Proteomic analysis of selective cytotoxic anticancer properties of flavonoids isolated from *Citrus platymamma* on A549 human lung cancer cells

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Abstract. *Citrus platymamma* Hort. ex Tanaka (Byungkyul in Korean) has been used in Korean folk medicine for the treatment of inflammatory disorders and cancer. However, the molecular mechanism underlying the anticancer properties of flavonoids isolated from *C. platymamma* (FCP) remains to be elucidated. Therefore, the present study attempted to identify the key proteins, which may be important in the anticancer effects of FCP on A549 cells using a proteomic approach. FCP showed a potent cytotoxic effect on the A549 human lung cancer cells, however, it had no effect on WI-38 human fetal lung fibroblasts at the same concentrations. Furthermore, 15 differentially expressed protein spots (spot intensities ≥2-fold change; $P<0.05$) were obtained from comparative proteome analysis of two-dimensional gel electrophoresis maps of the control (untreated) and FCP-treated A549 cells. Finally, eight differentially expressed proteins, one of which was upregulated and seven of which were downregulated, were successfully identified using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight tandem mass spectrometry and peptide mass fingerprinting analysis. Specifically, proteins involved in signal transduction were significantly downregulated, including annexin A1 (ANXA1) and ANXA4, whereas 14-3-3ε was upregulated. Cytoskeletal proteins, including cofilin-1 (CFL1), cytokeratin 8 (KRT8) and KRT79, and molecular chaperones/heat shock proteins, including endoplasmin, were downregulated. Proteins involved in protein metabolism, namely elongation factor Ts were also downregulated. Consistent with results of the proteome analysis, the immunoblotting results showed that 14-3-3ε was upregulated, whereas CFL1, ANXA4 and KRT8 were downregulated in the FCP-treated A549 cells. The majority of the proteins were involved in tumor growth, cell cycle, apoptosis, migration and signal transduction. These findings provide novel insights into the molecular mechanisms underlying FCP-induced anticancer effects on A549 cells.

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Introduction

Lung cancer remains a leading cause of cancer-associated mortality worldwide, and non-small cell lung cancer (NSCLC) is a predominant type, accounting for 75-80% of lung cancer cases (1,2). Although surgical resection, chemotherapy and radiotherapy are available, the long-term survival rates of patients with NSCLC remain poor. Therefore, a shift in paradigm is required from increasing the survival rates of patients with lung cancer patients to preventing lung cancer development. Previous investigations on cancer have focused on natural herbs in preventing or controlling cancer as an alternate therapy (3-5). In addition, natural substances and plant derivatives have been used to treat cancer patients with reduced toxicity. Previous reports have also suggested that flavonoids in fruit can enhance anticancer effects (6-10), indicating that these substances are suitable for chemoprevention. *Citrus platymamma* Hort. ex Tanaka (Byungkyul in Korean) has been used in Korean folk medicine for the treatment of various diseases, including cancer. Flavonoids, which has been reported in citrus species, including *C. platymamma,* exert

antiproliferative, anticancer, antioxidant, anti-inflammatory and antidiabetic activities (11-14). However, the molecular mechanism underlying the anticancer effect of flavonoids from *C. platymamma* (FCP) on lung cancer remains to be elucidated.

Proteomics is now an important area of investigation in various fields, including cancer biology. Proteome analysis has been applied in the investigation of various types of cancer *in-vitro* and *in vivo* (15-19), including lung cancer (20-22). Previously, the anticancer mechanisms of therapeutic agents have been elucidated by performing comparative proteome analysis on A549 cells (23-25). The present study was conducted to investigate the mechanism of the anti-cancer effect of FCP-treated A549 cells by examining the expression of proteins involved in cancer cell survival, apoptosis, differentiation, invasion, metastasis and metabolism. Increased understanding of the molecular mechanisms underlying the anticancer effects of FCP may provide novel insights in the prevention of lung carcinogenesis, which may assist in developing novel strategies not only to prevent cancer development, but also to improve quality of life for patients with lung cancer.

Our previous study demonstrated that FCP induced G2/M cell cycle arrest and apoptosis in A549 human lung cancer cells (26). Therefore, the present study aimed to identify the differentially expressed key proteins, which may underlie the anticancer effects of FCP on A549 cells, using a proteomic approach. In total, eight differentially expressed proteins were identified using two-dimensional gel electrophoresis, coupled with matrix-assisted laser desorption/ionization time-of-flight/time-of-flight tandem mass spectrometry (MALDI-TOF/TOF-MS) analysis; 14-3-3ε (YWHAE) was upregulated, and cofilin-1 (CFL1), annexin A1 (ANXA1), annexin A4 (ANXA4), endoplasmin (HSP90B1), cytoskeratin 8 (KRT8), elongation factor Ts (tsf) and uncharacterized protein (KRT79) were downregulated. The expression levels of YWHAE, CFL1, ANXA4 and KRT8 were also validated by immunoblotting. To date, this is first study, to the best of our knowledge, to use proteomic techniques to investigate the molecular mechanisms underlying the anticancer effects of FCP on A549 cells.

