Cell Cycle Arrest and Autophagy Induced by Compound Kushen Injection in sw620 and sw480 Colorectal Cancer Cells with p53 Mutation

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Abstract

Background Compound Kushen Injection (CKI) has been clinically used in China for 15 years to treat various types of solid tumors, including colorectal cancer. Here we examine cell cycle arrest, induced autophagy, and mutant p53 pathways perturbed by CKI in colorectal cancer cells. We and other groups have shown that CKI alters p53 gene expression patterns and suppresses proliferation in colorectal cancer cells. Methods We measured the effect of CKI on cell proliferation, cell cycle progression and autophagy in sw480 and sw620 colorectal cancer cells in vitro, and carcinogenesis and the progression of azoxymethane/dextran sodium sulfate-induced colorectal cancer in ICR mice in vivo. We also used RNA sequencing to analyze mRNA expression altered by CKI, and further validated the expression of mutant p53 and several genes in the cell cycle pathway using reverse transcriptase-quantitative PCR and western blotting. Using network pharmacology (BATMAN-TCM database), we have also predicted the active ingredients in CKI involved in regulating the expression of mutant p53. Results We show evidence that CKI significantly suppressed proliferation and cell cycle progression, and induced autophagy of sw480 and sw620 cells in vitro; it also inhibited the development of inflammatory colorectal cancer in vivo. We also show that the down-regulated expression of mutant p53 and adjustments in several key genes related closely to cell-cycle progression. Furthermore, N-oxysophocarpine, lupenone, and geranylacetone were predicted to be the active ingredients of CKI involved in the down-regulated expression of mutant p53. Conclusion Our results indicate that CKI likely acts as a potential anti-cancer therapeutic agent that targets the cell cycle pathway, suggesting a key role in the development of a novel subsidiary therapeutic approach against mutant p53 in patients with colorectal cancer.

Background

Colorectal carcinoma (CRC) remains one of the most common types of human malignancies [1]. CRC is one of the main causes of mortality due to the lack of effective treatment and its unclear pathogenesis. In addition to surgery, fluorouracil-based chemotherapy is widely used to treat advanced colon cancer, but toxicity and side effects are harmful, which seriously affects the survival of patients [2]. Hence, exploring new and effective drugs is required to combat the disease. Traditional Chinese Medicines (TCMs), including Compound Kushen Injection (CKI), have outstanding potential in the prevention and treatment of cancers, particularly gastrointestinal cancers [3-5].

CKI is extracted from two herbs, Kushen (*Radix Sophorae Flavescentis*) and Baituling (*Rhizoma Smilacis Glabrae*), with the primary components being oxymatrine and matrine. CKI has been extensively used to treat cancer patients in a Chinese clinical setting in combination with chemotherapy for more than 15 years. Randomized controlled trials show significant efficacy of CKI in the treatment of advanced cancer especially colorectal cancer [6]. It has been demonstrated that CKI decreased the growth of colorectal cancer [7], hepatic carcinoma[8], human breast cancer [9], and gastric cancer cells [10]*in vitro*. Despite extensive studies, the molecular mechanisms underlying the CKI-induced growth inhibition of CRC cells remain to be fully elucidated.

As reported, p53 plays an important role in protecting the integrity of the genome, being described as the “guardian of the genome” [11]. The tumor suppressor, p53, is important in mediating cell responses to various stresses, predominantly by inducing or suppressing a number of genes involved in cell behaviors, including cell proliferation, cell cycle arrest and autophagy [7, 12]. Previous studies have shown that p21 up-regulation was attributable to cell cycle phase arrest and that p53 was implicated [13]. Nevertheless, a mutation of the *p53* gene is found in approximately 50% of tumors, especially in CRC specimens [7]. According to official reports from the American Type Culture Collection, a mutation exists in codon 273 of the *p53* gene, resulting in the elevated expression of p53 protein in sw480 and sw620 cells. Thus, there is a constant need to identify novel drugs that are involved in inhibiting the expression of mutant p53. Studies have shown that the chemical components of TCM can act on the p53 signaling pathway to regulate cell cycle progression, suggesting a role in countering human colorectal cancer [7].

