Jinfukang induces cellular apoptosis through activation of *Fas* and *DR4* in A549 cells

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Abstract. The traditional Chinese medicine Jinfukang (JFK) has been shown as a valuable drug to treat non-small cell lung cancer (NSCLC). Previously, it was reported that JFK-induced epigenetic alteration is involved in anti-lung cancer activity. In the present study, the effect of JFK on lung cancer cell lines was examined with the aim to further understand the underlying mechanisms of JFK-induced anti-lung cancer activity by transcriptome profiling analysis. JFK was observed to decrease lung cancer cell viability and simultaneously induce cellular morphology alteration. Additionally, this causes cell cycle arrest and apoptosis in A549 cells. The present RNA-seq analysis identified 5,281 genes with differential expression ($P<0.05$). Gene ontology analysis indicated that genes involved in the cell cycle pathway are downregulated, including cyclin-dependent kinase 2, cyclin-dependent kinase 4, cyclin B1 and cyclin A2, and apoptosis-associated genes are upregulated, including *Fas*, death receptor 4 (*DR4*), tumor protein P53 binding protein 2 and BCL2 interacting protein 3 like. Particularly, the present results indicate knockdown of *Fas* and *DR4* attenuates JFK-induced apoptosis in A549 cells. Overall, the present study suggests JFK induces cellular apoptosis through activation of *Fas* and *DR4* in A549 cells and provides an insight for understanding the antitumor mechanisms of this Chinese traditional medicine.

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Abbreviations: JFK, Jinfukang formula; NSCLC, non-small cell lung cancer; TCM, Traditional Chinese Medicine; RNA-Seq, RNA sequencing; CCK8, Cell Counting Kit 8; PI, propidium iodine; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes

Key words: Jinfukang formula, traditional Chinese medicine, apoptosis, cell cycle arrest, non-small cell lung cancer, transcriptome

Introduction

Lung cancer is one of the most malignant cancers, with the highest morbidity and mortality among all cancers in the world (1). Non-small cell lung cancer (NSCLC) is closely associated with a long history of tobacco smoking and accounts for ~80% of all lung cancer cases (2). The primary treatment for patients with locally advanced or metastatic NSCLC includes systemic chemotherapy (alone or in combination with radiation therapy) and targeted biological therapy (3). However, lung cancer cells frequently show significant resistance to chemotherapeutic drugs (4). Therefore, it is urgent to develop alternative therapeutic strategies against lung cancer.

Traditional Chinese Medicine (TCM) has been effectively used against diseases for thousands of years in China (5,6). Indeed, numerous Chinese medicinal herbs show promising effects in the treatment of cancers (7). Jinfukang (JFK), an Astragalus-based herbal formula consisting of 12 herbs (Table I), has been reported to have anti-tumor efficacy against NSCLC (8-10). It has been approved by the Chinese Food and Drug Administration as a drug against NSCLC. Although a previous study reported that JFK could inhibit proliferation and promote apoptosis of cancer cells in rats (9), the underlying mechanisms remain elusive.

As the majority of TCMs consist of various components, it is necessary to utilize high-throughput strategies to delineate the underlying mechanisms (11). Previous studies have shown that cDNA profiling may be effectively used to

characterize the antitumor mechanism by measuring gene expression changes (12,13). With the emergence of RNA-seq, this approach has provided a better platform to investigate the mechanisms of various drugs due to its performance in robustness and effectiveness (14,15).

A previous study indicated that JFK-induced epigenetic alteration is involved in anti-lung cancer activity (16). In present study, the alteration in cellular growth and apoptosis upon JFK treatment was examined in human lung cancer cell lines, and RNA-seq analysis was performed to characterize the JFK-induced transcriptome. Among the genes whose expression was modulated by JFK, the present study further characterized those involved in the apoptosis pathway to understand how JFK exerts its effects on lung cancer cells.

Materials and methods

Preparation of JFK. JFK contains 12 herbals described in Table I, and these raw herbs were obtained from the pharmacy dispensary of Longhua Hospital, Shanghai University of Traditional Chinese Medicine (Shanghai, China). The names of herbs have been verified via www.theplantlist.org. Voucher specimens were deposited at the herbarium in Shanghai Jiao Tong University. The raw components of JFK (20 g) were minced and extracted with 200 ml 70% ethanol at 80˚C for 1 h according to the composition in Table I. Chromatographic and mass spectrographic finger prints for JFK ethanol extract were examined via LC-MS. The ethanol extract was filtered through a 0.45 μm syringe filter and diluted to various concentrations used in the present study.

Cell culture. The human lung cancer A549, NCI-H1975, NCI-H1650 and NCI-H2228 cell lines were maintained with RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere containing 5% CO_2.

Cell viability analysis. Human lung cancer cell lines were treated with various concentrations of JFK extract (0, 0.041, 0.054, 0.081, 0.108, 0.162 and 0.216 mg/ml) for 48 h at 37˚C, and then the cell viability was evaluated using Cell Counting Kit 8 (CCK8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) by spectrophotometric plate reader (Omega Bio-Tek, Inc., Norcross, GA, USA). All cell viability results were tested by three independent experiments.

Morphological changes and DAPI staining. To evaluate the effects of JFK extract on cell growth, $1x10^5$ A549 cells were seeded in each well of 6-well plates, and exposed to JFK extract at a half maximal inhibitory concentration (IC50; determined by CCK8 assay) for 48 h. The morphological changes in A549 cells were observed with a phase-contrast microscope (Model Ti-E; NIS4.0; Nikon, Tokyo, Japan). A549 cells were fixed, stained with DAPI and observed using fluorescence microscopy (Model Ti-E; NIS4.0; Nikon).

