6 and 12 hours, the percentage of cells in the G2/M phase was 42.1 and 44.8% respectively in KLE cells and 44.16 and 45.90% in the Hec1 A cells (Figures 2, 3).

Furthermore, the G2/M arrest induced by fisetin was observed to occur in a time- and dose-dependent
manner. The number of cells at G2/M phase following 12 hours exposure was higher than 6 hours exposure to fisetin in both KLE and Hec1 A. However, as in cell viability, the cell cycle arrest was more pronounced in Hec1 A than KLE cells. In our study, fisetin exposure induced significant increase in G2/M fraction and this was accompanied by a decrease in G0/G1 and S phase cells. This indicates that the cell inhibitory effect of fisetin against the proliferation of KLE and Hec1 A cells is correlated with cell-cycle arrest.

Cyclins, cyclin-dependent kinases (CDKs) and their inhibitors regulate cell cycle progression and arrest. Cyclin B1 and Cdc2 form a complex and co-operate to promote the G2/M phase transition. We investigated whether exposure to fisetin at 20-100 µM for 12 hours affected the expression of cyclin B1 and Cdc2 in cancer cells. Treatment with fisetin considerably suppressed the levels of cyclin B1 and induced a remarkable increase in the phosphorylation of Cdc2 at Tyr 15 (Figure 4). The expression of cyclin B1 decreased nearly 2-fold on exposure to fisetin at 100 µM. Enhanced levels of phosphorylation of Cdc2 at Tyr 15 suppresses its kinase activity and reduces the expression levels of cyclin B1 and subsequently leads to the inactivation of Cdc2-cyclinB1 kinase complex, thereby causing cell cycle arrest. Further, Cdc25C phosphatase regulates the phosphorylation state of Cdc2 at Tyr 15. The phospho-

Figure 3: Influence of fisetin on cell cycle in Hec1 A cells

Exposure to fisetin at (20 -100 µM) for 6 hours (A) and 12 hours (B) induces G2/M cell cycle arrest in a time and dose-dependent manner

Cyclins, cyclin-dependent kinases (CDKs) and their inhibitors regulate cell cycle progression and arrest. Cyclin B1 and Cdc2 form a complex and co-operate to promote the G2/M phase transition. We investigated whether exposure to fisetin at 20-100 µM for 12 hours affected the expression of cyclin B1 and Cdc2 in cancer cells. Treatment with fisetin considerably suppressed the levels of cyclin B1 and induced a remarkable increase in the phosphorylation of Cdc2 at Tyr 15 (Figure 4). The expression of cyclin B1 decreased nearly 2-fold on exposure to fisetin at 100 µM. Enhanced levels of phosphorylation of Cdc2 at Tyr 15 suppresses its kinase activity and reduces the expression levels of cyclin B1 and subsequently leads to the inactivation of Cdc2-cyclinB1 kinase complex, thereby causing cell cycle arrest. Further, Cdc25C phosphatase regulates the phosphorylation state of Cdc2 at Tyr 15. The phospho-
Figure 4: Fisetin modulates the expressions of cell cycle regulatory proteins
Fisetin at various concentrations modulates the expressions of cell cycle regulatory proteins in KLE (A, B) and Hec1 A cells (C, D) and causes G2/M cell cycle arrest. Values are presented as mean ± S.D from 3 individual experiments. *represents statistical significance at p<0.05 compared against control as determined by one-way ANOVA.
Figure 4: Fisetin modulates the expressions of cell cycle regulatory proteins (Cont.)

Fisetin at various concentrations modulates the expressions of cell cycle regulatory proteins in KLE (A, B) and Hec1 A cells (C, D) and causes G2/M cell cycle arrest. Values are presented as mean ± S.D from 3 individual experiments. *represents statistical significance at p<0.05 compared against control as determined by one-way ANOVA
Phosphorylation of Cdc25C following treatment with fisetin at 20-100 µM was determined. The level of p-Cdc25C (Ser 216) was notably increased on fisetin treatment (Figure 4) while the levels of Cdc25C remained not much altered. The results suggest that fisetin-induced G2/M arrest is associated with the negative regulation of cyclin B1 and Cdc2 in Hec1A and KLE cells.