In preliminary studies, the antitumor efficacy of CKI in human colon cancer cells and the role mutant p53 played in the antitumor activity of CKI were investigated. We found that after CKI treatment, colon cancer cell proliferation and autophagy were induced. Gene and protein expression analyses showed the suppression of mutant p53, which is involved in cell cycle arrest and autophagy behavior. Furthermore, three ingredients making up CKI were identified as acting on mutant p53 by network pharmacology analysis. Our results indicate that CKI likely acts as a potential anti-cancer therapeutic agent that targets the cell cycle pathway, suggesting a key role in the development of a novel subsidiary therapeutic approach against mutant p53 in patients with colorectal cancer.

Methods

Cell Culture

The human colorectal cancer cell lines, SW480 (Catalog No. TCHu172) and SW620 (Catalog No. TCHu101) were purchased from the Cell Bank of the Chinese Academy of Sciences(Shanghai, China). Cells were maintained in DMEM media (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 IU/mL penicillin, and 100 μg/mL streptomycin. All cells were incubated at 37°C in a humidified incubator with 5% CO_2. Media were changed every second or third day.

Cell Proliferation Assays

The cell viability of SW480 and SW620 cells after CKI treatment was characterized after a CCK-8 (Cell Counting Kit-8) assay. Briefly, we adjusted the cell concentration to 1×10^6 cells/mL, then seeded 100 μL of SW480 and SW620 cells (5,000 cells per well) into 96-well flat-bottomed plates, respectively. We then added 10 mL of CKI to each well, with the final drug concentration at 2 mg/mL (based on the total alkaloid concentration in CKI). CKI were purchased from Shanxi Zhendong Pharmaceutical Co., Ltd. (20161113, changzhi, shanxi, China). The plates were incubated for 24 or 48 h. Ten μL of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added to each well of the plates and mixed thoroughly. Subsequently, the plates were incubated for another 2h, and optical densities were obtained by reading

the plates at 450 nm with a 96-well micro test spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). Each concentration of CKI was assayed three times in the same plate. Matched control cells received DMEM cell culture medium and 5-Fu (10 mg/mL) instead of CKI. By comparing untreated control cells with CKI-treated cells, we investigated the effect of CKI on cell proliferation.

Flow Cytometry Assays

For cell cycle analysis, SW480 and SW620 cells (1×10^6 cells/well) were seeded into six-well plates, cultured in completed culture medium overnight, and then exposed to CKI (2 mg/mL) for 24 h. Nuclear DNA was analyzed using a BD Cycletest™ Plus DNA Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Stained cells were detected and quantified with a FACS Flow Cytometer (BD Biosciences). All FACS results were analyzed with FlowJo software (Version 7.6).

Autophagy Assays

Autophagy activity was analyzed by Cell Meter Autophagy assay. First, SW480 and SW620 cells were adjusted to a concentration of 2×10^4 cells/mL, then 100 μL of this solution (2,000 cells per well) was seeded into 96-well flat-bottomed plates and cells incubated overnight. At the same time, a non-induced negative control cell population at the same density as the induced population was cultured for every labeling condition. The medium was then removed, 100 μL of Autophagy Blue™ working solution (AAT Bioquest Co., CA, USA) was added to each well, and the cells incubated at 37°C in a 5% CO_2 incubator for 1h. The cells were washed with wash buffer three times. Finally, the fluorescent intensity was monitored with a fluorescence microscope using a DAPI channel.