Cell cycle analysis. Briefly, A549 cells were harvested by trypsin with no EDTA (Thermo Fisher Scientific, Inc.), and washed twice with PBS. The cells were fixed with cold 70% ethanol overnight, and then stained with a solution consisting of 20 μg/ml propidium iodine (PI) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 0.1% TritonX-100 (Sigma-Aldrich; Merck KGaA), 100 μg/ml RNase A (Fermentas; Thermo Fisher Scientific, Inc.) for 15 min at 37˚C in the dark. Cell cycle progression was then analyzed by flow cytometry (BD LSRFortessa; BD Biosciences, Franklin Lakes, NJ, USA) and ModFit LT software (version 3.2; Verity Software House, Topsham, ME, USA).

Cell apoptosis analysis. Determination of phosphatidyl serine (PS) and membrane integrity was performed using the Annexin V-FITC/PI Apoptosis kit (Beijing Zoman Biotechnology Co., Ltd., Beijing, China). In brief, A549, NCI-H1975, NCI-H1650 and NCI-H2228 cells were harvested by trypsin (no EDTA) and washed twice with PBS, then stained with Annexin V-FITC/PI and analyzed by flow cytometry as aforementioned. PI positive cells were designated as end stage apoptotic cells, and FITC-positive cells were designated as early stage apoptotic cells.

RNA-seq library construction. RNA-seq assays were performed in A549 cells without and with JFK treatment for 48 h (0.054 mg/ml), as described in our recent study (17). Briefly, total RNA was extracted by TRIzol (Thermo Fisher Scientific, Inc.), and further treated with DNase to remove genomic DNA contamination. Isolation of mRNA was performed with Oligotex mRNA Mini kit (Qiagen, Hilden, Germany) and then used for RNA-seq library construction with NEBNext Ultra Directional RNA Library Prep kit for Illumina (New England BioLabs, Inc., Ipswich, MA, USA), which was subjected to Illumina sequencing. The raw sequencing data are available in the EMBL database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-3592.

Analysis of differentially expressed genes. The raw sequencing reads were mapped to the reference genome (hg19) by TopHat. Cufflinks was used to characterize the differential transcription pattern (18). The present study has taken biases in library preparation into account. The gene expression level was measured by reads per kilobase of transcript per million reads mapped.

Functional annotation and pathway analysis. The Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resource was used to annotate gene functions and pathways (19,20).

Quantification of mRNA level. Transcription level of genes of interest was evaluated using quantitative reverse transcription-quantitative polymerase chain reaction (RT-qPCR) with SYBR-Green PCR Master Mix (Qiagen). RT-qPCR was performed according to our previous studies (13,16,17). All primer sequences used for RT-qPCR are presented in Table II.

Western blot analysis. Cells were washed with PBS and collected in a 1.5 ml tube. A549 cells were lysed using the procedures essentially the same as described previously (13). The total protein extracts were obtained following

Table I. Composition of JFK formula.

Botanical name	Herbal name	Common name	Chinese name	Ratio	Voucher no.
Astragalu smembranaceus (Fish.) Bunge	Radix astragali	Milkvetch root	Huang-qi	8	SJTU-JFK140121-1
Glehnia littoralis F. Schmidt ex Miq.	Radix glehniae	Coastal glehnia root	Bei-sha-shen	8	SJTU-JFK140121-2
Asparagus cochinchinensis (Lour.) Merr	Radix asparagi	Cochinchinese asparagus root	Tian-men-dong	2	SJTU-JFK140121-3
Ligustrum lucidum W.T. Aiton	Fructus ligustri lucidi	Glossy privet fruit	Nv-zhen-zi	2	SJTU-JFK140121-4
Selaginella doederleinii Hieron.	Herba selaginellae	Spikemoss	Shi-Shang-Bai	8	SJTU-JFK140121-5
Paris polyphylla Smith var. yunnanensis (Franch.) Hand Mazz	Rhizoma paridis yunnanensis	Yunnan manyleafparis rhizome	Chong-lou	3	SJTU-JFK140121-6
Epimedium sagittatum (Siebold&Zucc.) Maxim	Folium epimedii	Shorthornedepimedium	Yin-yang-huo	2	SJTU-JFK140121-7
Gynostemma pentaphyllum (Thunb.) Makino	Herbal gynostemmatis	Five leaf gynostemma herb	Jiao-gu-lan	2	SJTU-JFK140121-8
Cornus officinalis Siebold & Zucc.	Fructus corni	Asiatic cornelian cherry fruit	Shan-zhu-yu	2	SJTU-JFK140121-9
Salvia chinensis Benth.	Herba salviae chinensis	Chinese sage	Shi-jian-chuan	8	SJTU-JFK140121-10
Ophiopogon japonicas (Thunb.) Ker Gawl	Radix ophiopogon	Dwarf lilyturf	Mai-dong tuber	2	SJTU-JFK140121-11
Trigonella foenum-graecum L.	Semen trigonella	Common fenugreek seed	Hu-lu-ba	2	SJTU-JFK140121-12

JFK, Jinfukang.

Table II. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

		Primer sequence	
Gene	GenBank	Forward (5'-3')	Reverse (5'-3')
CDK2	[NG_034014]	TACACCCATGAGGTGACTCG	GTCCCCAGAGTCCGAAAGAT
CDK4	[NM_000075]	CCCGAAGTTCTTCTGCAGTC	CTGGTCGGCTTCAGAGTTTC
CCNA2	[NM_001237]	GGTACTGAAGTCCGGGAACC	AAGATCCTTAAGGGGTGCAA
CCNB1	[NM_172301]	TGTGGATGCAGAAGATGGAG	TTTGGTCTGACTGCTTGCTC
DR4	[NM_003844]	TGCAGGTCGTACCTAGCTCA	GGACACAACTCTCCCAAAGG
Fas	[NG_009089]	ATTGCTCAACAACCATGCTG	CCAATCCCTTGGAGTTGATG
TP53BP2	[NG_029950]	TCCTTGGTCATTCAGGCTTC	GGCCAACTGGATGGATTTTA
BNIP3L	[NM_004331]	CAACAACAACTGCGAGGAAA	TTGCTGCTGTTCATGGGTAG
GAPDH	[NG_007073]	GCACCGTCAAGGCTGAGAAC	ATGGTGGTGAAGACGCCAGT