Materials and methods

Chemical and reagents. The A549 human lung cancer cells and WI-38 normal human fetal lung fibroblast cells were obtained from the Korea Cell Line Bank (Seoul, Korea). RPMI 1640 medium was purchased from GE Healthcare Life Sciences Hyclone Laboratories (Logan, UT, USA). Fetal bovine serum (FBS) and antibiotics (streptomycin/penicillin) were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). 5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Materials and chemicals used for electrophoresis were obtained from Bio-Rad Laboratories, Inc, (Hercules, CA, USA). FCP was provided by Animal Bio-Resources Bank (Jinju, Korea). Antibodies targeting CFL1 (cat. no. AB3831), ANXA4 (cat. no. ABC885) and KRT8 (cat. no. MAB1611) and β-actin (cat. no. MABT825) were purchased from EMD Millipore (Billerica, MA, USA). Antibody targeting YWHAE (cat. no. BS6109) was obtained from Bioworld Technology, Inc. (St. Louis Park, MN, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (ALX-211-205TS-C100) and anti-rabbit IgG (ADI-SAB-301-J) were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). All other chemicals used in the present study were purchased from Amresco, Inc. (Solon, OH, USA) and Sigma-Aldrich. All chemicals used were of the highest grade commercially available.

Cytotoxicity assay. The A549 cells and WI-38 cells ($1x10^5$) were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin in a humidified incubator with 5% CO_2 in air 37˚C. The cells were cultured in 12-well plates and incubated overnight. Subsequently, the cells were treated with 0, 100, 200, 300, 400 and 500 μg/ml of FCP for 24 h. Following incubation, 100 ml of 0.5 mg/ml MTT solution was added to each well and incubated for 3 h at 37˚C in the dark. The formazan contained in the cells was solubilized by the addition of 500 ml of DMSO, and the absorbance was measured at 540 nm using an enzyme-linked immunosorbent assay plate reader.

Preparation of the total cellular extract for 2-DE. The total proteins were extracted from the A549 cells in the untreated (control) and FCP-treated groups. Following incubation with FCP, the cells were lysed in lysis buffer, containing 7 M urea, 2 M thiourea and 4% (w/v) CHAPS, on ice for 1 h. The lysates were then centrifuged at 13,000 x g for 15 min at 4˚C, and the collected supernatant was stored at -80˚C until analysis. The total protein was used for 2-DE. The protein concentration was determined using the Non-Interfering™ protein assay kit (G-Biosciences, St. Louis, MO, USA), in accordance with the manufacturer's protocol.

2-DE and image analysis. An equal quantity (150 μg) of protein per sample was loaded onto a 18 cm linear IPG strip (pH 4-7; Amersham Biosciences; Uppsala, Sweden) for first-dimensional isoelectrofocusing, which was followed by 12% second dimension sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on an Ettan DALT II system (Amersham Biosciences). The gels were stained with silver nitrate, as described previously with modifications (27), and three independent gels were used in triplicate. Briefly, gels were incubated in fixation solution (50% ethanol and 5% acetic acid) for 15 h, washed once with 30% ethanol for 15 min followed by three times with distilled water for 5 min each. The gels were stained with silver nitrate (0.3%) in the dark for 25 min at room temperature. The gels were subsequently rinsed with water three times and developed with solution containing 3% sodium carbonate, 0.02% sodium thiosulfate and 0.05% formalin. The gels were scanned and image analysis was performed using Progenesis Samespots software (Nonlinear Dynamics, Newcastle, UK). Using this software, the differentially expressed spots were identified by automatic matching of the detected protein spots. Those spots differing significantly ($P<0.05$) in their intensities (fold-change ≥2), in the FCP-treated A549 cells were used for further analysis.

MALDI-TOF/ TOF MS analysis. Selected protein spots were excised manually from the 2-DE gel, and protein digestion was performed (28) with modifications. Briefly, the excised gel pieces were washed with 100 μl 100 mM NH_4HCO_3 for 5 min and then dehydrated in 100 μl acetonitrile for 10 min. Following drying in a lyophilizer (SFDSM06; Samwon Freezing Engineering Co., Busan, South Korea), the gel pieces were rehydrated in 5-10 μl 50 mM NH_4HCO_3 containing 20 ng/μl trypsin (Promega Corporation, Madison, WI, USA) on ice. After 45 min, the trypsin solution was removed and replaced with 10-20 μl 50 mM NH_4HCO_3 without trypsin, and digestion was performed for a minimum of 16 h at 37˚C. Subsequently, the peptide mixtures were targeted onto a MALDI-TOF/TOF plate and analyzed using a Voyager-DE STR mass spectrometer (Applied Biosystems; Thermo Fisher Scientific, Inc.), equipped with delay ion extraction.

Database search. The proteins were identified using the Mascot program (http://www.matrixscience.com). The Swissprot database and peptide mass fingerprinting (PMF) data were used to identify matching proteins. The following parameters were used for the database searches: Taxonomy, *Homo sapiens* (human); cleavage specificity, trypsin with one missed cleavage permitted; peptide tolerance of 100 ppm for the fragment ions; permitted modifications, fixed cysteine carbamidomethylation, variable oxidation of methionine. Protein scores >84 were considered statistically significant (P<0.05).