Animal Model Experiments

For *in vivo* tumor growth assays, 5-week-old ICR male mice were purchased from Beijing Vital River Laboratory Animal Technology Co. and housed in a dedicated specific pathogen-free facility at the Laboratory Animal Center of Peking University People's Hospital. Time-course observations during AOM/DSS (azoxymethane/dextran sodium sulfate)-induced mouse colorectal carcinogenesis were conducted to determine when colonic tumors occurred in the inflamed colon of mice that received 2% DSS after AOM initiation. Male ICR mice received a single intraperitoneal injection of AOM (10 mg/kg body weight; Sigma-Aldrich, St Louis, MI, USA) and followed by one week administration of 2% DSS (MP Biomedicals, Irvine, CA, USA) in drinking water, after which the mice were maintained on regular water for 28 days. Then, the mice in the model group were randomly divided into three groups (n = 6/each group). At week seven, the three groups of mice were injected intraperitoneally with 200 mL NaCl (original injection), 200 mL 5-Fu (5-fluorouracil, 4 mg/mL), and 200 mL CKI (original injection), respectively. The weight of mice was measured twice a day with a platform scale. At about 4 weeks after drug intervention, the mice were sacrificed by carbon dioxide euthanasia method. Briefly, after the mice were put into the cage, the power of the pressure reducer was switched on and the valve of the carbon dioxide cylinder was slowly opened. The pressure was kept to 6Mpa and the flow rate to 1.3L/ min till the mice were confirmed death, and the gas cylinder valve was then shut down. Following, the colorectal tissues of the mice were

removed and photographed. Tissue samples were fixed overnight in 10% paraformaldehyde, embedded in paraffin, and sectioned for histological analysis. The colorectal tissues of mice, which were fixed by formaldehyde and embedded in paraffin, were sectioned at a 4-mm thickness and stained with hematoxylin and eosin according to standard protocols.

All animal studies were performed in accordance with rules and guidelines concerning the use and care of laboratory animals and approved by the Animal Care Committee of Peking University People's Hospital.

Validation of Results by Real Time RT-qPCR Assays

SW480 and SW620 cells were incubated at 37°C in a humidified incubator after treatment with CKI (2 mg/mL) for 24 h. At the same time, phosphate-buffered saline (PBS) was used as a control. Total RNA was extracted using an RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and cDNA was synthesized with a SuperScript Synthesis Reagent Kit (Invitrogen, Carlsbad, CA, USA). Reverse transcription quantitative PCR (RT-qPCR) analyses for mRNA levels of p53, p21, CHEK1, RBL1 in SW480 cells and p53, p21, YWHAZ, BUB3, WEE1 in SW620 cells were performed using KAPA SYBR Green FAST Universal qPCR kit (Roche, Basel, Switzerland) on a Bio-Rad CFX96TM Real-Time PCR System. The program was run as follows: initial denaturation was at 94°C for 2 min. The following steps were reiterated for 44 cycles: denaturation at 94°C for 30 sec; annealing at 60°C for 30 sec; and extension at 72°C for 45 sec. The final extension was set at 72°C for 5 min. β-actin was identified as a suitable internal control, and the results were expressed as the threshold cycle (Ct). The relative quantification of target transcripts was determined by a comparative Ct method (ΔΔCt) according to the manufacturer's protocol. The $2^{-\Delta\Delta Ct}$ method was used to analyze relative changes in gene expression. Control PCR experiments in the absence of reverse transcription were performed to confirm that total RNA was not contaminated with genomic DNA. The primers used are listed in Table 1.

Western blot analysis

To analyze the relative expression of cell cycle proteins, western blot analysis was performed. The cells of the two colorectal cancer cell lines, SW480 and SW620, were collected in the logarithmic growth phase, and the cell concentration was adjusted to 5×10^5 cells/mL. CKI (2 mg/mL) was added to each cell culture, and cells were incubated for 24h. The cells were then collected, washed twice with cold PBS, and harvested in 500 mL cell lysis buffer (containing incubation buffer, 20% Triton X-100, proteinase inhibitor, sodium fluoride, and sodium orthovanadate solution) for 30 min on ice, briefly mixed, and centrifuged at 14,000×g at 4°C for 30 min. The total protein concentration was determined by a BCA Protein Assay Kit (Thermo Scientific Pierce, Waltham, MA, USA). Equal amounts of cellular proteins were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with Tris-buffered saline (TBS) buffer containing 5% skim milk powder at room temperature for 1h, and then incubated with primary antibodies, including rabbit polyclonal antibody against p53 (1:1000; Proteintech, Rosemont, IL, USA), p21 (1:1000; Cell