centrifugation (18,000 x g for 15 min, at 4˚C). The protein concentration was evaluated by Pierce BCA protein assay kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Protein samples (~50 μg) were fractionated by SDS-PAGE (10% polyacrylamide gels) and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The protein-binding membrane was incubated in PBS containing 0.1% Tween-20 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 60 min at 4˚C. 5% skim milk was used to block non-specific binding for 3 h at 4˚C. This was followed by incubation overnight at 4˚C with primary antibody. The primary antibodies used in the present

study included; cyclin B1 (*CCNB1*; cat no. D160234-0100; dilution, 1:250; Sangon Biotech Co., Ltd., Shanghai, China), cyclin-dependent kinase 4 (*CDK4*; cat no. 11026-1-AP; dilution, 1:100; Proteintech Group, Inc., Wuhan, China), death receptor 4 (*DR4*; cat no. 24063-1-AP; dilution, 1:100; Proteintech Group, Inc.), *Fas* (cat no. D220092-0100; dilution, 1:550; Sangon Biotech Co., Ltd.), and GAPDH (cat no. 5174S; dilution, 1:2,000; Cell Signaling Technology, Inc., Danvers, MA, USA). The membrane with protein antibody complexes were washed with PBS containing 0.1% Tween-20, incubated for 2 h with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (cat no. 14708S; dilution, 1:15,000; Cell Signaling Technology, Inc.) at 4˚C. Immunodetection was performed using ECL reagent (Santa Cruz Biotechnology Inc., Dallas, TX, USA) and images were captured and analyzed using the LI-COR Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE, USA). The protein amounts were semi-quantified by analyzing blot intensity using GAPDH as loading control.

siRNA and transfection. siRNA duplexes were designed to downregulate *DR4* and *Fas*. The *DR4*-targeting siRNA sequence was 5'-CCUUCAAGUUUGUCGUCGUdTdT-3' and the *Fas*-targeting siRNA sequence was 5'-GUGCAG AUGUAAACCAAACdTdT-3'. siRNAs for *in vitro* transfection were obtained from Shanghai Gene Pharma Co., Ltd. (Shanghai, China). Transfection was performed using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's instructions. Briefly, A549 and NCI-H2228 cells were transfected with 50 nmol/l of siRNAs directed against *Fas* and *DR4* respectively, and incubated for 6 h at 37˚C.

Statistical analysis. Data are presented as the mean ± standard deviation. Comparisons between the groups were examined by standard one-way analysis of variance ANOVA, SPSS for Windows 14.0 software package (SPSS, Inc., Chicago, IL, USA), followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

JFK induces cell growth arrest and DNA damage in lung cancer cells. To examine the effects of JFK on lung cancer cell growth, A549, NCI-H1975, NCI-H1650 and NCI-H2228 cells were treated with JFK at various concentrations (0, 0.041, 0.054, 0.081, 0.108, 0.162 and 0.216 mg/ml) for 48 h and cell viability was assessed. The cell viability of these human lung cancer cell lines decreases significantly in a concentration-dependent manner following JFK treatment (Fig. 1A). Additionally, JFK was observed to exert an increased inhibition effect on A549 cells compared with other three human lung cancer cell lines, with a IC50 of ~0.054 mg/ml. JFK treatment (0.054 mg/ml) for 48 h reshapes a large number of A549 cells to be distinctly flat and enlarged in size (Fig. 1B) and makes the nucleus condensed and disintegrated (Fig. 1C), indicating multiple effects of JFK on A549 cells. Based on these observations, A549 was next focused on and the antitumor mechanisms of JFK were characterized.

JFK leads to cell cycle arrest and cell apoptosis in A549 cells. To further investigate whether JFK cytotoxicity involves cell cycle arrest, a flow cytometry assay was performed to characterize alterations of the cell cycle. As shown in Fig. 1D, cell cycle analysis indicates that a significant portion of A549 cells accumulates in G1 and G2 phase upon JFK (0.054 mg/ml) treatment, suggesting that JFK could induce G1/S and G2/M transition arrest. To examine whether JFK triggers apoptosis in A549 cells, the extent of apoptotic cells was investigated by flow cytometry, and it was observed that numerous A549 cells were induced to undergo apoptosis after exposure to JFK for 48 h (Fig. 1E). This phenomenon not only presents in early apoptosis, but also presents in total apoptosis (Fig. 1F). Thus, the cell apoptotic rates dramatically increase upon JFK treatment.

JFK-associated transcriptome analysis in A549 cells. Given the observations of anti-proliferation and pro-apoptosis effects induced by JFK, the present study subsequently aimed to understand the underlying molecular mechanisms by transcriptome-wide differential gene expression analysis through RNA-seq. In total, 13,130,096 and 10,433,359 reads were generated from JFK-treated and untreated A549 cells, 54 and 66% of which were uniquely mapped to the human genome, corresponding to 13,720 and 17,519 expressed genes, respectively (Table III). Compared with the control, 5,281 differentially expressed genes ($P<0.05$) were identified, with 2,777 (52.6%) downregulated and 2,504 (47.4%) upregulated (Fig. 2A).

Gene ontology analysis of differentially expressed genes. Gene Ontology (GO) analysis of the genes with JFK-induced differential expression was performed using DAVID (19) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways database (20). The top ten GO items were listed in Table IV, in which apoptosis-associated and cell cycle-associated biological processes are statistically enriched in the upregulated and downregulated genes, respectively. For the gene sets with differential expression, KEGG pathway analysis was performed. Similarly, it was observed that the cell cycle and apoptosis pathways are also included in the top 10 enriched pathways (Fig. 2B and C). These results suggest that JFK could induce growth inhibition potentially through repressing pathways of cell cycle, nuclear division and translation pathways. Simultaneously, it could induce apoptosis in A549 cells partially through activating apoptosis, cell death and protein transport pathways.

