

Aspirin inhibits the proliferation of human uterine leiomyoma cells by downregulation of K-Ras-p110 α interaction

MIN GAO^{1*}, KAI-MIN GUO^{2*}, YING-MEI WEI³, MING-MING MA⁴,
JIA-RONG CAI⁵, TING-TING XIA⁶ and QING-JIAN YE⁷

¹Department of Pharmacy, The Sixth Affiliated Hospital of Sun Yat-Sen University, Guangzhou, Guangdong 510655;

²Department of Obstetrics and Gynecology, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, Guangdong 510623; ³Department of Neurology, Sun Yat-sen Memorial Hospital of Sun Yat-sen University, Guangzhou, Guangdong 510120;

⁴Department of Pharmacology, Cardiac and Cerebral Vascular Research Center, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou, Guangdong 510080;

Departments of ⁵Urology, ⁶Infertility and Sexual Medicine, and ⁷Gynecology, The Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, Guangdong 510630, P.R. China

Received March 15, 2017; Accepted August 14, 2017

DOI: 10.3892/or.2017.5915

Abstract. Aspirin has been confirmed as an effective antitumor drug in various cancers. However, the relationship between aspirin and uterine leiomyoma is still underexplored. Here, we explored the effects of aspirin on human uterine leiomyoma cells and provide insights into the underlying mechanisms. Cell Counting Kit-8 (CCK-8) and flow cytometry analysis showed that aspirin treatment inhibited cell proliferation and promoted cell cycle arrest at G0/G1 phase in a dose- and time-dependent manner of human uterine leiomyoma cells. Further studies revealed that aspirin blocked the interaction between K-Ras and p110 α by co-immunoprecipitation and immunofluorescence. Western blotting demonstrated K-Ras-p110 α interaction was required for the effects of aspirin-induced inhibition on cell growth and cell cycle transition via cell cycle regulators, including cyclin D1 and cyclin-dependent kinase 2 (CDK2). PI3K/Akt/caspase signaling pathway was involved in human uterine leiomyoma cell growth under aspirin treatment. Taken together, these results suggest that aspirin inhibited human uterine leiomyoma cell growth by regulating K-Ras-p110 α

interaction. Aspirin which targeting on interaction between K-Ras and p110 α may serve as a new therapeutic drug for uterine leiomyoma treatment.

Introduction

Uterine leiomyoma (fibroid) is one of the most common benign smooth muscle tumors with an estimated incidence of 75% in reproductive aged women and ~25% of fertile women bear clinical symptoms, such as heavy or abnormal uterine bleeding, pelvic pain and infertility (1-3). Although surgical therapies, including myomectomy and hysterectomy, are frequently used for the management in fibroids, medical therapies are considered as the first-line treatment. Current medical managements, such as gonadotropin-releasing hormone (GnRH) analogs, selective estrogen receptor modulators and ulipristal acetate, are used for reducing fibroid size and improving severe symptoms (1,4).

Aspirin, the most commonly used non-steroidal anti-inflammatory agent for the treatment of fever, pain or other inflammatory conditions, has been identified as a potential chemopreventive drug supported by epidemiological data or clinical trials (5-8). The accumulated evidence shows aspirin was related with the reduced risks of breast, lung, colorectal, skin, prostate and endometrial cancer (5,9-11). A study showed using a continuous low dose of aspirin daily decreased the risk of cancer distant metastasis (12). Several anti-proliferation mechanisms of aspirin involve inhibition of WNT- β -catenin signaling, suppression of PI3K/Akt signaling, modulation of Bax and Bcl-2 activation, and regulation of prostaglandin (PG) synthesis and catabolism (8,13-16). Although the antitumor effects of aspirin are associated with cell proliferation, the relationship between aspirin and uterine leiomyoma remains unclear.

Kirsten rat sarcoma-2 viral (v-Ki-ras2) oncogene homologue (K-Ras) mutations represent genetic defect found in

Correspondence to: Dr Qing-Jian Ye, Department of Gynecology, The Third Affiliated Hospital of Sun Yat-Sen University, 600 Tianhe Road, Guangzhou, Guangdong 510630, P.R. China
E-mail: yeqingjian@163.com

Dr Min Gao, Department of Pharmacy, The Sixth Affiliated Hospital of Sun Yat-Sen University, 26 Erheng Road of Yuan Village, Guangzhou, Guangdong 510655, P.R. China
E-mail: gaomin20040520@163.com

*Contributed equally

Key words: human uterine leiomyoma cell, aspirin, proliferation, K-Ras-p110 α interaction

various human cancers. Activated K-Ras signaling contributed to promoting tumor initiation and cell proliferation (17,18). Previous studies supported the idea that simultaneous activation of phosphatidylinositol 3-kinase (PI3K) was essential for K-Ras-induced tumorigenesis (19-21). It was indicated that a functional interaction K-Ras and PI3K p110 α (PI3K catalytic subunit) in host tissue was required for the growth-permissive environment for the tumor (22,23). Di Magliano *et al* (24) demonstrated that K-Ras activation could be inhibited by aspirin. Several studies demonstrate that PI3K mutation serves as a predictive molecular biomarker for adjuvant aspirin therapy (25,26). Thus, K-Ras or PI3K associated signaling pathway may be a key target of aspirin therapy for uterine leiomyoma.

In this study, we used human uterine leiomyoma cells to investigate the anti-proliferation function of aspirin and investigated the underlying mechanisms.

Materials and methods

Cell culture. Human uterine leiomyoma (UtLM) cells (GM10964) were obtained from Coriell Institute for Medical Research (Camden, NJ, USA). Cells were incubated in culture medium: Medium 199 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), endothelial cell growth supplement (PromoCell, Heidelberg, Germany), heparin (Sigma-Aldrich, St. Louis, MO, USA), 10⁵ U/l penicillin and 100 mg/l streptomycin (Gibco) at 37°C, in 5% CO₂. Upon reaching 70-80% confluence, cells were subcultured with 0.1% trypsin with 0.02% EDTA (Gibco). Human uterine leiomyoma cells were used for the following experiments by aspirin (Sigma-Aldrich) treatment.

Reagents. Aspirin with a purity exceeding 98% was purchased from Sigma-Aldrich. It was dissolved in distilled water at the desired concentrations.

Cell viability assay. Cell viability was assessed by Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan). Cells (5x10³ cells/well) were seeded in a 96-well plate. Plates were pre-incubated for 24 h in a humidified incubator. Then the cells were serum starved for another 24 h. Forty-eight hours after different treatments, 10% CCK-8 solution was added to each well of the plate and the plates were incubated for 2 h in the incubator. The absorbance was measured at 450 nm by BioTek microplate reader (Winooski, VT, USA).

Flow cytometry. Cell cycle was evaluated by flow cytometry. After appropriate treatment, cells were harvested, rinsed twice with phosphate-buffered-saline (PBS) and fixed in 70% ethanol for 24 h. Cells were incubated with 100 μ l RNase A solution (KeyGen Biotech, Nanjing, China) for 30 min at 37°C and were added with 400 μ l PI solution (KeyGen Biotech) for staining for 30 min in the dark. DNA content of cell distribution was analyzed at 488 nm by flow cytometry (EPICS XL-MCL; Beckman Coulter, Miami, FL, USA).

Western blot analysis. Total proteins were prepared from cultured human uterine leiomyoma cells. Protein was extracted

with RIPA lysis buffer (Beyotime Biotechnology, Nantong, China) and protease inhibitor cocktail (Sigma-Aldrich). The protein content was evaluated using BCA Protein assay kit (Beyotime Biotechnology) and bovine serum albumin as the standard. Equal amounts of total protein were boiled and separated on SDS-PAGE gels (Beyotime Biotechnology) and transferred to PVDF membrane (Millipore, Bedford, MA, USA). The membranes were incubated with the blocking solution at room temperature for 1 h. Then the membranes were incubated with primary antibodies against K-Ras (mouse monoclonal, 1:1,000; Sigma-Aldrich), p110 α (rabbit monoclonal, 1:1,000; Cell Signaling Technology, Beverly, MA, USA), p85 (rabbit monoclonal, 1:1,000; Cell Signaling Technology), p-p85 (Tyr-458, rabbit polyclonal, 1:1,000; Cell Signaling Technology), Akt (rabbit polyclonal, 1:1,000; Cell Signaling Technology), p-Akt (Ser-473, rabbit polyclonal, 1:1,000; Cell Signaling Technology), cyclin D1 (rabbit monoclonal, 1:1,000; Cell Signaling Technology), CDK2 (rabbit monoclonal, 1:1,000; Cell Signaling Technology), caspase-3 (rabbit monoclonal, 1:1,000; Cell Signaling Technology), caspase-9 (rabbit monoclonal, 1:1,000; Cell Signaling Technology), caspase-8 (rabbit monoclonal, 1:1,000; Cell Signaling Technology), caspase-12 (rabbit monoclonal, 1:1,000; Cell Signaling Technology), PARP (rabbit monoclonal, 1:1,000; Cell Signaling Technology), α -tubulin (mouse monoclonal, 1:1,000; Sigma-Aldrich), his (mouse monoclonal, 1:1,000; Sigma-Aldrich), flag (rabbit monoclonal, 1:1,000; Sigma-Aldrich) for 24 h at 4°C. Then the membranes were subsequently probed with respective secondary antibodies (1:2,000, Cell Signaling Technology) for 1 h at room temperature. The protein band signals of target bands were detected by Bio-Rad Molecular Imager ChemiDoc XRS plus System (Bio-Rad, Richmond, CA, USA). Quantification of band intensities was measured via ImageJ software (National Institute of Health, Bethesda, MD, USA).