Signaling, Danvers, MA, USA) and mouse monoclonal anti-β-actin (1:3000; Sigma-Aldrich) overnight at 4°C. The blots were washed three times in TBS with Triton X-100 and incubated with horse radish peroxidase-conjugated secondary antibody (1:1000; ZSGB Biotechnology, Beijing, China) for 1h at room temperature. Blots were then visualized with enhanced chemiluminescence reagent (Thermo Fisher Scientific, Waltham, MA, USA). All grey values of the protein bands were performed using custom ImageJ (National Institutes of Health; http://rsb.info.nih.gov/ij/) script.

Prediction of Ingredients Acting on p53 in CKI

BATMAN-TCM is the first tool in bioinformatics analysis for studying the molecular mechanism of TCMs by predicting potential targets for ingredients in TCMs (http://bionet.ncpsb.org/BATMAN-TCM). After that, compounds were entered as search queries in the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP; http://lsp.nwu.edu.cn/tcmsp.php.) and in the Traditional Chinese Medicine Integrated Database (TCMID; http://www.megabionet.org/tcmid/search/). The following parameter settings were used: Target Prediction's Score cutoff was 20; Target Analysis's Adjusted *p*-value was less than 0.05.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.0. In this study, all experiments were repeated at least three times. Student's *t*-test was used to evaluate the differences between two groups. All *p* values were two-sided and differences between the groups were identified as statistically significant at three levels: $p<0.05$, $p<0.01$, and $p<0.001$.

Results

CKI inhibits colon cancer cell proliferation *in vitro*

To determine the effect of CKI on the proliferation of sw480 and sw620 colorectal cancer cells, we used the CCK-8 assay to measure cell viability after treatment with CKI (2 mg/mL, based on the total alkaloid concentration in CKI). The proliferation ofsw480 and sw620 cells were dramatically inhibited when treated with CKI ($p< 0.05$ for both) or 5-Fu(fluorouracil; $p< 0.01$ for both) compared with untreated cells(Figure 1).

CKI induces colon cancer cell cycle progression arrest *in vitro*

To characterize whether the effect of CKI on the proliferation of sw480 and sw620 colorectal cancer cells was relevant to cell cycle arrest, both cell lines were labeled with propidium iodide to detect cell cycle progression by flow cytometry. After CKI treatment, the sw480 cell cycle was significantly inhibited in the G0/G1 phase compared with the control ($p< 0.001$), and the sw620 cell cycle was arrested in the G2/M phase (Figure 2; $p< 0.05$).These data indicate that CKI inhibits colon cancer cell proliferation by curbing cell cycle transition, with*in situ* cancer and metastatic cancer cells blocked in different cell cycle phases.

CKI induces autophagy of SW480 and SW620 colon cancer cells *in vitro*

The observed cell cycle arrest induced by CKI provided a strong clue for studying the fate of colon cancer cells following exposure to the examined drugs. Recent studies have demonstrated that nuclear p53 negatively regulates the process of autophagy, which is a conserved catabolic pathway, by mediating the turnover of intracellular organelles and protein complexes. We next investigated whether CKI induced autophagic cell death in such colon cancer cells. For this purpose, we measured vesicles labeled with Autophagy Blue™, a widely used specific autophagosome marker, to analyze the activity of autophagy. As depicted in Figure 3, both sw480 and sw620 cells showed significant changes in morphology, compared with those cells cultured in medium containing serum, after starvation in serum-free medium or CKI incubation for 24 h ($p< 0.001$ for both). In addition, cells treated with CKI in culture medium with serum showed obvious autophagic vacuoles after staining with Autophagy Blue™. These results indicate that autophagy may be a mechanism by which CKI induces cell death in colon cancer cells.