JFK modulates the expression of genes involved in cell cycle and apoptosis. For gene sets with a differential expression pattern determined by RNA-seq analysis, cell cycle-associated genes, including *CDK2*, *CDK4*, *CCNB1* and cyclin A2 (*CCNA2*), and apoptosis-associated genes, including *Fas*, *DR4*, tumor protein P53 binding protein 2 (*TP53BP2*) and BCL2 interacting protein 3 like (*BNIP3L*), for further validation (Fig. 3A). The transcriptional activities of selected genes were measured by RT-qPCR, and the results were shown in Fig. 3B and C. These results confirm that JFK represses cell cycle-associated genes, and activates apoptosis-associated genes as well. Among these selected genes, the alterations in

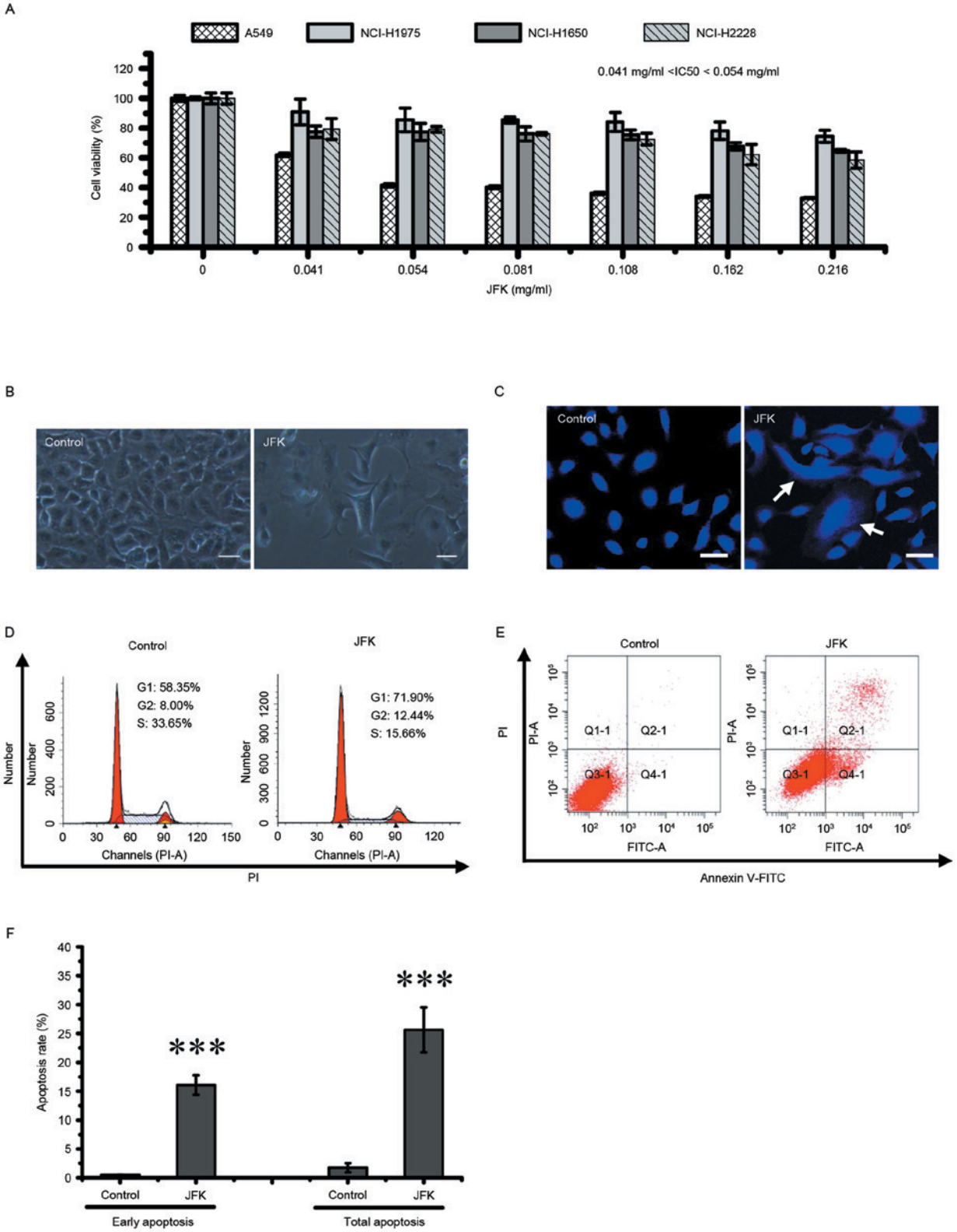

Figure 1. Effect of JFK on cytotoxicity in lung cancer cells. (A) A549, NCI-H1975, NCI-H1650 and NCI-H2228 cells were treated with JFK (0, 0.041, 0.054, 0.081, 0.108, 0.162 and 0.216 mg/ml, respectively) for 48 h, and examined for viability. Data are expressed as the mean ± SD (n=3). (B) A549 cells were exposed to JFK (0.054 mg/ml) for 48 h. Morphological alteration was examined by phase-contrast microscopy. Scale bar, 50 μm. (C) A549 cells were stained with DAPI for detecting nucleus changes using fluorescence microscopy. Scale bar, 25 μm. (D) Cell cycle distribution was evaluated by flow cytometry. (E) Apoptotic cells were determined by flow cytometry after exposure to JFK for 48 h. (F) Ratios of early apoptosis and total apoptosis were analyzed basing on flow cytometric detection. Data are expressed as the mean ± SD (n=3). ***P<0.001. SD, standard deviation; JFK, Jinfukang; PI, propidium iodide; FITC, fluorescein isothiocyanate; IC50, half maximal inhibitory concentration.

protein level of *CDK4, CCNB1, Fas* and *DR4* were examined by western blot analysis. As shown in Fig. 3D and E, protein levels of *CDK4* and *CCNB1* decrease and those of *Fas* and *DR4* increase after JFK (0.054 mg/ml) treatment.