Co-immunoprecipitation. The cell lysates were extracted using non-denaturing lysis buffer. Co-immunoprecipitation (co-IP) was done using the Thermo Scientific Pierce co-IP kit. The antibodies were first immobilized for 2.5 h using AminoLink Plus coupling resin. The resin was then washed and incubated with cell lysate overnight. Then the resin was washed and protein eluted using elution buffer. Samples were resolved on SDS-PAGE gels (Beyotime Biotechnology) and transferred onto PVDF membranes (Millipore). The bound proteins were determined by immunoblotting with the indicated antibodies.

Plasmid transfection. Human uterine leiomyoma cells were seeded at a density of \sim 1x10⁵/ml. After 24 h, pEnter-his vector, pEnter-his-K-Ras and pEnter-flag-p110 α (Vigene Biosciences, Jinan, China) were transfected into the cells with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Plasmids and Lipofectamine 2000 reagent were diluted in serum and antibiotics free Opti-MEM[®] I (Invitrogen) to form the transfection complexes, respectively, for 20 min at room temperature and then added to the cells. After incubation for 6 h at 37°C, transfection complexes were replaced with DMEM containing 20% fetal calf serum. Cells were prepared for following experiments after 48 h.

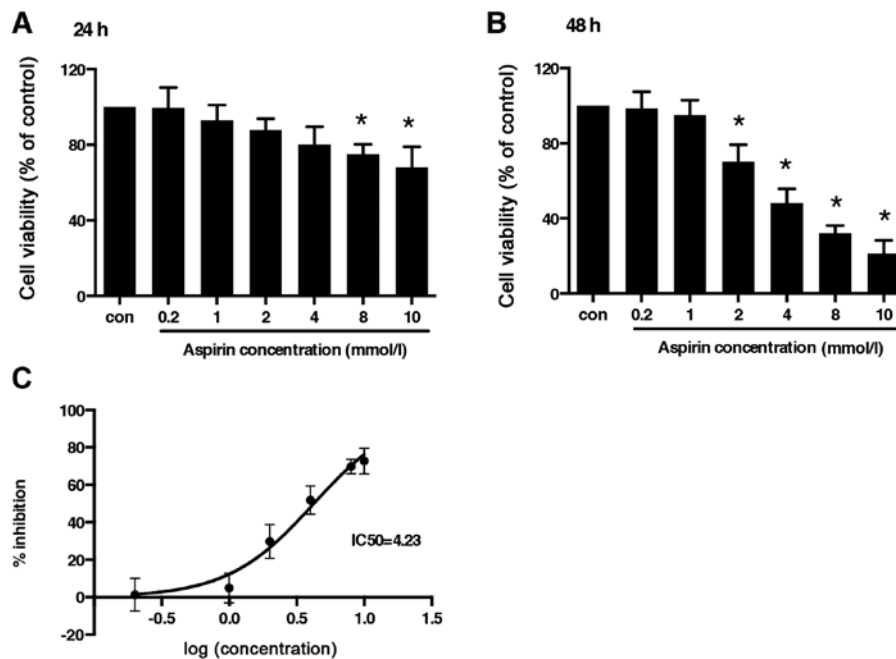


Figure 1. Aspirin inhibits human uterine leiomyoma cell growth. CCK8 analysis was used to measure the cell viability of human uterine leiomyoma cells after treatment with aspirin (0.2, 1, 2, 4, 8 or 10 mmol/l) for 24 h (A) and 48 h (B) (n=5-6, *P<0.05 vs con). (C) Inhibitory dose-response curves to determine the IC₅₀ for aspirin on cell viability after 48-h treatment was calculated by non-linear regression using four-parameter logistic curves on GraphPad Prism 5 software (n=5-6, *P<0.05 vs con). All values represent mean \pm SD.

siRNA transfection. The siRNA duplexes against K-Ras gene synthesized by Ribobio (Guangzhou, China) were transiently transfected with Lipofectamine 2000 reagent (Invitrogen). Scramble RNA was used as negative control. Scramble RNA and K-Ras siRNA strands and Lipofectamine 2000 reagent were diluted in serum and antibiotic-free DMEM to form the transfection complexes for 10 min at room temperature and then were added to cells. After incubation for 6 h at 37°C, transfection complexes were replaced with DMEM containing 20% fetal calf serum (Gibco). Cells were prepared for following experiments after 48 h. The siRNA sequences for K-Ras are as follows: negative control: sense-5'-UUCUCCGAACGUGU CACGU-3'; antisense-5'-ACGUGACACGUUCGGAGAA-3'. KRAS siRNA: sense-5'-GUCUCUUGGAUAUUCUGA-3'; antisense-5'-UCGAGAAUAUCCAAGAGAC-3'.

Immunofluorescence analysis. Immunofluorescence was performed to detect the effects of aspirin on the p110 α /K-Ras interaction. Human uterine leiomyoma cells cotransfected with his-K-Ras and flag-p110 α in confocal well were incubated with primary his and flag antibodies (1:100, Sigma-Aldrich) at 4°C overnight, followed by the incubation with secondary Cy3 anti-mouse or FITC anti-rabbit antibodies (1:200, Beyotime Biotechnology) at room temperature for 1 h. Nuclei were stained with Hoechst (1:200, Beyotime Biotechnology) for 5 min. Cells were photographed by confocal system (Olympus, Tokyo, Japan).

Statistical analysis. Raw data were applied directly in statistical analysis. Statistical analysis was calculated using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Collected data were presented as the mean \pm SD. Datasets with three or more groups were analyzed by one-way

ANOVA. Comparisons of two groups were analyzed by Student's two-tailed t-test. The level for statistical significance was 0.05.

Results

Aspirin inhibits cell proliferation in human uterine leiomyoma cells. Human uterine leiomyoma (UtLM) cells (GM10964) obtained from Coriell Institute for Medical Research have been used widely for investigating cell proliferation of uterine leiomyoma in research as *in vitro* modeling system (27,28). Therefore, we used this cell line for investigating the effects of aspirin on uterine leiomyoma.

To investigate the effects of aspirin on the growth of human uterine leiomyoma cells, human uterine leiomyoma cells were exposed to six different concentrations of aspirin (0.2, 1, 2, 4, 8 and 10 mmol/l) for 24 and 48 h. CCK-8 analysis was used to assess the effects of aspirin on cell viability. Aspirin induced a dose- and time-dependent inhibition in cell viability (Fig. 1). After treatment with 8 and 10 mmol/l aspirin for 24 h, the cell number decreased to 75.09 \pm 5.18 and 68.12 \pm 10.82% respectively, compared with control group (Fig. 1A). The cell number decreased to 70.28 \pm 8.98, 48.12 \pm 7.65, 32.23 \pm 3.87 and 21.32 \pm 6.91% under the treatment with 2, 4, 8 and 10 mmol/l aspirin for 48 h, respectively (Fig. 1B). The half maximal inhibitory concentration (IC₅₀) (Fig. 1C) of aspirin for human uterine leiomyoma cells was 4.23 mmol/l at 48 h of incubation which was selected in the following experiments.