CKI inhibits azoxymethane/dextran sodium sulfate-derived mouse colon tumors progression *in vivo*

A time-course observation during azoxymethane/dextran sodium sulfate (AOM/DSS)-induced mouse colorectal carcinogenesis was conducted to determine whether CKI can play an anticancer role in advanced colorectal cancer. As shown in the schematic chart in Figure4A, ICR mice were treated with AOM/DSS to establish colon tumors, and then injected with CKI or 5-Fu (fluorouracil) and sacrificed. As exhibited by hematoxyl in and eosin staining (Figure 4B), the untreated colorectal cancer model group showed obvious tumor lesions (NaCl group), where the center of the large intestinal gland became larger and irregular. The mice in the 5-Fu intervention group showed healthy tissue morphology and had no tumor focus. The histocyte morphology of mice treated with CKI was much better than that of the untreated colorectal cancer model group. The results suggest that CKI may inhibit tumor progression in advanced colorectal cancer compared with untreated colorectal cancer tissues.

CKI alters the expression of cell cycle regulatory factors in sw480 and sw620 cells.

To investigate the mechanism of CKI regulating the cell cycle, an RNA-sequencing (Seq) approach was used to examine the expression of genes known to be important for cell cycle G0/G1 and G2/M phase transition. We used a reverse transcription quantitative PCR approach to certify RNA-Seq results, including the expression of p53, p21, CHEK1, RBL1, YWHAZ, BUB3 and WEE1 mRNAs in sw480 and sw620 cells. We found that after CKI treatment, the mRNA abundance of p53 was significantly suppressed and that of p21 was significantly increased in sw620 cells ($p< 0.001$ for both). However, only p53 was significantly reduced in sw480 cells ($p< 0.001$; Figure 5A, 5B). In order to verify the results of mRNA expression studies, we evaluated expression levels of cell cycle regulatory proteins by western blot analysis. Immunoblot results in Figure 5C and 5D show that CKI significantly caused the low protein expression of p53 in both sw480 and sw620 cell lines compared with untreated control ($p< 0.05$, $p< 0.01$, respectively). In contrast, the expression of p21 protein after CKI treatment was inconsistent in the two cell lines, which was suppressed in sw480 cells ($p< 0.01$) but significantly enhanced in sw620 cells ($p<$

0.001). These results revealed that CKI could alter the expression of cell cycle regulatory factors in colon cancer cells *in vitro*.

N-oxysophocarpine, lupenone, and geranylacetonemay be the bioactive ingredients in CKI that act on p53

To predict the potential compounds that may be involved in regulating p53, we tested two herbs of CKI using the BATMAN-TCM database. We found that Kushen consisted of 146 compounds, and that N-oxysophocarpine, lupenone, and geranylacetone were predicted to be the active ingredients that acted on p53. However, among the 33 components of Baituling, no potential active substance acting on p53 was predicted (Table 2). These results suggest that in for CKI activity, Kushen plays a more important role in regulating p53 than Baituling.

Discussion

Compound Kushen Injection (CKI) is a traditional Chinese medicinal preparation that is widely used in clinics. Several recent meta-analyses were the most comprehensive analyses undertaken of currently available data regarding the treatment of patients with colon cancer and other advanced cancers with CKI combined with radiotherapy [6,14,15]. In these clinical reports, CKI enhanced the efficacy and reduced the toxic side effects of chemotherapeutic drugs since CKI improves immune function in patients with advanced cancer, thereby enhancing therapeutic efficacy [16]. In addition, many studies have found that CKI can directly inhibit the growth of tumor cells *in vitro* [8, 9, 17]. Our research also confirms that CKI inhibits colon cancer cell proliferation *in vivo* and tumor growth *in vitro*. However, the mechanism involved in the antitumor effect of CKI is still unknown.