Table III. The statistics of RNA-seq data sets.

Group	Total reads	Unique mapping reads	Mapping rate, %	Expressed gene number
Control	10433359	6908569	66	17519
JFK	13130096	7035144	54	13720

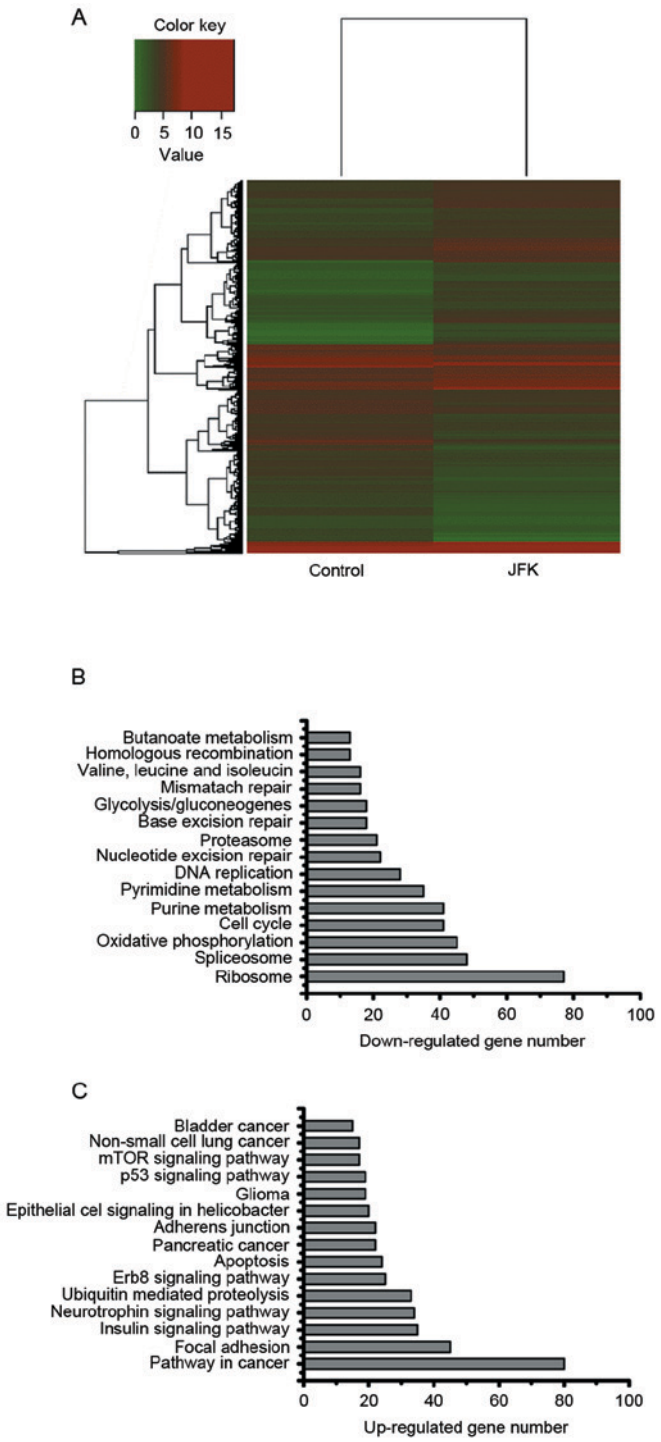

Figure 2. Functional classification and pathway analysis of differentially expressed genes. (A) Heat map of RNA-seq analysis in A549 cells with or without JFK (0.054 mg/ml) treatment. In total, 5,281 genes were identified as differentially expressed (P<0.05). (B and C) KEGG pathway analysis of (B) upregulated and (C) downregulated genes. JFK, Jinfukang.

Table IV. GO analysis of differentially expressed genes.

A, Downregulated

Gene ID	Function	Count	P-value
GO:0006414	Translational elongation	85	6.79×10^{-57}
GO:0006412	Translation	140	6.93×10^{-39}
GO:0007049	Cell cycle	226	2.69×10^{-32}
GO:0022402	Cell cycle process	178	7.65×10^{-30}
GO:0000278	Mitotic cell cycle	132	7.16×10^{-28}
GO:0000279	M phase	120	2.39×10^{-26}
GO:0022403	Cell cycle phase	134	1.00×10^{-23}
GO:0000087	M phase of mitotic cell cycle	88	5.95×10^{-22}
GO:0000280	Nuclear division	87	6.22×10^{-22}
GO:0007067	Mitosis	87	6.22×10^{-22}

B, Upregulated

Gene ID	Function	Count	P-value
GO:0046907	Intracellular transport	150	5.33×10^{-15}
GO:0015031	Protein transport	166	1.36×10^{-14}
GO:0045184	Establishment of protein localization	167	1.51×10^{-14}
GO:0008104	Protein localization	182	1.28×10^{-13}
GO:0010941	Regulation of cell death	165	1.09×10^{-11}
GO:0042981	Regulation of apoptosis	163	1.32×10^{-11}
GO:0043067	Regulation of programmed cell death	164	1.55×10^{-11}
GO:0016265	Death	149	3.69×10^{-11}
GO:0008219	Cell death	148	4.29×10^{-11}
GO:0034613	Cellular protein localization	95	4.45×10^{-10}

GO, gene ontology.

Activation of Fas and DR4 contributes to JFK-induced pro-apoptotic effect in A549 cells. To explore whether *Fas* and *DR4* mediate the JFK-induced apoptosis in A549 cells, lastly siRNA assays were performed to suppress the expression of *Fas* and *DR4* in JFK-treated A549 cells. As shown in Fig. 4A and B, the apoptotic rates (including early apoptosis rate and total apoptosis rate) significantly decrease when *Fas* and *DR4* were knocked down. The protein expression levels of *Fas* and *DR4* also drop when *Fas* and *DR4* were knocked down (Fig. 4C). These results indicate that activation of *Fas* and *DR4* is partially involved in JFK-induced apoptosis.