Effect of aspirin on cell cycle in human uterine leiomyoma cells. To investigate the underlying mechanisms of cell growth inhibition induced by aspirin in human uterine leiomyoma cells, we examined the effect of aspirin on cell cycle

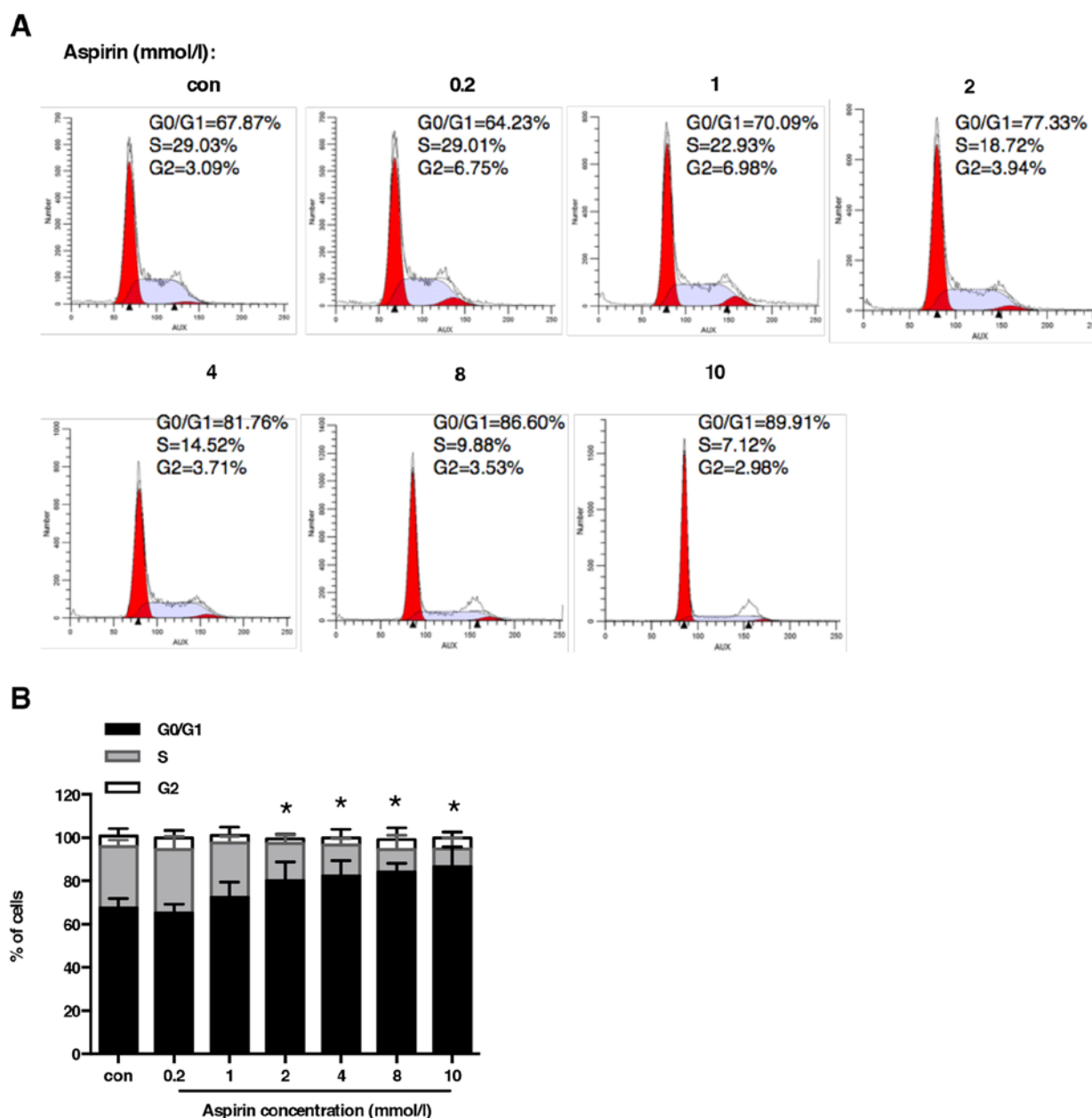


Figure 2. Aspirin inhibits G1/S transition in human uterine leiomyoma cells. (A) Flow cytometry analysis of cell cycle distribution induced by various concentrations of aspirin for 48 h. (B) Percentage of cell number in different phases in cell cycle was determined by quantitative analysis ($n=6-7$, $^*P<0.05$ vs con). All values represent mean \pm SD.

distribution. Different concentrations of aspirin (0.2, 1, 2, 4, 8 and 10 mmol/l) were used for 48 h in human uterine leiomyoma cells (Fig. 2). The percentage of cells in the G0/G1 phase increased from 67.45 ± 4.45 to 80.01 ± 8.80 , 82.33 ± 7.09 , 84.12 ± 4.15 and $86.72 \pm 8.98\%$ in aspirin treatment of 2, 4, 8 and 10 mmol/l respectively. Aspirin decreased the cell population in the S phase from 28.45 ± 3.03 to 17.23 ± 4.09 , 14.19 ± 3.19 , 10.47 ± 6.53 and $8.12 \pm 5.17\%$ in concentrations of 2, 4, 8 and 10 mmol/l respectively. The data suggested aspirin inhibited the cell entrance from the G0/G1 phase to the S phase.

Aspirin inhibits human uterine leiomyoma cell proliferation through blocking the interaction between K-Ras and p110 α . As reported, K-Ras was involved in various carcinomas and the interaction between PI3K p110 α and Ras was required

in tumorigenesis (11). Our results illustrated that the phosphorylation of PI3K p85 and Akt as well as the expression of cell cycle associated protein, including cyclin D1 and CDK2 were decreased under the treatment of aspirin (4.23 mmol/l) for 48 h (Fig. 3A). However, the protein expression of K-Ras and PI3K p110 α did not alter compared with control group (Fig. 3A). Then the K-Ras-p110 α interaction was detected (Fig. 3B). The interaction between K-Ras and p110 α was decreased significantly under treatment of aspirin. In cells transfected with flag-tagged p110 α and his-tagged K-Ras plasmids, co-IP and confocal images also showed that aspirin reduced the interaction between p110 α and K-Ras (Fig. 3C and D). These findings implied that aspirin inhibited human uterine leiomyoma cell growth via reducing the interaction between p110 α and K-Ras.

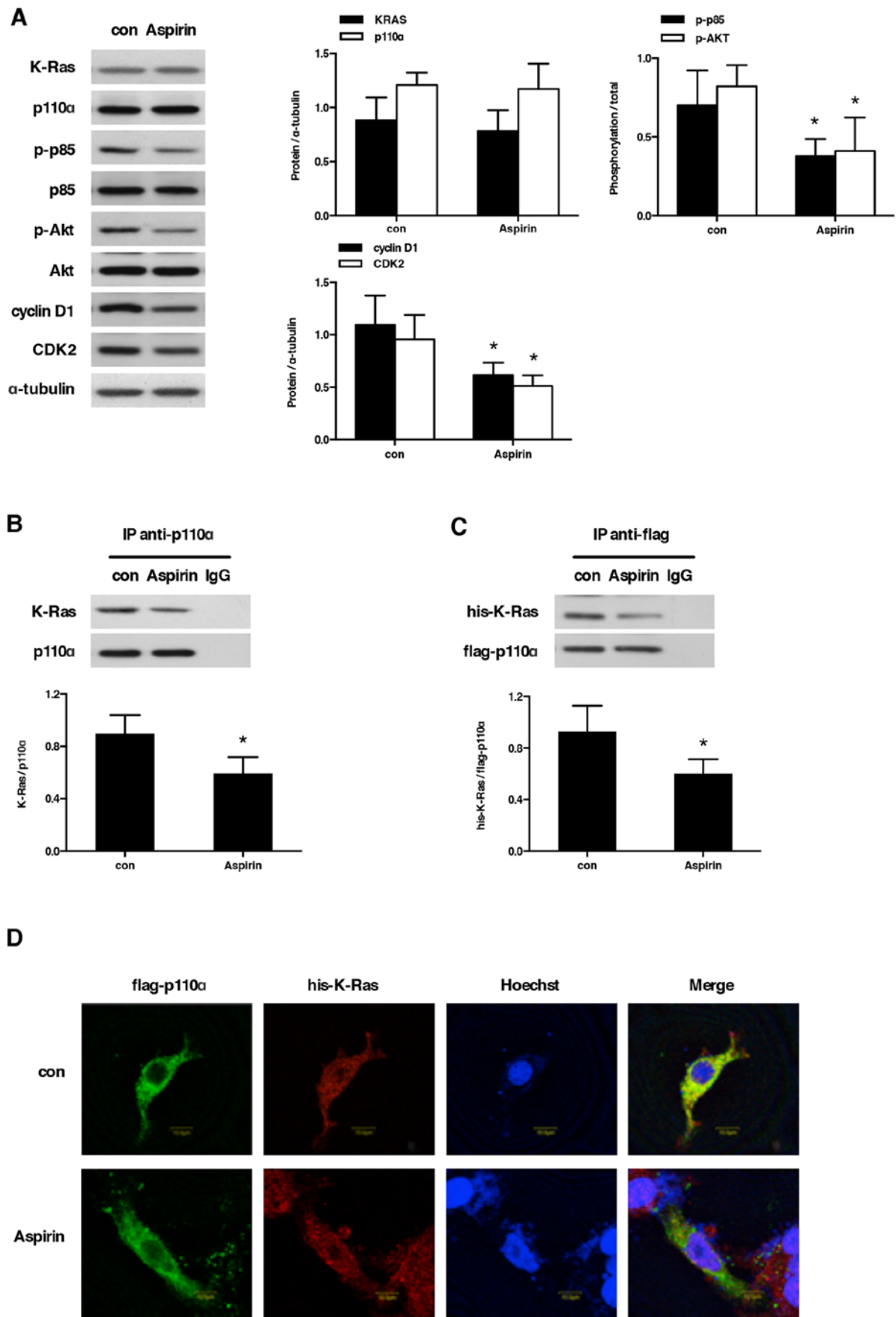


Figure 3. Aspirin blocks K-Ras-p110α interaction in human uterine leiomyoma cells. (A) Western blot analysis of protein expression of human uterine leiomyoma cells under aspirin treatment (4.23 mmol/l) for 48 h. Human uterine leiomyoma cell lysates were harvested for co-immunoprecipitated assays to detect the endogenous (B) and exogenous (C) interaction (n=5, *P<0.05 vs con). (D) Confocal images showed K-Ras-p110α interaction induced by aspirin. Green is for flag-p110α and red is for his-K-Ras and yellow is for merge of green with red which accounted for the K-Ras-p110α interaction (n=3). All values represent mean ± SD.