Western blot analysis. The A549 cells ($2x10^5$) were cultured in 6-well plates and incubated with FCP (363 μg/ml) for 24 h. The cell lysates was prepared, and 30 μg of proteins were separated by 12% SDS-PAGE and transferred onto a PVDF membrane. The blots were blocked with 5% nonfat dry milk for 1 h at room temperature and then incubated with primary antibodies overnight (dilution, 1:1,000), followed by incubation with HRP-conjugated goat anti-mouse IgG (dilution, 1:1,000) for 2 h at room temperature. The signal was visualized using ECL detection reagent (GE Healthcare Life Sciences) and quantified by densitometry using the Image J (http://rsb.info.nih.gov) program. The densitometry readings of the bands were normalized to the expression of β-actin. The experiment was repeated three times.

Gene ontology (GO) analysis. GO analysis was performed using the Agbase database (http://www.agbase.msstate.edu/), as previously described (29). GO annotations were obtained from GORetriever by submitting the spot identities. The annotation results were summarized based on the GOA and whole proteome GOSlim set using GOSlimViewer (agbase.msstate.edu/cgi-bin/tools/goslimviewer_select.pl).

Statistical analysis. All statistical analyses were performed using SPSS software (SPSS for Windows, ver. 10.0; SPSS, Inc. Chicago, IL, USA). The data are presented as the mean ± standard deviation of at least three independent experiments. The statistical significance between the control and test groups was determined using one-way analysis of variance followed by Student's *t*-test. P<0.05 was considered to indicate a statistically significant difference.

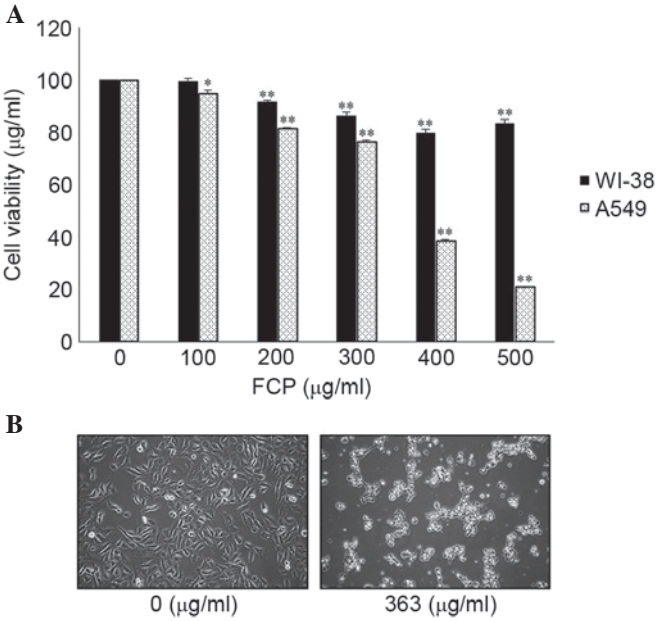

Figure 1. Cytotoxic effects of FCP against WI-38 cells and A549 human lung cancer cells. The cells were incubated at indicated concentrations of FCP for 24 h. The cell viability was analyzed using a 5-diphenyltetrazolium bromide assay. (A) FCP showed growth inhibitory and cytotoxic effects against A549 cells in a dose-dependent manner. No cytotoxic effects were found against the WI-38 cells. (B) Morphological changes in the FCP-treated A549 cells were visualized using optical microscopy (magnification, x400). The data are shown as the mean ± standard deviation of three independent experiments (*P<0.05 and **P<0.01, vs. control). FCP, flavonoids isolated from *Citrus platymamma*.

Results

FCP specifically inhibits the proliferation of A549 human lung cancer cells. To investigate the anticancer activity of FCP, A549 human lung cancer cells and WI-38 human fetal lung fibroblasts were treated with the indicated concentrations (0-500 μg/ml) of FCP for 24 h. FCP inhibited the growth of the A549 cells in a dose-dependent manner, however, no definite cytotoxic effects were found in the WI-38 cells (Fig. 1A) and cytotoxicity was negligible at concentrations of ≥200 μg/ml. These results showed that FCP exhibited a level of specific cytotoxicity towards cancer cells. The 50% inhibitory concentration for FCP treatment was found to be ~363 μg/ml. In addition, morphological changes, including cell shrinkage and density, and decreased cell numbers were observed in the FCP-treated A549 cells (Fig. 1B). These results suggested that the FCP specifically inhibited the A549 lung cancer cells.

2-DE analysis and identification of differentially expressed proteins using MALDI-TOF/TOF-MS. Subsequently, to investigate the mechanism underlying the inhibitory effects of FCP on A549 cell proliferation, 2-DE gel analysis was performed. The representative 2-DE patterns of the untreated (control) and FCP-treated A549 cells were obtained by resolving 150 μg of total proteins on IPG strips (18 cm; pH 4-7) in the first dimension and 12% SDS-PAGE in the second dimension (Fig. 2). In total, 15 differentially expressed protein spots were identified (≥2-fold; P<0.05) using Progenesis

Table I. List of differentially expressed proteins in A549 cells treated with flavonoids isolated from *Citrus platymamma*, identified using MALDI-TOF/TOF-MS analysis.