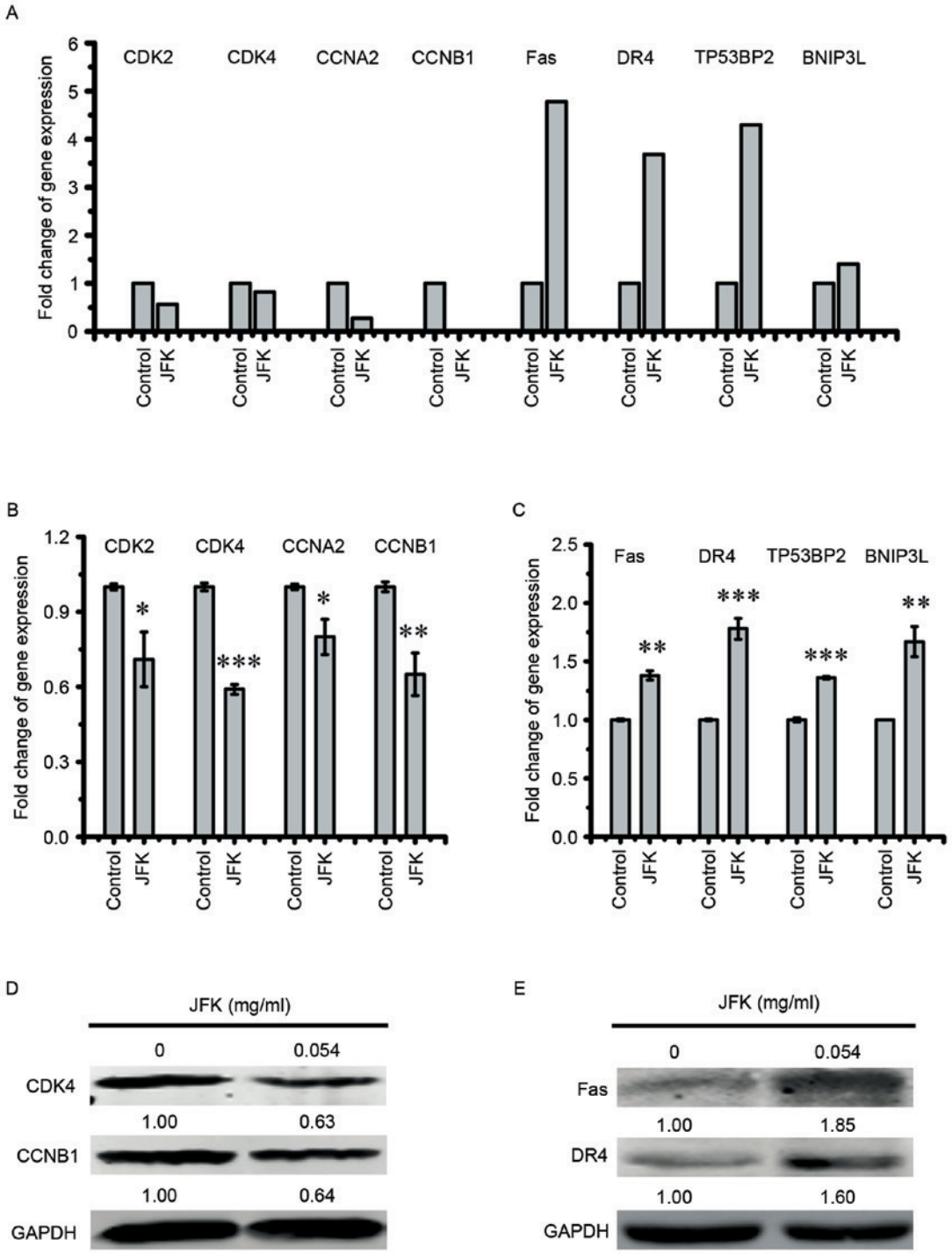

Figure 3. Expression validation of genes involved in the cell cycle and apoptosis upon JKF treatment in A549 cells. (A) Fold-change of cell cycle and cell apoptosis-associated gene expression from RNA-seq results. (B and C) A549 cells were treated with JFK (0 or 0.054 mg/ml) for 48 h, and then the mRNA levels of cell cycle and apoptosis-associated genes were examined by reverse transcription-quantitative polymerase chain reaction. The data are expressed as the mean ± standard deviation (n=5). *P<0.05, **P<0.01 and ***P<0.001. (D and E) A549 cells were treated with or without JFK (0.054 mg/ml) for 48 h, and then the protein levels of *CDK4, CCNB1, Fas* and *DR4* were examined by western blot analysis. CDK4, cyclin-dependent kinase 4; CDK2, cyclin-dependent kinase 2; CCNB1, cyclin B1; CCNA2, cyclin A2; TP53BP2, tumor protein P53 binding protein 2; BNIP3L, BCL2 interacting protein 3 like; JFK, Jinfukang.

Discussion

Herbal intervention has been widely used for the treatment and prevention of various diseases for thousands of years in China (21). The majority of known anticancer drugs are derived from herbal plants (22). JFK, a type of Astragalus-based herbal formula, has been reported to be clinically effective in NSCLC patients (8,10,23). In the present study, the anticancer mechanisms of JFK were explored. The cellular behavior of lung cancer cell lines treated with JFK were examined, and it was found that JFK could induce cellular growth arrest and the cell cycle is regulated by a complex cascade of events and mediated by cell cycle regulatory proteins, including cyclins and CDKs (24). CDK2 and CDK4 are two