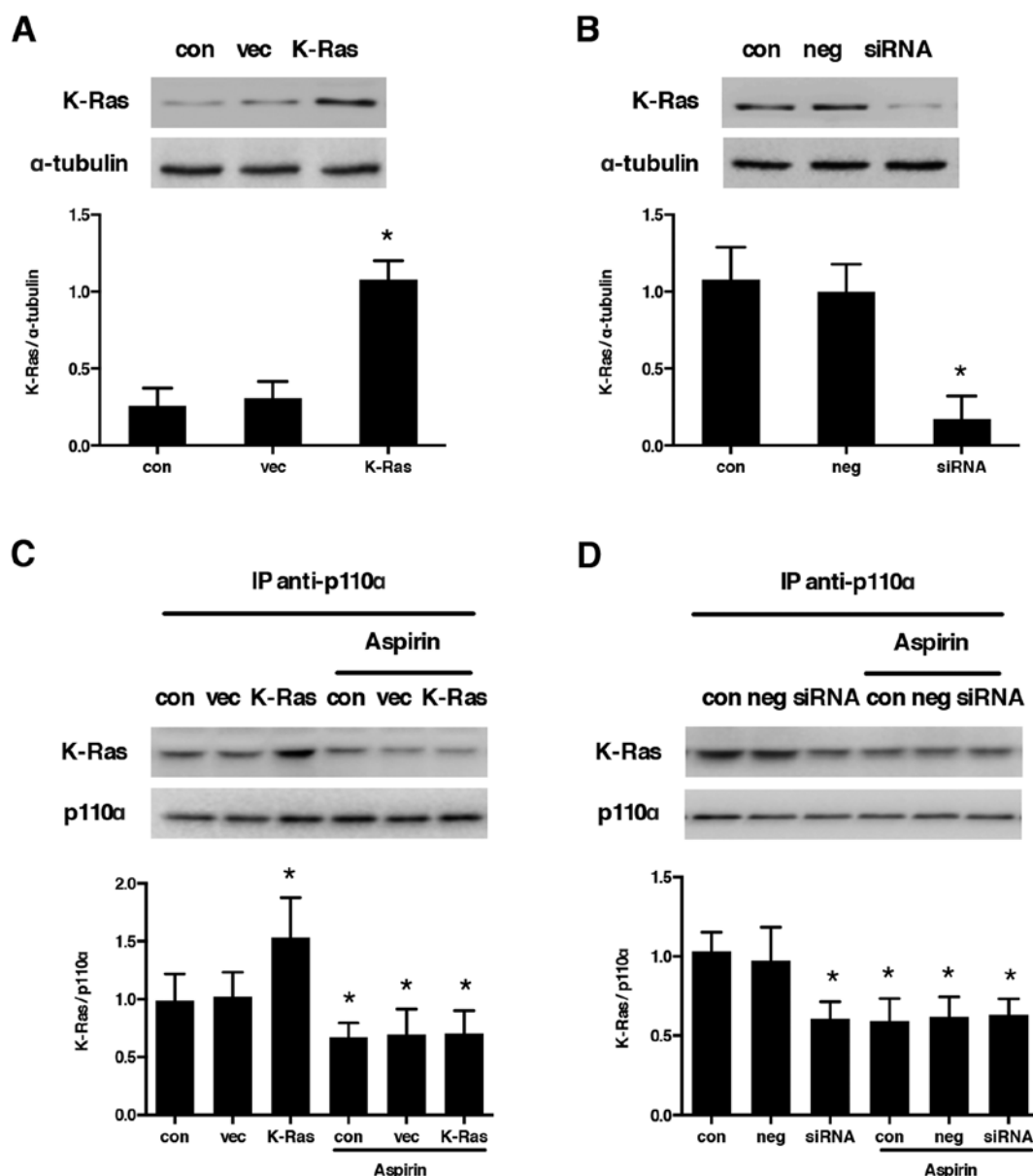


Figure 4. K-Ras-p110 α interaction is regulated by K-Ras expression under aspirin treatment. (A) Western blot analysis showed that pEnter-his-K-Ras plasmid transfection increased K-Ras expression in human uterine leiomyoma cells (n=6, *P<0.05 vs con). (B) Anti-K-Ras siRNA (40 mmol/l) transfection for 48 h significantly decreased endogenous K-Ras protein expression in human uterine leiomyoma cells (n=6, *P<0.05 vs con). (C) The interaction between K-Ras and p110 α was increased in K-Ras overexpressed cells which was inhibited by aspirin treatment (n=6, *P<0.05 vs con). (D) The K-Ras-p110 α interaction was blocked by K-Ras knockdown and aspirin had no further inhibitory effects (n=6, *P<0.05 vs con). All values represent mean \pm SD.

Effect of K-Ras on K-Ras-p110 α interaction under aspirin treatment. Western blotting showed that pEnter-K-Ras plasmid transfection increased K-Ras expression and anti-K-Ras siRNA (40 mmol/l) transfection significantly decreased endogenous K-Ras protein expression in human uterine leiomyoma cells (Fig. 4A and B).

The association between K-Ras and p110 α was remarkably increased by K-Ras overexpression which could be inhibited by aspirin treatment (4.23 mmol/l) for 48 h (Fig. 4C). In K-Ras deletion cells, the interaction between K-Ras and p110 α was blocked and aspirin had no more inhibitory effect on K-Ras-p110 α interaction (Fig. 4D). The results showed the K-Ras-p110 α interaction was in inhibitory status under aspirin treatment no matter how the K-Ras protein expression was changed.

Aspirin inhibits cell proliferation via blocking the interaction of K-Ras with p110 α . In human uterine leiomyoma cells with increased K-Ras-p110 α interaction induced by K-Ras overexpression, the cell viability was enhanced to 132.19 \pm 4.34% which was significantly reduced to 52.91 \pm 7.87% under the treatment of aspirin (4.23 mmol/l) for 48 h (Fig. 5A). Whereas K-Ras knockdown, with blocked K-Ras-p110 α interaction, reduced the cell growth to 55.63 \pm 8.98% compared with control cultures and aspirin did not have further inhibitory effects (Fig. 5B).

Cell cycle distribution was quantified by flow cytometry. The S-phase cell fraction in K-Ras overexpressed cells, with increased K-Ras-p110 α interaction, was increased from 28.45 \pm 4.01 to 36.77 \pm 6.09% and the G0/G1-phase cell fraction was reduced from 65.59 \pm 7.52 to 58.73 \pm 4.67%, while the