Spot no.	Accession number[a]	Protein name (gene name)[a]	Swiss-Prot entry name[a]	Theoretical[a]/ experimental[c] Mr (kDa)	Theoretical[a]/ experimental[c] pI value	Sequence coverage (%)/peptides matched	Mascot score[b]	Fold change[c]	P-value (*t*-test[c])
1	F6XWG3	Uncharacterized protein (KRT79)	F6XWG3_HORSE	58.10/32	7.62/6.22	27/16	90	3.3↓	0.010
2	P62258	14-3-3 protein epsilon (YWHAE)	1433E_HUMAN	26.91/37	4.92/4.48	44/13	141	3.8↑	0.015
3	P09525	Annexin A4 (ANXA4)	ANXA4_HUMAN	36.09/35	5.84/5.93	52/32	342	7.4↓	0.021
4	S7MQH4	Cofilin-1 (CFL1)	S7MQH4_MYOBR	18.73/16	8.22/6.54	63/11	105	14.3↓	0.025
5	G8XJK6	Elongation factor Ts (tsf)	G8XJK6_MYCHR	33.24/44	5.98/6.48	50/17	122	3.0↓	0.027
6	P08712	Endoplasmin (HSP90B1)	ENPL_MESAU	46.88/54	4.96/4.9	30/16	118	6.1↓	0.029
7	G1QL21	Annexin n=1 (ANXA1)	G1QL21_NOMLE	38.89/29	6.57/5.28	30/13	97	2.7↓	0.036
8	P05787	Keratin, type II cytoskeletal 8 (KRT8)	K2C8_HUMAN	53.67/79	5.52/5.58	43/29	430	3.7↓	0.046

[a]Accession number, protein name, theoretical molecular weight (Da) and pI from the Swiss-Prot database, identified using MALDI-TOF/TOF-MS. [b]Score is -10xlog(p), where p is the probability that the observed match is a random event, Protein scores >84 are considered to be significant (P<0.05). [c]Experimental Mr and pI, fold-change (≥2) and *t*-test P-values (P<0.05) were obtained using Progenesis Samespots software. Arrows ↑ and ↓ indicate upregulation and downregulation, respectively. MALDI-TOF/TOF-MS, matrix-assisted laser desorption/ionization time-of-flight/time-of-flight tandem mass spectrometry; MR, molecular weight; pI, isoelectric point.

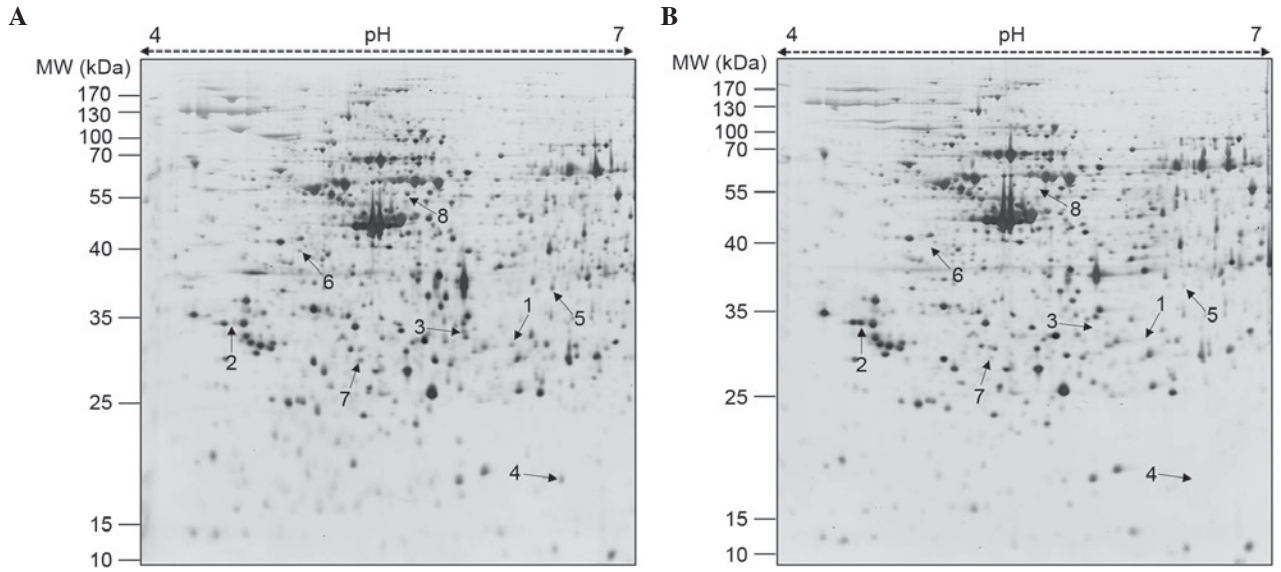

Figure 2. Two-dimensional gel electrophoresis proteome maps of the control and FCP-treated A549 cells. The (A) control and (B) FCP-treated (363 μg/ml) A549 cells were incubated with the indicated concentrations of FCP for 24 h. The total proteins were separated on 18 cm linear IPG strips (pH 4-7) in the first dimension and 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis in the second dimension. The gels were silver stained. The numbered arrows indicate protein spots successfully identified by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight tandem mass spectrometry The experiments were performed in triplicate. FCP, flavonoids isolated from *Citrus platymamma*.