Cell cycle control is one of the major regulatory mechanisms of cell growth, and abnormal regulation of the cell cycle is a marked characteristic of cancer cells [18]. Cellular proliferation is regulated primarily by regulation of the cell cycle to monitor DNA integrity, which consists of four distinct sequential phases (G0/G1, S, G2, and M) [19]. The present study has demonstrated that after CKI treatment, the sw480 cell cycle was significantly inhibited in the G0/G1 phase, while the sw620 cell cycle was arrested in G2/M phase. The discrepancy in CKI-mediated cell cycle control between sw480 and sw620 cells may be due to differences in genetic back grounds or signaling networks between the two cell lines. Although we have confirmed that CKI can regulate cell cycle progression, the mechanism is unclear.

In response to various cellular signals, p53 becomes activated to function as a transcription factor that transcribes a program of genes to accomplish a number of different functions, such as activating DNA repair, inducing cell cycle arrest, and initiating cell autophagy [11, 12]. The upregulation of p53 protein can initiate p21-dependent cellular growth arrest. $P21^{CIPI/WAF1}$ protein, a potent cyclin-dependent kinase (CDK) inhibitor, binds to and inhibits the activity of cyclin–CDK2 complexes, and thus functions as a regulator of cell cycle progression. Expression of this gene is tightly controlled by the tumor suppressor protein, p53 [20]. Thus, the p53-p21 axis is always regarded as a classic pathway for G2/M phase arrest induced by DNA damaging agents [13]. However, when p53 mutates, the original function of p53 is

inhibited. This was supported by our findings obtained from sw480 and sw620 cells, which expressed elevated levels of mutant p53 protein.

Sw480 was originally established from a primary adenocarcinoma of the colon. It has a G->A mutation in codon 273 of the *p53* gene resulting in an Arg->His substitution, and a C->T mutation in codon 309 resulting in a Pro->Ser substitution. In comparison, sw620 was initiated from a lymph node in the same manner as sw480 was initiated from the primary adenocarcinoma it was derived from the previous year [21]. Our results showed that after CKI treatment, mutant p53 expression decreased and p21 expression increased in sw620 cells, in which G2/M growth was arrested. However, we identified a distinct mechanism underlying CKI regulation in that the G0/G1 phase was growth arrested rather than the G2/M phase in sw480 cells, while mutant p53 expression was down-regulated without affecting p21 protein expression. An explanation for this differential effect of p21 upon CKI treatment mayrely on multiple p53 downstream-targeted genes in addition to p21 following CKI treatment. We demonstrated that CKI may inhibit colorectal cancer proliferation by down-regulating mutant p53 protein expression, thereby regulating cell cycle progression. The detailed molecular mechanisms involved in the effect of CKI on colorectal cancer are currently under investigation in our laboratory.

Autophagy is an evolutionarily conserved lysosomal self-digestion process. Autophagy plays a dual role in tumorigenesis and cancer therapy. With respect to cancer, many links exist between autophagy and p53. Wild-type p53–mediated adenosine monophosphate-activated protein kinase activation resulted in cytoprotective autophagy in response to the DNA-damaging drug, topotecan, in human colon cancer cells. Autophagy sensitized colon cancer cells with wild-type p53 were inhibited by topotecan treatment; however, autophagy inhibition attenuated the anti-tumour effect of topotecan treatment in p53 mutant or knockout colon cancer cells, both *in vitro* and *in vivo* [22]. In the present study, we show that the human colon cancer cell lines with mutant p53, sw480 and sw620, undergo autophagic cell death following treatment with CKI. These findings suggest that p53 may modulate autophagy and that the p53 status could determine cell fate following anticancer drug-induced autophagy and help maintain autophagic homeostasis.