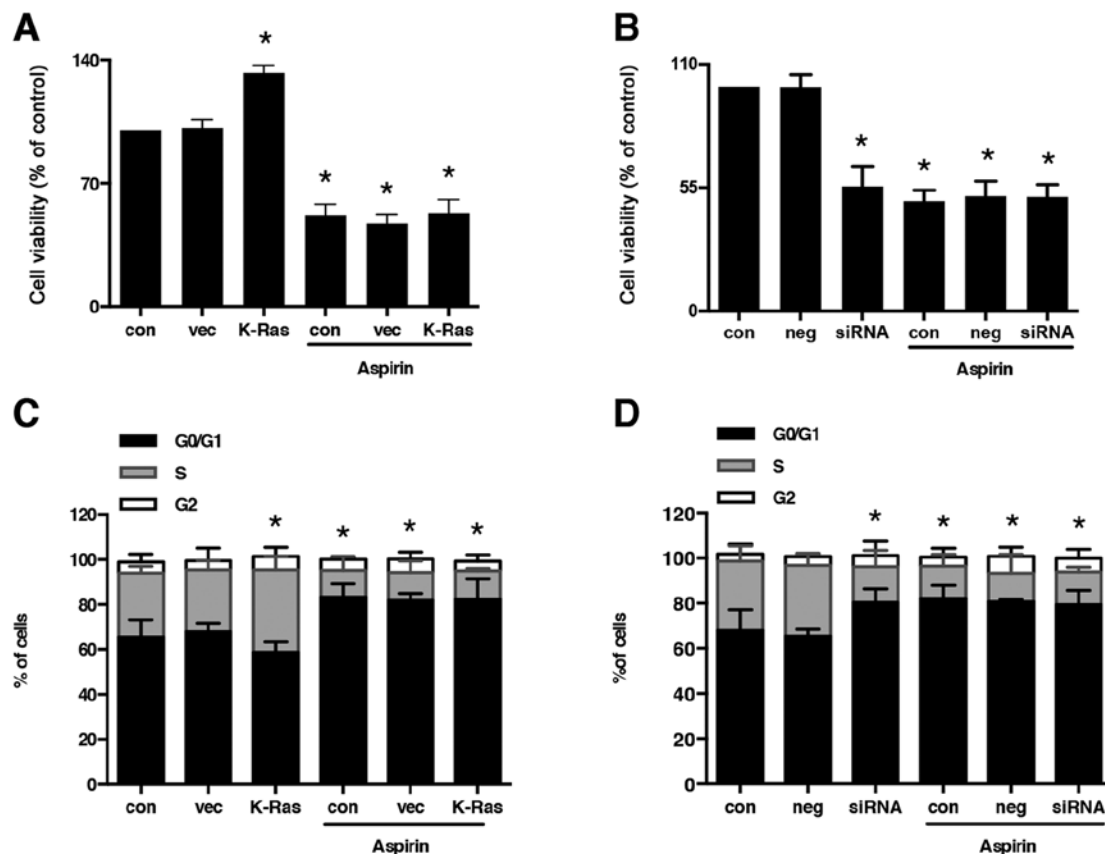


Figure 5. Effects of K-Ras-p110 α interaction on cellular proliferation under aspirin treatment. (A) CCK8 results showed that K-Ras overexpression promoted cell growth which was inhibited by aspirin treatment (n=5, *P<0.05 vs con). (B) K-Ras deficiency inhibited cellular proliferation and aspirin had no more inhibitory effects (n=6, *P<0.05 vs con). (C) Flow cytometry analysis showed that G1/S transition was promoted by K-Ras overexpression which was inhibited by aspirin treatment (n=6, *P<0.05 vs con). (D) K-Ras knockdown inhibited the cell cycle transition with no more inhibitory effects under aspirin treatment (n=6-7, *P<0.05 vs con). All values represent mean \pm SD.

S-phase cell fraction was reduced to $12.67 \pm 1.09\%$ and the G0/G1-phase cell fraction was increased to $82.34 \pm 9.01\%$ under aspirin treatment (Fig. 5C). Furthermore, in K-Ras knockdown cells, with reduced K-Ras-p110 α interaction, the S-phase cell fraction was reduced from 30.73 ± 6.78 to $15.79 \pm 7.22\%$ and the G0/G1-phase cell fraction was increased from 67.98 ± 9.09 to $80.37 \pm 6.01\%$ and aspirin did not have further effects on cell cycle arrest (Fig. 5D). Our data indicated that the inhibitory effects on cell viability and cell cycle transition of aspirin treatment was dependent on the blockage of the interaction of K-Ras with p110 α in human uterine leiomyoma cells.

Effect of K-Ras-p110 α interaction on cell cycle regulators under treatment of aspirin. Cell cycle transition is positively regulated by cyclins and cyclin-dependent kinases. To explore the molecular mechanism by which K-Ras-p110 α interaction affects G1/S transition, we analyzed the proteins regulating cell cycle progression including cyclin D1 and CDK2. We found that the expression of cyclin D1 and CDK2 was enhanced in K-Ras overexpression group in which the interaction of K-Ras and p110 α was increased while the protein expression was remarkably reduced under aspirin treatment (Fig. 6A). In addition, K-Ras deletion made reduction in expression of cyclin D1 and CDK2 compared with control group and aspirin had no more effects in reduction of cyclin D1 and CDK2 that was attributed to the reduced association of K-Ras and

p110 α (Fig. 6B). The data suggested that aspirin-induced inhibition on cell growth and cell cycle transition via cell cycle regulators was K-Ras-p110 α interaction-dependent.

PI3K/Akt signaling pathway was involved in aspirin-induced inhibitory effects on cell proliferation. In K-Ras overexpressed cells with the increase in K-Ras-p110 α interaction, the levels of phosphorylated PI3K p85 and phosphorylated Akt were enhanced while the phosphorylated protein expression was decreased in aspirin treatment (Fig. 7A). On the contrary, in K-Ras knockdown cells with reduced K-Ras-p110 α interaction, the protein expression of phosphorylated p85 and Akt was reduced and aspirin did not have further inhibitory effects (Fig. 7B). Total proteins of PI3K p85 and Akt had no significant differences among the groups. Taken together, these results demonstrated that aspirin inhibited PI3K/Akt pathway by targeting K-Ras-p110 α interaction.

Inhibition of PI3K/AKT pathway by aspirin enhances caspase activation. Caspase family members have been proved to be associated with PI3K/AKT signaling pathway in cell growth (29,30). Therefore, we further determined the effects of aspirin on caspase activation. In K-Ras overexpression cells, the levels of cleaved caspase-3, -9, -8, -12 and cleaved PARP protein expression were increased significantly by aspirin treatment (Fig. 8A). On the other hand, in K-Ras knockdown

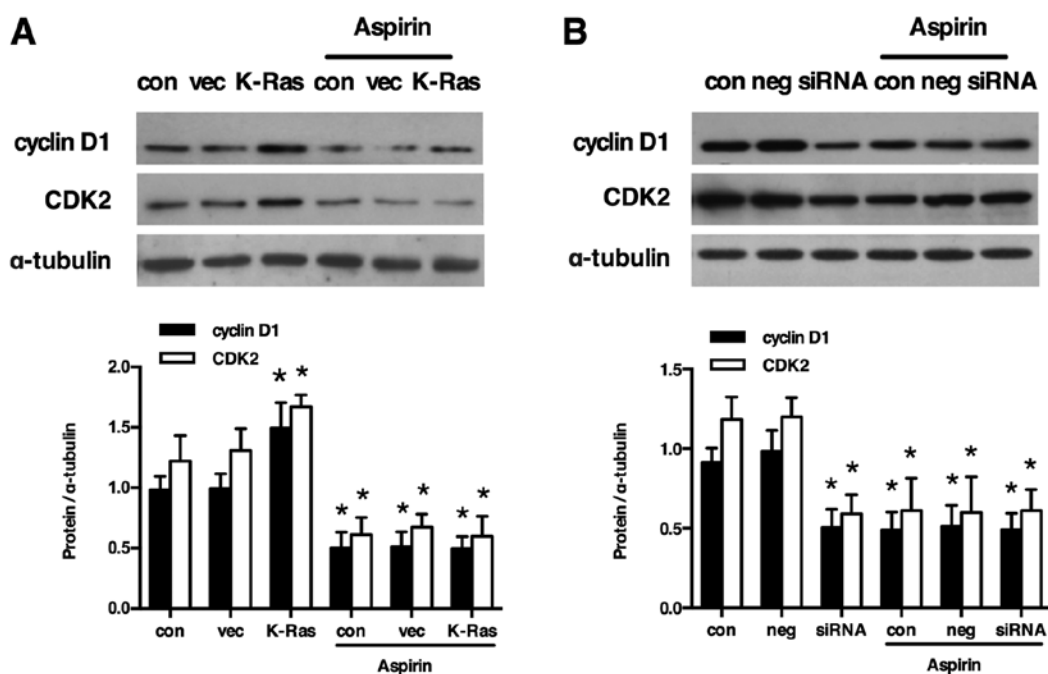


Figure 6. Effects of K-Ras-p110 α interaction on cell cycle regulatory proteins. (A) Representative western blot images and densitometric analysis showed that K-Ras cDNA transfection significantly increased cyclin D1 and CDK2 expression which was reduced by aspirin treatment in human uterine leiomyoma cells ($n=5$, $^*P<0.05$ vs con). (B) Knockdown of K-Ras reduced protein expression of cyclin D1 and CDK2 which aspirin had no more effects on ($n=6$, $^*P<0.05$ vs con). All values represent mean \pm SD.