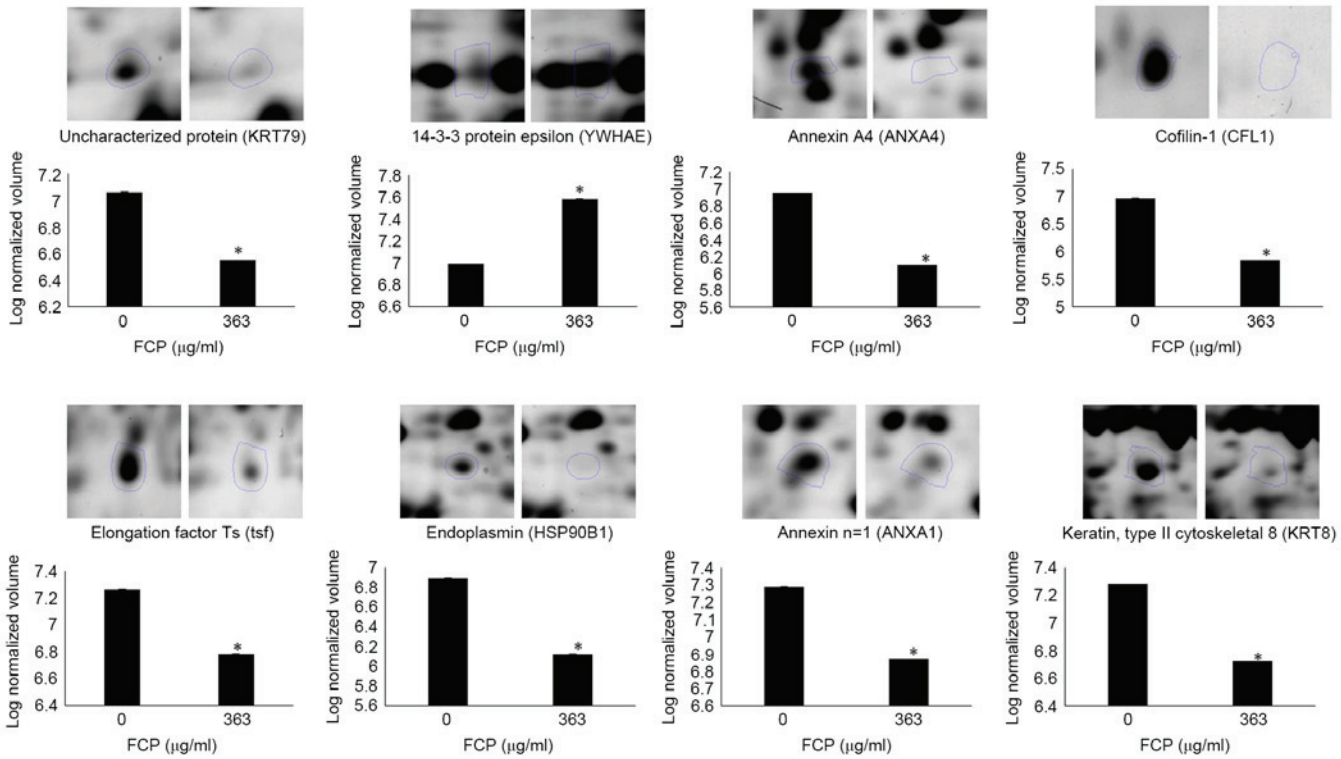

Figure 3. Expression profiles of enlarged two-dimensional gel electrophoresis spots identified in FCP-treated A549 cells. Gels were scanned and image analysis was performed using Progenesis Samespots software. The spots differing significantly in their intensities (fold-change ≥2) in FCP-treated A549 cells, compared with untreated control cells. The data are shown as the mean ± standard deviation of three independent experiments (*P<0.05 vs. control). FCP, flavonoids isolated from *Citrus platymamma*.

Samespots image analysis software (version 4.0). Finally, eight differentially expressed proteins, one upregulated and seven downregulated, were detected in the FCP-treated A549 cells using MALDI-TOF/TOF-MS analysis and database searching (Fig. 2 and Table I). Specifically, YWHAE was upregulated, and ANXA4, CFL1, tsf, HSP90B1, ANXA1, KRT8 and KRT79 were downregulated in the FCP-treated A549 cells, compared with the control cells (enlarged 2-DE images in Fig. 3). The annotation of all identified proteins with their corresponding Swissprot accession number, experimental and theoretical