Even though we have shown that CKI affects the regulation of the cell cycle, which important components are involved in regulating p53 are not well understood. Chinese herbal compounds contain substantial natural products and are valuable resources that have enabled the identification of novel anticancer agents [23].Of these, alkaloids have demonstrated promising anti-cancer effects. These compounds have also been shown to elicit effects on a variety of different targets, including regulators associated with cell cycle progression, cell apoptosis and the inhibition of drug resistance [24]. The current study was performed to investigate the potential ingredients that contribute to the regulation of mutant p53. Predictive data by BATMAN-TCM revealed that N-oxysophocarpine, lupenone, and geranylacetone, which are present in Kushen (*Radix Sophorae Flavescentis*), were the active ingredients that acted on mutant p53. However, the exact bioactive ingredients need to be further characterized. Since mutant p53 is overexpressed in patients with CRC and promotes tumor growth *in vivo*, the inhibition of mutant p53 may be a useful strategy for the treatment of mutant p53-overexpressing CRC in patients.

CKI, including its active ingredients that are predicted to act on mutant p53, likely acts as potential anti-cancer therapeutic agent that targets the cell cycle pathway. This suggests that CKI can play a key role in the development of a novel subsidiary therapeutic approach against mutant p53 colorectal cancer in patients.

Conclusions

In this study we suggest that N-oxysophocarpine, lupenone, and geranylacetone, which were active components of CKI, may act as antitumor agents against colorectal cancer by regulating cell cycle progression through a mutant p53 signaling pathway, thereby inhibiting the proliferation of sw480 and sw620 cells and promoting autophagy. Although further experiments are required to verify candidate targets and active ingredients, our findings offer a novel approach to counter colorectal cancer.

Abbreviations

CKI: Compound Kushen Injection **CRC:** Colorectal carcinoma **TCMs:** Traditional Chinese Medicines **FBS:** Fetal bovine serum **CCK-8:** Cell Counting Kit-8 **AOM/DSS:** Azoxymethane/dextran sodium sulfate **5-Fu:** 5-Fluorouracil **PBS:** Phosphate-buffered saline **RT-qPCR:** Reverse transcription quantitative PCR **TBS:** Tris-buffered saline **TCMSP:** Traditional Chinese Medicine Systems Pharmacology Database **TCMID:** Traditional Chinese Medicine Integrated Database **CDK:** Cyclin-dependent kinase.

Declarations

Ethics approval and consent to participate

These experiments have passed ethics approval and consent to participate. All animal experimental procedures were reviewed and approved by the Animal Care and Use Committee of People's Hospital of Peking University, and animal care was conducted in accordance with institutional guidelines.

Consent for publication

Not applicable.

Availability of data and materials

All data support the results can be found in the manuscript. Details of the data are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

WD-Y and SJ-W designed the research study. JS, DW, YZ, QM, ML and XL-H performed the biological research and analyzed the data. JS and JY-C performed the chemical research and analyzed the data. XY contributed essential research facilities. JS, DW and YZ wrote the manuscript. All authors read and approved the final manuscript.