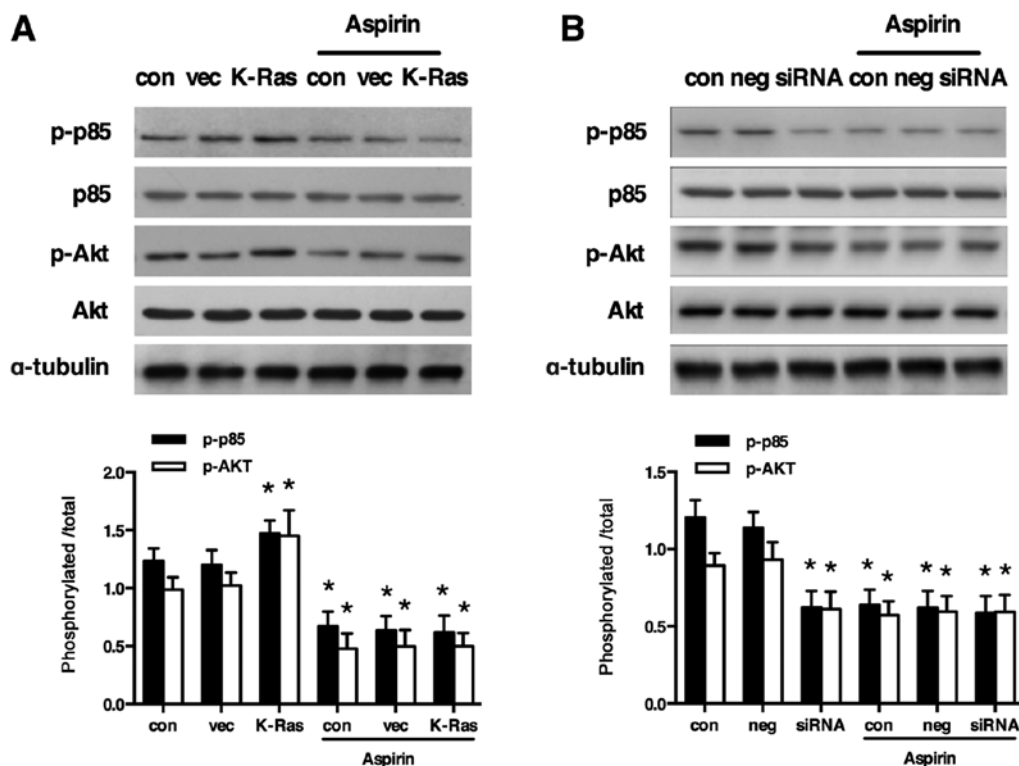


Figure 7. Aspirin inhibits human uterine leiomyoma cell proliferation through K-Ras/PI3K/Akt signaling pathway. (A) Western blot analysis showed K-Ras cDNA transfection significantly increased the phosphorylation of PI3K p85 and Akt which was inhibited by treatment of aspirin ($n=5$, $^*P<0.05$ vs con). (B) K-Ras deletion reduced the protein expression of phosphorylated PI3K p85 and Akt with no more inhibitory effects under aspirin treatment in human uterine leiomyoma cells ($n=7-8$, $^*P<0.05$ vs con). All values represent mean \pm SD.

cells with reduced K-Ras-p110 α interaction, the protein expression of cleaved caspase-3, -9, -8, -12 and cleaved PARP were increased and aspirin did not have further enhancement

effects (Fig. 8B). Our results indicated that aspirin enhanced caspase activation through the inhibition of PI3K/AKT signaling pathway.

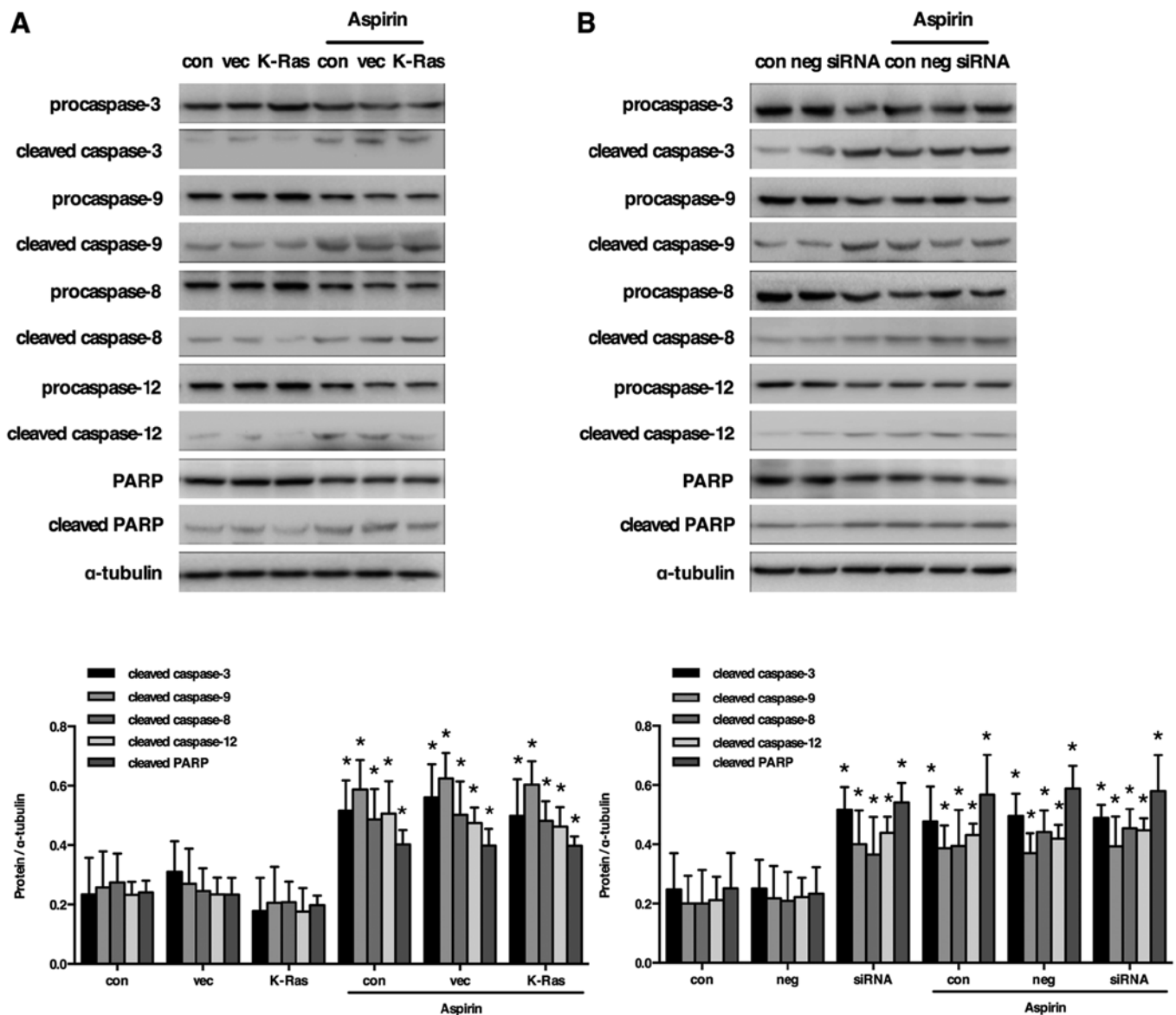


Figure 8. Caspase activation and PARP cleavage induced by aspirin are dependent on K-Ras/PI3K/Akt signaling pathway. (A) Western blot results showed in K-Ras overexpression cells, the expression of cleaved caspase-3, -9, -8, -12, and PRAP were increased under aspirin treatment (n=4, *P<0.05 vs con). (B) Western blot results showed that cleaved caspase-3, -9, -8, -12, and PRAP were increased in K-Ras deletion human uterine leiomyoma cells and aspirin had no more enhancement effects on caspase activation and PARP cleavage (n=4-5, *P<0.05 vs con). All values represent mean \pm SD.

Discussion

In the present study, we found that aspirin inhibits the proliferation and promote cell cycle arrest via cell cycle regulators of human uterine leiomyoma cells. These effects are at least in part by downregulation of K-Ras-p110 α interaction and the subsequent modulation of PI3K/Akt/caspase signaling pathway.

Recent studies have demonstrated a wide variety of positive effects of aspirin on cancer therapy, however, little is known about the effects of aspirin on uterine leiomyoma. Thus, we showed that aspirin inhibited human uterine leiomyoma cell proliferation *in vitro*. The dose of aspirin is varied depend on different diseases, such as 75-325 mg/day for cardiovascular disease, \leq 325 mg/day for osteoarthritis and rheumatoid arthritis, and low dose of 75-300 mg/day for colorectal cancer and endometrial cancer (8,31-33). In

addition, *in vitro* experiments, the effective doses of aspirin in most studies in colon, prostate or breast cancer cells vary from 1-10 mmol/l (26,34,35). Our data suggested that the IC₅₀ value of aspirin for cell growth inhibition was 4.23 mmol/l. Moreover, Juárez Olguín *et al* (36) demonstrated that it was safe to reach the maximum serum concentration of aspirin as high as 10 mmol/l, indicating that the dose of 4.23 mmol/l in aspirin use was safe and feasible and was used for the following experiments.