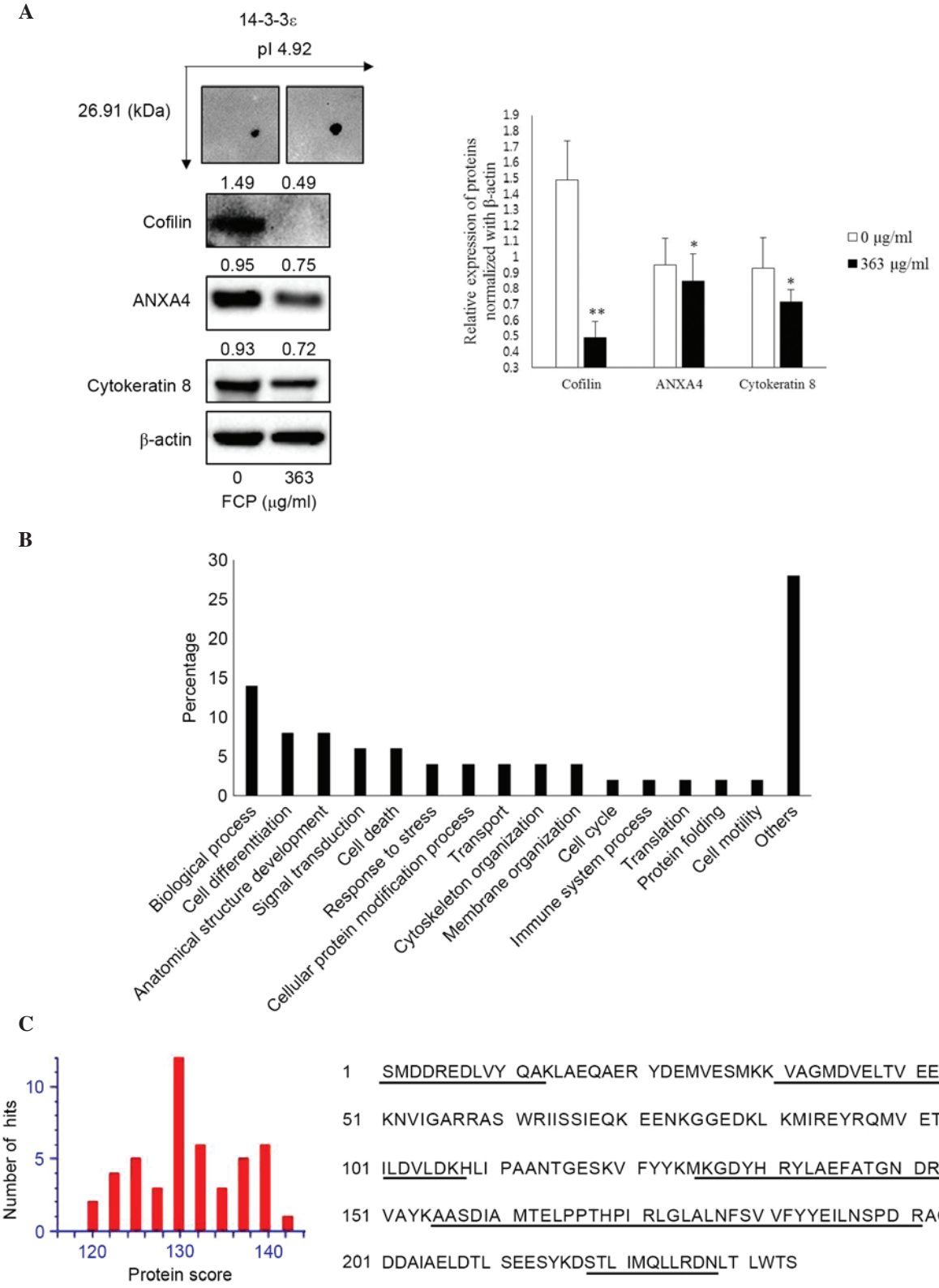

Figure 4. Validation of the results from two-dimensional gel electrophoresis using immunoblotting. The cells were incubated at indicated concentrations of FCP for 24 h. (A) Expression levels of 14-3-3ε, cofilin, ANXA4 and cytokeratin 8 were assayed using western blot analysis. (B) Gene ontology analysis of the identified proteins, according to their biological function, using the GOSlimViewer tool at Agbase (http://www.agbase.msstate.edu/). (C) Top hits of protein scores for spot no. 2 (14-3-3ε) when MSDB was searched with peptide mass fingerprinting and matched peptides (shown in red), with 44% coverage. The experiments were performed in triplicate (*P<0.05 and **P<0.01, vs. control). FCP, flavonoids isolated from *Citrus platymamma*; ANXA4, annexin A4.

molecular weight, experimental and theoretical isoelectric point, sequence coverage and number of peptide matches, Mascot score, expression and statistical values are shown in Table I. Based on protein functions, the identified proteins were divided into the following categories: Cytoskeletal proteins (CFL1, KRT8 and KRT79), signal transduction