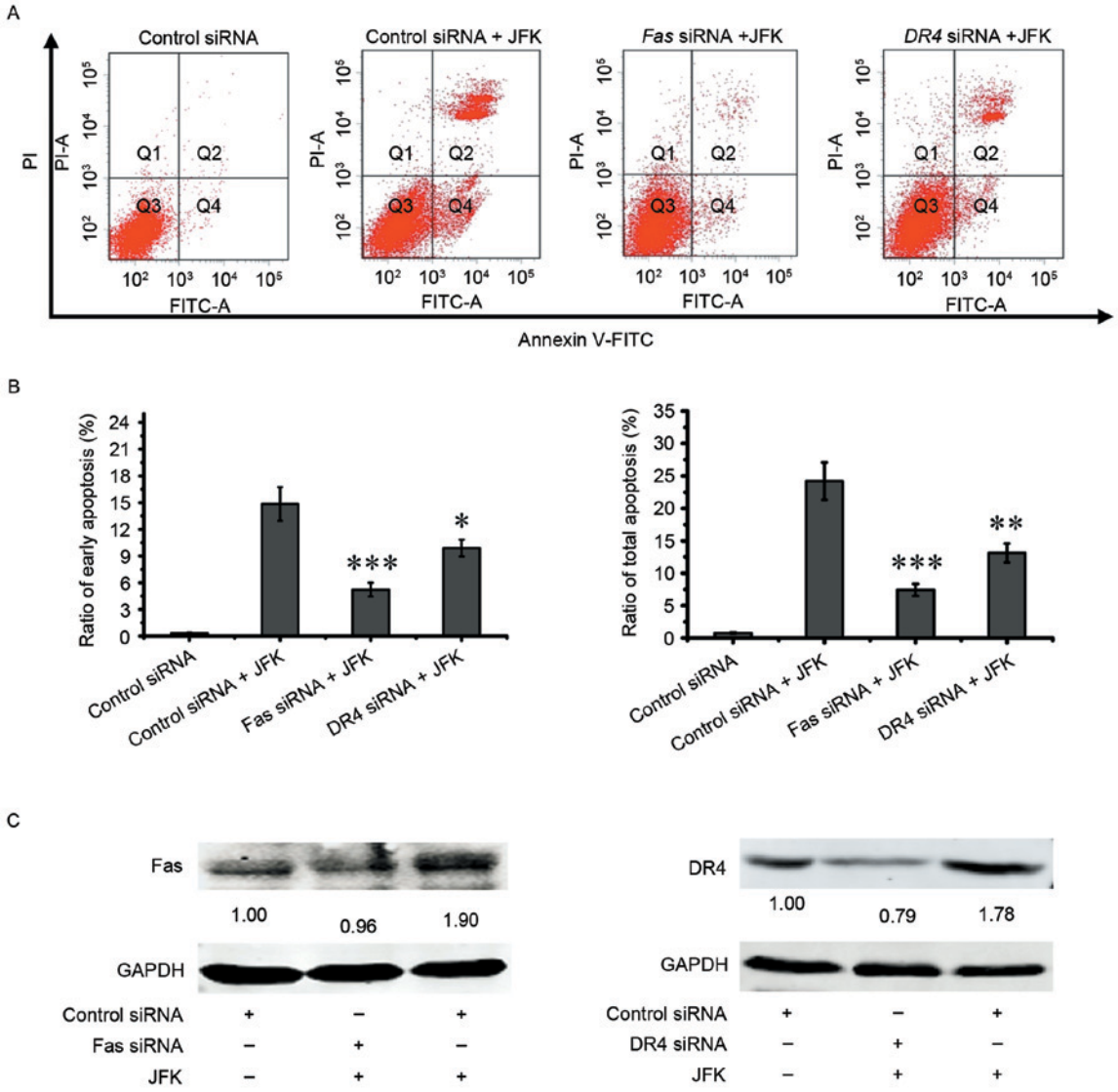

Figure 4. Knockdown of *DR4* and *Fas* attenuates the pro-apoptotic effect of A549 cells induced by JFK. *Fas* and *DR4* in A549 cells were knocked down, and the cells were exposed to JFK (0.054 mg/ml) for 48 h. (A) The cell apoptosis ratio was examined by flow cytometry. (B) Ratios of early apoptosis and total apoptosis were analyzed basing on flow cytometry detection. Data are expressed as the mean ± standard deviation (n=3), *P<0.05, **P<0.01 and ***P<0.001. (C) *Fas* and *DR4* protein expression levels were determined by western blot analysis. DR4, death receptor 4; siRNA, small interfering RNA; JFK, Jinfukang; PI, propidium iodide; FITC, fluorescein isothiocyanate.

key members of the cyclin-dependent kinase family, whose activities are restricted to the G1-S phase, and are essential for the G1-Stransition (25,26). CCNA2 is a critical regulatory factor that can interact with CDK kinases for regulating G1-S transition and G2-M transition (27). CCNB1 usually has an important role in mitosis, and indicates a poor prognosis when overexpressed in NSCLC (28). The present results show that A549 cells could be induced to a state of G1 and G2 phase enrichment upon JFK treatment (Fig. 1D), accompanying downregulation of cell cycle-associated genes, including *CDK2*, *CDK4*, *CCNB1* and *CCNA2* (Figs. 3A and 4B). These results suggest that JFK leads to A549 cell cycle arrest through the suppression of the cell cycle pathway.

Induction of apoptosis in tumor cells is one of the most common anticancer mechanisms in cancer therapies (29). The death receptor apoptotic pathway has previously been proposed as an anti-cancer drug target in human lung cancer (30). *Fas* and *DR4*, two members of the death receptor family, have a vital role during the process of apoptosis. *TP53BP2* induces apoptosis through the mitochondrial death pathway (31). *BNIP3L* has been reported to promote cell death via apoptosis (32). In the present study, it was found the expression activities of *Fas* and *DR4* in A549 cells are upregulated in both mRNA and protein levels after JFK treatment. *TP53BP2* and *BNIP3L* are also activated by JFK (Fig. 3A and C). Particularly, it was observed that downregulation of *Fas* and *DR4* could attenuate JFK-induced apoptosis in A549 cells (Fig. 4). However, this phenomenon was not observed to occur in the human lung cancer NCI-H1650 and NCI-H228 cell lines, although JFK also exerts an inhibitory viability effect on these two cell lines (Fig. 5A-C). JFK enhances the transcription of *DR4* and *Fas* in NCI-H2228 cells, but induces marginal apoptosis in siRNA knockdown assays (Fig. 5D-F), suggesting JFK-induced apoptosis is cellular context-dependent.