PI3K along with Ras family small GTPases, mediating cell growth, differentiation, proliferation and multiple other cellular processes, are one of the most important early signaling components (37,38). Ras small GTPases have been shown to directly associate with p110 catalytic subunits of PI3K through a Ras binding domain (RBD) and then activate the following PI3K/Akt pathway. It is reported that Ras is indispensable for the full activation of class I PI3Ks (39).

Rao *et al* illustrated that low-dose aspirin inhibited the pancreatic carcinoma activity in Kras^{G12D/+} transgenic mice (40). Then we testified that aspirin dramatically blocked the interaction between K-Ras and p110 α . These results indicated that aspirin might inhibit the proliferation of human uterine leiomyoma cells through reduction in K-Ras-p110 α interaction.

The K-Ras protein has been considered as a major target in the discovery of antitumor drugs because K-Ras mutation is commonly observed in various cancers and it lies at the apex of numerous growth regulatory cascades (41-43). Knockdown of K-Ras suppressed tumor growth while overexpression of K-Ras led to various cancers (44,45). It has been reported that several K-Ras inhibitors, including farnesyltransferase inhibitors, have been explored to lead to inhibition of wild-type K-Ras activity, growth and development (46). We found that in human uterine leiomyoma cells, K-Ras overexpression could increase the interaction of K-Ras and p110 α while K-Ras knockdown could inhibit the K-Ras-p110 α interaction. Then we used the strategy of K-Ras overexpression or K-Ras knockdown to modulate the interaction of K-Ras with p110 α . In order to verify the role of K-Ras-p110 α interaction which may perform as a potential target of aspirin, we assessed the cell growth, cell cycle transition, the expression of cell cycle regulators and the activation of the following PI3K/Akt signaling pathway upon overexpression or knockdown of K-Ras in human uterine leiomyoma cells. In the present study, it was demonstrated that the inhibition in cell proliferation along with the G1/S phase transition by aspirin treatment could be observed in K-Ras overexpressed cells. Furthermore, K-Ras knockdown did not have further inhibitory effects on cell growth induced by aspirin. These data demonstrated that the anti-proliferation effects of aspirin on human uterine leiomyoma cells was K-Ras-p110 α interaction-dependent. Further studies found that in K-Ras overexpressed cells, expression of cyclin D1 and CDK2 along with the upstream kinases of phosphorylated PI3K p85 and Akt were reduced while K-Ras deletion did not cause further inhibitory effects under aspirin treatment. Caspase activity has been shown to take part in modulating cell proliferation and apoptosis. Several recent observations suggest caspases exert important functions beyond that of cell apoptosis, including cellular proliferation and cell cycle progression (47,48). Previous reports have suggested that the activation of caspases, including caspase-3, caspase-8 and caspase-9, can be regulated by PI3K/AKT pathway (30,49,50). Our data showed that in K-Ras overexpressed human uterine leiomyoma cells, the caspase activation could be increased by aspirin. Otherwise, K-Ras knockdown increased caspase activation while aspirin did not have further enhancement effects on caspase activation. These findings illustrated that the proliferation inhibition effects of aspirin on human uterine leiomyoma cells was dependent on K-Ras-p110 α interaction, followed by modulation of PI3K/Akt/caspase signaling pathway.

In conclusion, aspirin inhibits the proliferation of human uterine leiomyoma cells *in vitro*. Specifically, it inhibited cell proliferation and promoted cell cycle arrest. Furthermore, the status of downregulation of the K-Ras-p110 α interaction, reduction of PI3K/Akt activation significantly affected the efficacy of aspirin, which resulted in caspase activation and

cyclin D1 and CDK2 degradation and eventually, cell cycle arrest and cell growth inhibition. It is noteworthy that the inhibition of K-Ras-p110 α interaction may play the most critical role. This study suggested that aspirin might be a kind of therapeutic agent for uterine leiomyoma treatment, and targeting K-Ras-p110 α interaction might provide new strategies to treat uterine leiomyoma.

Acknowledgements

This study was supported by National Natural Science Foundation of China (no. 81302771), Natural Science Foundation of Guangdong Province (no. 2014A030313087), Science and Technology program of Guangzhou City (no. 201607010255), and the Fundamental Research Funds for the Central Universities (no. 17ykzd02).

References

- Islam MS, Protic O, Giannubilo SR, Toti P, Tranquilli AL, Petraglia F, Castellucci M and Ciarmela P: Uterine leiomyoma: Available medical treatments and new possible therapeutic options. *J Clin Endocrinol Metab* 98: 921-934, 2013.
- Mehine M, Kaasinen E, Mäkinen N, Katainen R, Kämpjärvi K, Pitkänen E, Heinonen HR, Bützow R, Kilpivaara O, Kuosmanen A, *et al*: Characterization of uterine leiomyomas by whole-genome sequencing. *N Engl J Med* 369: 43-53, 2013.
- Eltoukhi HM, Modi MN, Weston M, Armstrong AY and Stewart EA: The health disparities of uterine fibroid tumors for African American women: A public health issue. *Am J Obstet Gynecol* 210: 194-199, 2014.
- Drayer SM and Catherino WH: Prevalence, morbidity, and current medical management of uterine leiomyomas. *Int J Gynaecol Obstet* 131: 117-122, 2015.
- Bosetti C, Rosato V, Gallus S, Cuzick J and La Vecchia C: Aspirin and cancer risk: A quantitative review to 2011. *Ann Oncol* 23: 1403-1415, 2012.
- Rothwell PM, Price JF, Fowkes FG, Zanchetti A, Roncaglioni MC, Tognoni G, Lee R, Belch JF, Wilson M, Mehta Z, *et al*: Short-term effects of daily aspirin on cancer incidence, mortality, and non-vascular death: Analysis of the time course of risks and benefits in 51 randomised controlled trials. *Lancet* 379: 1602-1612, 2012.
- Thun MJ, Jacobs EJ and Patrono C: The role of aspirin in cancer prevention. *Nat Rev Clin Oncol* 9: 259-267, 2012.
- Drew DA, Cao Y and Chan AT: Aspirin and colorectal cancer: The promise of precision chemoprevention. *Nat Rev Cancer* 16: 173-186, 2016.
- Johnson CC, Hayes RB, Schoen RE, Gunter MJ, Huang WY and Team PT: PLCO Trial Team: Non-steroidal anti-inflammatory drug use and colorectal polyps in the Prostate, Lung, Colorectal, And Ovarian Cancer Screening Trial. *Am J Gastroenterol* 105: 2646-2655, 2010.
- Gamba CA, Swetter SM, Stefanick ML, Kubo J, Desai M, Spaunhurst KM, Sinha AA, Asgari MM, Sturgeon S and Tang JY: Aspirin is associated with lower melanoma risk among postmenopausal Caucasian women: The Women's Health Initiative. *Cancer* 119: 1562-1569, 2013.
- Veitonmäki T, Tammela TL, Auvinen A and Murtola TJ: Use of aspirin, but not other non-steroidal anti-inflammatory drugs is associated with decreased prostate cancer risk at the population level. *Eur J Cancer* 49: 938-945, 2013.
- Rothwell PM, Wilson M, Price JF, Belch JF, Meade TW and Mehta Z: Effect of daily aspirin on risk of cancer metastasis: A study of incident cancers during randomised controlled trials. *Lancet* 379: 1591-1601, 2012.
- MacDonald BT, Tamai K and He X: Wnt/beta-catenin signaling: Components, mechanisms, and diseases. *Dev Cell* 17: 9-26, 2009.
- Park IS, Jo JR, Hong H, Nam KY, Kim JB, Hwang SH, Choi MS, Ryu NH, Jang HJ, Lee SH, *et al*: Aspirin induces apoptosis in YD-8 human oral squamous carcinoma cells through activation of caspases, down-regulation of Mcl-1, and inactivation of ERK-1/2 and AKT. *Toxicol In Vitro* 24: 713-720, 2010.