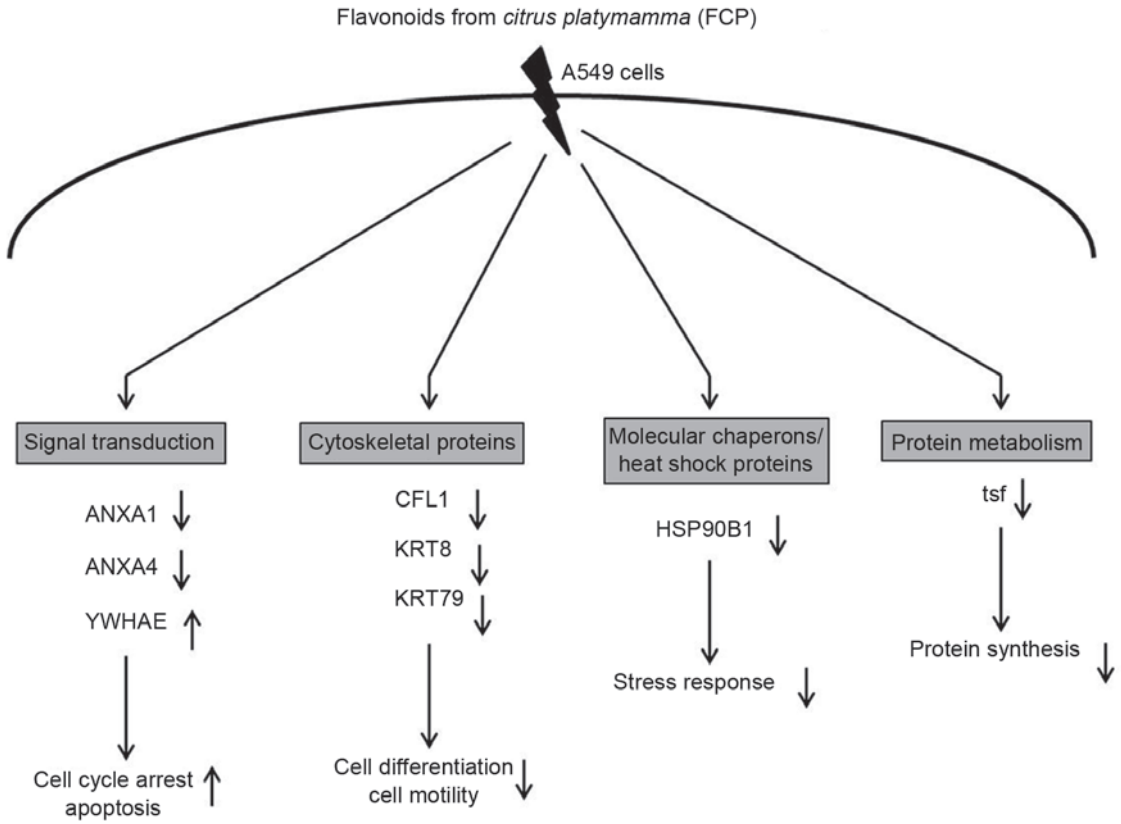

Figure 5. Biological role of eight differentially expressed proteins identified in FCP-treated A549 cells using two-dimensional gel electrophoresis coupled with matrix-assisted laser desorption/ionization time-of-flight/time-of-flight tandem mass spectrometry analysis. Specifically, proteins involved in signal transduction were significantly downregulated, including ANXA1 and ANXA4, whereas YWHAE was upregulated. Cytoskeletal proteins, including CFL1, KRT8 and KRT79, and molecular chaperones/heat shock proteins, including HSP90B1, were downregulated. Proteins involved in protein metabolism, namely tsf, were also downregulated. The majority of these proteins were involved in tumor growth and progression, cell cycle, stress response and apoptosis. (↑ indicates upregulation of protein and ↓ indicates downregulation of protein). ANXA, annexin A; YWHAE, 14-3-3ε; CFL1, cofilin-1; KRT8, cyroskeratin 8; KRT79, uncharacterized protein; HSP90B1; endoplasmin; tsf, elongation factor Ts.

(YWHAE, ANXA1 and ANXA4), molecular chaperons/heat shock proteins (HSP90B1) and protein metabolism (tsf). The identified proteins were predominantly involved in tumor growth, cell cycle, apoptosis, migration and signal transduction.

Validation of differential expressed proteins using western blot analysis. The immunoblotting was performed to confirm the expression of proteins, which were identified in the FCP-treated A549 cells using proteome analysis. The results showed that YWHAE was significantly upregulated, whereas CFL1, ANXA4 and KRT8 were significantly downregulated in the FCP-treated A549 cells, compared with the control ($P<0.05$; Fig. 4A). These findings suggested that the results of the immunoblotting were consistent with those of the comparative proteomic analysis.

GO analysis. The GO terms for biological processes were examined for all eight identified proteins. The most notable functional categories in terms of the protein expression pattern are shown in Fig. 4B. The highest associations were with biological processes (14%; GO:0008150). Another 8% of the associations were with cell differentiation (GO:0030154) and anatomical structure development (GO:0048856), whereas 6% were associated with signal transduction (GO:0,007165) and cell death (GO:0008219). Of the remainder, 4% were associated with response to stress (GO:0006950), cellular protein modification process (GO:0006464), cytoskeleton organization (GO:0007010), membrane organization (GO:0061024) and transport (GO:0006810).