Chk1/2 kinases act up-stream of Cdc25C (Matsuoka et al., 1998). As shown in Figure 4, fisetin markedly up-regulated the phosphorylation status of Chk1/2 kinases. The elevated expression of Chk1/2 kinases was observed to occur in a dose-dependent manner in both Hec1A and KLE cells. Thus could be suggested that the loss of Cdc25C phosphatase activity observed can result from the phosphorylation of inhibitory sites by Chk1/2.

ATM kinases are central in triggering cellular responses to DNA damage/genotoxic stress in eukaryotic cells (Kastan and Lim, 2000; Shiloh, 2003) and are known to activate Chk1 and Chk2 (Khanna et al., 2001). We examined the expression of p-ATM on exposure to fisetin. Fisetin treatment of cells for 12 hours resulted in a strong increase in the levels of p-ATM protein, which showed that ATM was activated. Further, fisetin caused increased serine 139 phosphorylation of histone H2AX that is phosphorylated by ATM upon its activation by phosphorylation. These results suggest that fisetin-induced G2/M phase arrest involves ATM checkpoint signalling.

Fisetin treatment markedly increased both p21 and p27 expressions in Hec1 A and KLE cells in a dose-dependent manner (Figure 5). The levels of p21 and p27 increased multifold on exposure to 100 µM fisetin when compared to the expression levels following treatment with 20 µM fisetin. In addition p21 and p27 expression was more pronounced in Hec1 A than KLE cells. Furthermore, p21 expression was higher than p27 upon fisetin treatment in both Hec1 A and in KLE cells.

Discussion

In our study, fisetin was observed to effectively modulate the levels of cyclin B1 and p-Cdc2. Fisetin induced marked elevations in the phosphorylated forms of Cdc25C which could have caused raised p-Cdc2 levels thus ultimately leading to their inactivation and cell cycle arrest.

Phosphorylation of Cdc25C is controlled by Chk1 and Chk2 (Bartek and Lukas, 2003). However activity of Chk1 and Chk2 are regulated by ATM, which in turn is activated in response to DNA damage. Activated ATM results in the activation of Chk1 and Chk2 by phosphorylation, and also stabilizes p53 by phosphorylation (Al Rashid et al., 2011).

Fisetin caused a significant increase in p-ATM levels...
thus indicating ATM activation which induces phospho- 
-rylation of Chk1 (Ser 345) and Chk2 (Ser 516) and also 
phosphorylation of Cdc25C (Ser 216) and Cdc2 (Tyr 15). 
Furthermore, in our study, fisetin exposure resulted in 
phosphorylation of H2AX (Histone H2A), a substrate of 
ATM kinases. H2AX phosphorylation is a major and 
early response to double-stranded DNA breaks (DSBs) 
(Fernandez-Capetillo et al., 2003). ATM kinase induces 
phosphorylation of ser 139 on the histone H2AX tail 
and the subsequent rapid formation of H2AX foci at the 
DSB sites (Redon et al., 2002). These observations 
suggest that fisetin may possibly cause a DNA damage 
response.

We also assessed the role of p21 and p27 in the 
response to fisetin-induced G2/M arrest. p21(WAF1/CIP1), a member of the 
cyclin dependent kinase inhibitor (CDKI) family plays a 
role in both the G1 and G2 checkpoints (Harper et al., 
1993; Ando et al., 2001). Fisetin at various concentra-
tions caused a significant increase in p21 and p27 
expression both in KLE and Hec1 A cells suggesting 
that it effectively blocks cell cycle progression.

Development of novel therapeutic agents is a critical 
need of today for the treatment and survival of patients 
with late-stage and recurrent endometrial cancer (Garai 
et al., 2006). Increasing amount of evidence suggests 
that certain phytochemicals have marked cancer 
chemopreventive properties (Waladkhani and Clemens, 
1998; Kelloff et al., 2000). Observations of the present 
investigation suggest that fisetin strongly inhibits cell 
cycle progression by causing G2/M cell cycle arrest. 
The findings of the study are in line with previous 
reports (Park et al., 2009; Shin et al., 2011; Lee et al., 
2013) demonstrating the significance of flavonoids inhib-
biting cell growth.

In conclusion, fisetin effectively causes G2/M cell cycle 
arrest by modulating the expression of vital cell cycle 
regulating proteins- cdc25C-cdc2 via ATM-Chk1/2 activation.

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