15. Wang D and Dubois RN: The role of COX-2 in intestinal inflammation and colorectal cancer. *Oncogene* 29: 781-788, 2010.
16. Ding JH, Yuan LY, Huang RB and Chen GA: Aspirin inhibits proliferation and induces apoptosis of multiple myeloma cells through regulation of Bcl-2 and Bax and suppression of VEGF. *Eur J Haematol* 93: 329-339, 2014.
17. Niault TS and Baccarini M: Targets of Raf in tumorigenesis. *Carcinogenesis* 31: 1165-1174, 2010.
18. Hong W, Abi-Raad R, Alomari AK, Hui P and Buza N: Diagnostic application of KRAS mutation testing in uterine microglandular proliferations. *Hum Pathol* 46: 1000-1005, 2015.
19. Lim KH and Counter CM: Reduction in the requirement of oncogenic Ras signaling to activation of PI3K/AKT pathway during tumor maintenance. *Cancer Cell* 8: 381-392, 2005.
20. Schubbert S, Bollag G, Lyubynska N, Nguyen H, Kratz CP, Zenker M, Niemeyer CM, Molven A and Shannon K: Biochemical and functional characterization of germ line KRAS mutations. *Mol Cell Biol* 27: 7765-7770, 2007.
21. Shieh A, Ward AF, Donlan KL, Harding-Theobald ER, Xu J, Mullighan CG, Zhang C, Chen SC, Su X, Downing JR, *et al*: Defective K-Ras oncoproteins overcome impaired effector activation to initiate leukemia in vivo. *Blood* 121: 4884-4893, 2013.
22. Castellano E, Sheridan C, Thin MZ, Nye E, Spencer-Dene B, Diefenbacher ME, Moore C, Kumar MS, Murillo MM, Grönroos E, *et al*: Requirement for interaction of PI3-kinase p110 α with RAS in lung tumor maintenance. *Cancer Cell* 24: 617-630, 2013.
23. Murillo MM, Zelenay S, Nye E, Castellano E, Lassailly F, Stamp G and Downward J: RAS interaction with PI3K p110 α is required for tumor-induced angiogenesis. *J Clin Invest* 124: 3601-3611, 2014.
24. di Magliano MP and Logsdon CD: Roles for KRAS in pancreatic tumor development and progression. *Gastroenterology* 144: 1220-1229, 2013.
25. Liao X, Lochhead P, Nishihara R, Morikawa T, Kuchiba A, Yamauchi M, Imamura Y, Qian ZR, Baba Y, Shima K, *et al*: Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. *N Engl J Med* 367: 1596-1606, 2012.
26. Turturro SB, Najor MS, Ruby CE, Cobleigh MA and Abukhdeir AM: Mutations in PIK3CA sensitize breast cancer cells to physiologic levels of aspirin. *Breast Cancer Res Treat* 156: 33-43, 2016.
27. Di X, Yu L, Moore AB, Castro L, Zheng X, Hermon T and Dixon D: A low concentration of genistein induces estrogen receptor- α and insulin-like growth factor-I receptor interactions and proliferation in uterine leiomyoma cells. *Hum Reprod* 23: 1873-1883, 2008.
28. Gao X, Yu L, Castro L, Tucker CJ, Moore AB, Xiao H and Dixon D: An essential role of p27 downregulation in fenvalerate-induced cell growth in human uterine leiomyoma and smooth muscle cells. *Am J Physiol Endocrinol Metab* 303: E1025-E1035, 2012.
29. Hafizi S, Ibraimi F and Dahlbäck B: C1-TEN is a negative regulator of the Akt/PKB signal transduction pathway and inhibits cell survival, proliferation, and migration. *FASEB J* 19: 971-973, 2005.
30. Barber AJ, Nakamura M, Wolpert EB, Reiter CE, Seigel GM, Antonetti DA and Gardner TW: Insulin rescues retinal neurons from apoptosis by a phosphatidylinositol 3-kinase/Akt-mediated mechanism that reduces the activation of caspase-3. *J Biol Chem* 276: 32814-32821, 2001.
31. Silverstein FE, Faich G, Goldstein JL, Simon LS, Pincus T, Whelton A, Makuch R, Eisen G, Agrawal NM, Stenson WF, *et al*: Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. *JAMA* 284: 1247-1255, 2000.
32. Campbell CL, Smyth S, Montalescot G and Steinhilb SR: Aspirin dose for the prevention of cardiovascular disease: A systematic review. *JAMA* 297: 2018-2024, 2007.
33. Verdoordt F, Friis S, Dehlendorff C, Albieri V and Kjaer SK: Non-steroidal anti-inflammatory drug use and risk of endometrial cancer: A systematic review and meta-analysis of observational studies. *Gynecol Oncol* 140: 352-358, 2016.
34. Pathi S, Jutooru I, Chadalapaka G, Nair V, Lee SO and Safe S: Aspirin inhibits colon cancer cell and tumor growth and down-regulates specificity protein (Sp) transcription factors. *PLoS One* 7: e48208, 2012.
35. Olivan M, Rigau M, Colás E, Garcia M, Montes M, Sequeiros T, Regis L, Celma A, Planas J, Placer J, *et al*: Simultaneous treatment with statins and aspirin reduces the risk of prostate cancer detection and tumorigenic properties in prostate cancer cell lines. *BioMed Res Int* 2015: 762178, 2015.
36. Juárez Olguín H, Flores Pérez J, Lares Asseff I, Loredó Abdalá A and Carbajal Rodríguez L: Comparative pharmacokinetics of acetyl salicylic acid and its metabolites in children suffering from autoimmune diseases. *Biopharm Drug Dispos* 25: 1-7, 2004.
37. Cantley LC: The phosphoinositide 3-kinase pathway. *Science* 296: 1655-1657, 2002.
38. Gupta S, Ramjaun AR, Haiko P, Wang Y, Warne PH, Nicke B, Nye E, Stamp G, Alitalo K and Downward J: Binding of ras to phosphoinositide 3-kinase p110 α is required for ras-driven tumorigenesis in mice. *Cell* 129: 957-968, 2007.
39. Yang HW, Shin MG, Lee S, Kim JR, Park WS, Cho KH, Meyer T and Heo WD: Cooperative activation of PI3K by Ras and Rho family small GTPases. *Mol Cell* 47: 281-290, 2012.
40. Rao CV, Mohammed A, Janakiram NB, Li Q, Ritchie RL, Lightfoot S, Vibhudutta A and Steele VE: Inhibition of pancreatic intraepithelial neoplasia progression to carcinoma by nitric oxide-releasing aspirin in p48(Cre/+)-LSL-Kras(G12D/+) mice. *Neoplasia* 14: 778-787, 2012.
41. Downward J: Targeting RAS signalling pathways in cancer therapy. *Nat Rev Cancer* 3: 11-22, 2003.
42. Gelb MH, Brunsfeld L, Hrycyna CA, Michaelis S, Tamanoi F, Van Voorhis WC and Waldmann H: Therapeutic intervention based on protein prenylation and associated modifications. *Nat Chem Biol* 2: 518-528, 2006.
43. Zimmermann G, Papke B, Ismail S, Vartak N, Chandra A, Hoffmann M, Hahn SA, Triola G, Wittinghofer A, Bastiaens PI, *et al*: Small molecule inhibition of the KRAS-PDE δ interaction impairs oncogenic KRAS signalling. *Nature* 497: 638-642, 2013.
44. Pylayeva-Gupta Y, Grabocka E and Bar-Sagi D: RAS oncogenes: Weaving a tumorigenic web. *Nat Rev Cancer* 11: 761-774, 2011.
45. Sunaga N, Shames DS, Girard L, Peyton M, Larsen JE, Imai H, Soh J, Sato M, Yanagitani N, Kaira K, *et al*: Knockdown of oncogenic KRAS in non-small cell lung cancers suppresses tumor growth and sensitizes tumor cells to targeted therapy. *Mol Cancer Ther* 10: 336-346, 2011.
46. Lerner EC, Zhang TT, Knowles DB, Qian Y, Hamilton AD and Sefti SM: Inhibition of the prenylation of K-Ras, but not H- or N-Ras, is highly resistant to CAAX peptidomimetics and requires both a farnesyltransferase and a geranylgeranyltransferase I inhibitor in human tumor cell lines. *Oncogene* 15: 1283-1288, 1997.
47. Los M, Strohm C, Jänicke RU, Engels IH and Schulze-Osthoff K: Caspases: More than just killers? *Trends Immunol* 22: 31-34, 2001.
48. Song R, Mahidhara RS, Zhou Z, Hoffman RA, Seol DW, Flavell RA, Billiar TR, Otterbein LE and Choi AM: Carbon monoxide inhibits T lymphocyte proliferation via caspase-dependent pathway. *J Immunol* 172: 1220-1226, 2004.
49. Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S and Reed JC: Regulation of cell death protease caspase-9 by phosphorylation. *Science* 282: 1318-1321, 1998.
50. Uriarte SM, Joshi-Barve S, Song Z, Sahoo R, Gobejishvili L, Jala VR, Haribabu B, McClain C and Barve S: Akt inhibition upregulates FasL, downregulates c-FLIPs and induces caspase-8-dependent cell death in Jurkat T lymphocytes. *Cell Death Differ* 12: 233-242, 2005.