Discussion

The present study focused on the differentially expressed proteins, which are involved in the behaviors of FCP-treated A549 cells using proteome techniques. Of 15 differentially expressed protein spots, eight proteins were successfully identified in the FCP-treated A549 cells using 2-DE coupled with MALDI-TOF/TOF-MS analyses (Fig. 2 and Table I). The identified proteins were predominantly involved in tumor growth and progression, and the apoptosis of A549 cells. These results indicated that FCP inhibited cell proliferation and induced cell death of the A549 cells by regulating those proteins. The results of the immuneoblotting confirmed that the expression of YWHAE was significantly upregulated, and the expression levels of CFL8, ANXA4 and KRT8 were significantly downregulated in the A549 cells following incubation with FCP (363 μg/ml) for 24 h. These data suggested that FCP inhibited the growth of A549 cells by altering the expression of proteins, which are involved in tumor growth

and progression. This finding is consistent with those of the previous study, demonstrating that FCP induces G2/M cell cycle arrest and apoptosis in A549 lung cancer cells.

The 14-3-3 proteins are a highly conserved protein family in eukaryotic cells, and comprise seven isoforms (β, ε, γ, η, σ, τ/θ and ζ), which are crucial for regulating multiple cellular processes, including signal transduction, cell cycle regulation, apoptosis DNA repair, cytoskeletal regulation, cellular metabolism, proliferation, transcription, and redox-regulation or the stress response (30,31). Among the 14-3-3 isoforms, the overexpression of YWHAE has been demonstrated in various types of human malignancy, including lung cancer (32,33). In addition, the reduced expression of YWHAE in gastric cancer is associated with gastric carcinogenesis (34). In the present study, the expression of YWHAE (spot no. 2) was significantly increased in the FCP-treated A549 cells (Fig. 3). Consistent with the 2-DE results, the expression of YWHAE was further confirmed by immunoblotting analysis (Fig. 4A). Figure. 4C shows the protein scores for the top hits for YWHAE when MSDB was searched with PMF and matched peptides with 44% coverage. However, the role of the YWHAE protein in apoptosis remains controversial; another study showed that non-steroidal anti-inflammatory drugs induce apoptosis by the suppression of 14-3-3ε YWHAE in colorectal cancer cells (35). Therefore, further detailed studies are needed regarding the role of the YWHAE protein in the anticancer effects of FCP on A549 cells.

The annexins, a family of phospholipid-binding proteins, involved in various physiological processes, including anticoagulation, anti-inflammatory, endocytosis and exocytosis, signal transduction, cell proliferation, differentiation and apoptosis (36,37). ANXA1 is a calcium-dependent phospholipid-linked protein, differentially expressed in different types of cancer (38). The upregulation of ANXA1 in patients with lung cancer is associated with a poor clinical outcome (39,40). In addition, Biaoxue *et al* (41) demonstrated that the co-overexpression of Hsp90-β and ANXA1 was associated with poor survival rates and lymphatic metastasis in patients with lung cancer patients. The increased expression of ANXA4 is associated with drug resistance to paclitaxel, a drug commonly used for the treatment of cancer (42). In addition, the elevated expression of ANXA4 is associated with advanced T stages in colorectal cancer and lymph node metastasis in human penile squamous cell carcinoma (43,44). In the present study, the expression levels of ANXA1, ANXA4 and HSP90B1 (spot nos. 7, 3 and 6, respectively) were significantly downregulated in FCP-treated A549 cells (Figs. 3 and 4A). These results indicated that FCP exerted anticancer effects in A549 cells by suppressing the ANXA1, ANXA4 and HSP90B1 proteins.

In the present study, the expression of CFL1 (spot no. 4) was significantly downregulated in the FCP-treated A549 cells (Figs. 3 and 4A). CFL1, the actin regulatory protein, is important in tumor growth and progression (45). It has been reported that CFL1 is involved in tumor progression in ovarian carcinoma, almost 64% of all ovarian tumors are positive for CFL1 (46). In prostate cancer, knockdown of CFL1 was reported to increase sensitivity to docetaxel, a chemotherapeutic agent (47). In addition, the expression of KRT8 (spot no. 8) was also downregulated in FCP-treated A549 cells (Figs. 3 and 4A). The increased expression of KRT8 was significantly associated with tumor progression, and decreased survival rates in patients with NSCLC (48). These data suggested that the downregulation of CFL1 and KRT8 may also be involved in the anticancer effect of FCP on A549 cells.

In conclusion, the present study demonstrated the anticancer effects of FCP on A549 human lung cancer cells using a proteomic approach. In the present study, eight differentially expressed proteins (YWHAE was upregulated; CFL1, ANXA1, ANXA4, HSP90B1, KRT8, Tsf and KRT79 were downregulated) were identified in the FCP-treated A549 cells, which were found to be involved in tumor growth, cell cycle, apoptosis, migration and signal transduction (Fig. 5). Furthermore, the expression levels of YWHAE, CLF1, ANXA4 and KTR8 were validated by immunoblotting. To the best of our knowledge, the present study was the first to use the proteomic technique to investigate the molecular mechanism in FCP- treated A549 cells. The findings of the present study improve understanding of the molecular mechanism underlying the selective growth inhibition of FCP on A549 cells, which may offer a therapeutic potential for the treatment of lung cancer.

Acknowledgements

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