Overall, the present study reported that JFK induces cellular growth arrest and apoptosis in lung cancer cells. The

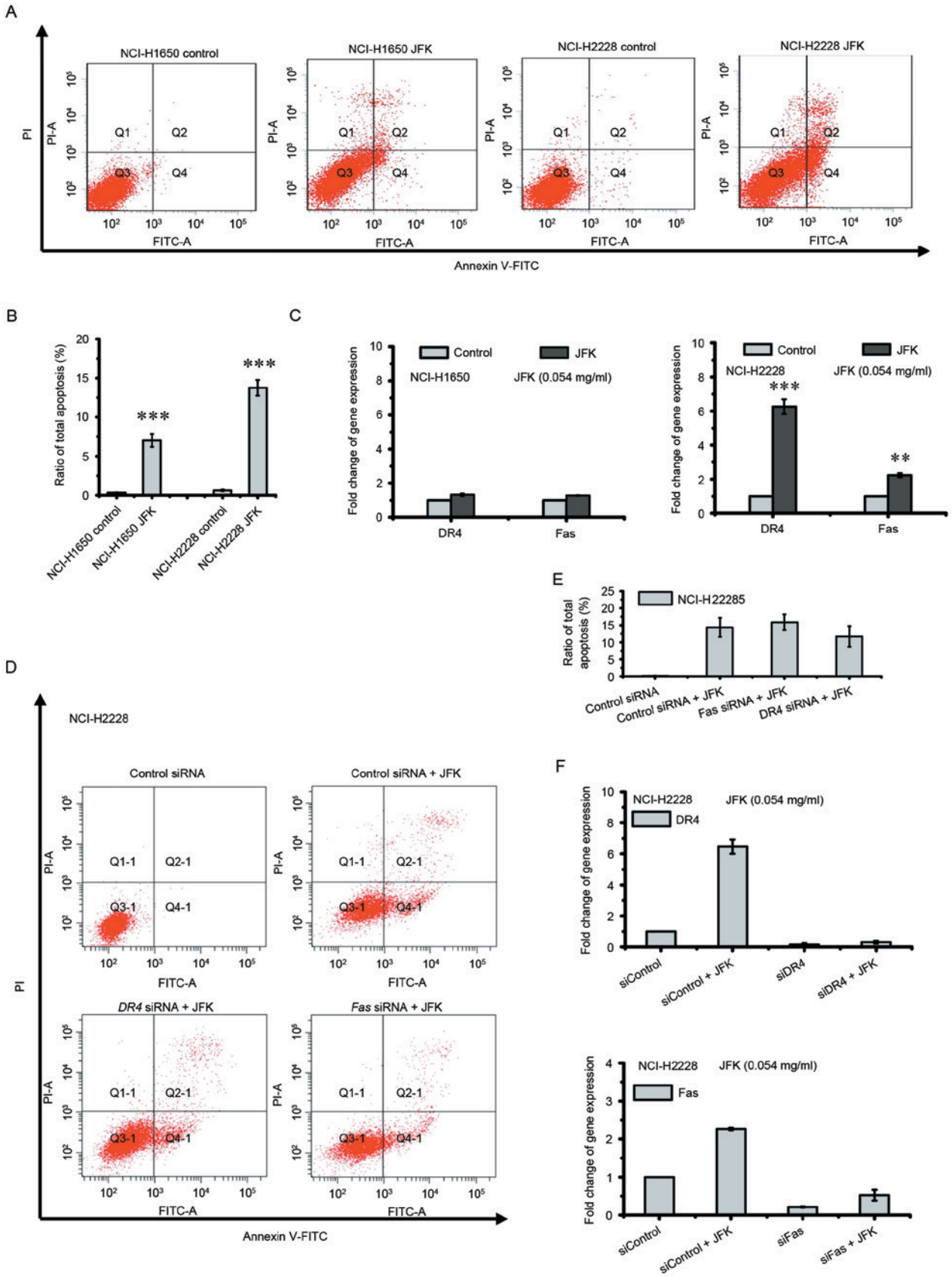

Figure 5. *DR4* and *Fas* are not involved in JFK-induced apoptosis in NCI-H1650 and NCI-H2228 cells. (A) The apoptotic cells were detected by flow cytometry, after JFK (0.054 mg/ml) treatment for 48 h in NCI-H1650 and NCI-H2228 cells. (B) Apoptosis rate was analyzed based on flow cytometry. Data were expressed as the mean ± SD (n=3), ***P<0.001. (C) mRNA levels were examined by reverse transcription-quantitative polymerase chain reaction. Data were expressed as the mean ± SD (n=3), **P<0.01 and ***P<0.001. (D and E) *DR4* and *Fas*-knockdown NCI-H2228 cells were treated with JFK (0.054 mg/ml) for 48 h, and then the percentage of apoptosis was determined by flow cytometry. Data are expressed as the mean ± SD (n=3). (F) *DR4* and *Fas* knockdown NCI-H2228 cells were treated with JFK (0.054 mg/ml) for 48 h, and then the mRNA levels were examined by reverse transcription-quantitative polymerase chain reaction. Data are expressed as the mean ± SD (n=3). SD, standard deviation; DR4, death receptor 4; JFK, Jinfukang; siRNA, small interfering RNA; PI, propidium iodide; FITC, fluorescein isothiocyanate.

transcriptome profiling analysis identified 5,281 genes whose expression was modulated by JFK and it was demonstrated that JFK contributes to A549 cellular apoptosis partially through activation of *DR4* and *Fas*. The present results provide new insight for understanding how this TCM formula exerts its effects against lung cancer.

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