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Figures

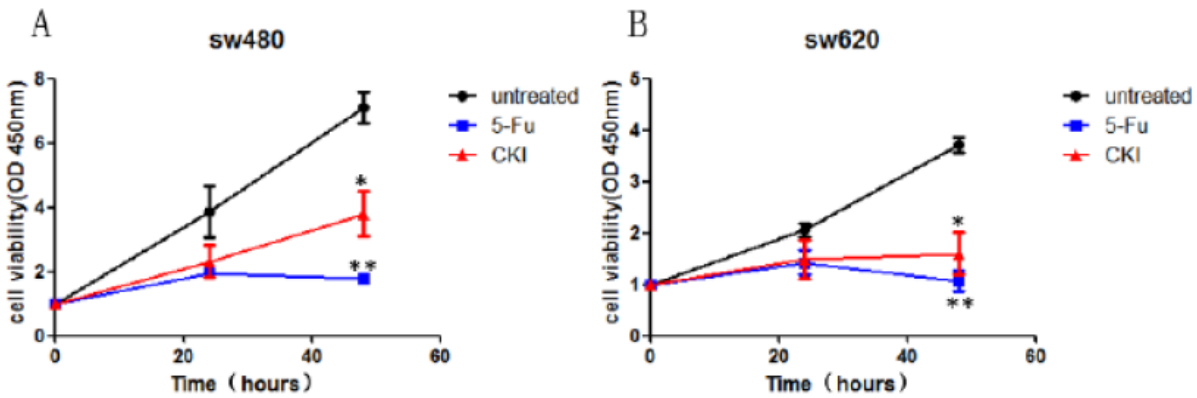

Figure 1

CKI inhibits colon cancer cell proliferation in vitro. A. Sw480 and B.sw620 cell viabilities under different treatments were measured using a CCK-8 assay. Each data point represents the mean± standard deviation (SD) from three independent experiments. *p<0.05, **p<0.01 compared with untreated group. CKI, Compound Kushen Injection; 5-Fu, fluorouracil

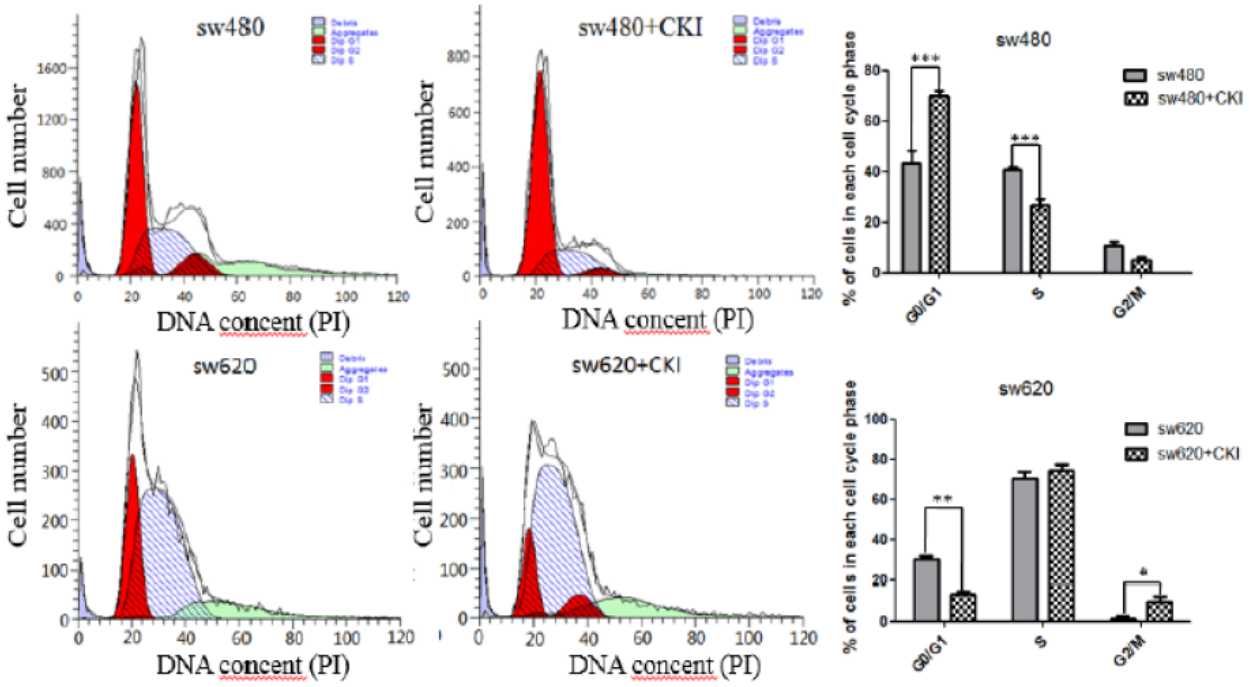

Figure 2

CKI induces colon cancer cell cycle progression arrest in vitro. Flow cytometry assays were performed to analyze the cell cycle in sw480 and sw620 cells after CKI treatment. Values at different stages of the cell cycle represent the mean± standard deviation (SD) from three independent experiments. *p<0.05, **p<0.01, ***p<0.001 compared with control. CKI, Compound Kushen Injection; PI, propidium iodide; concent, concentration

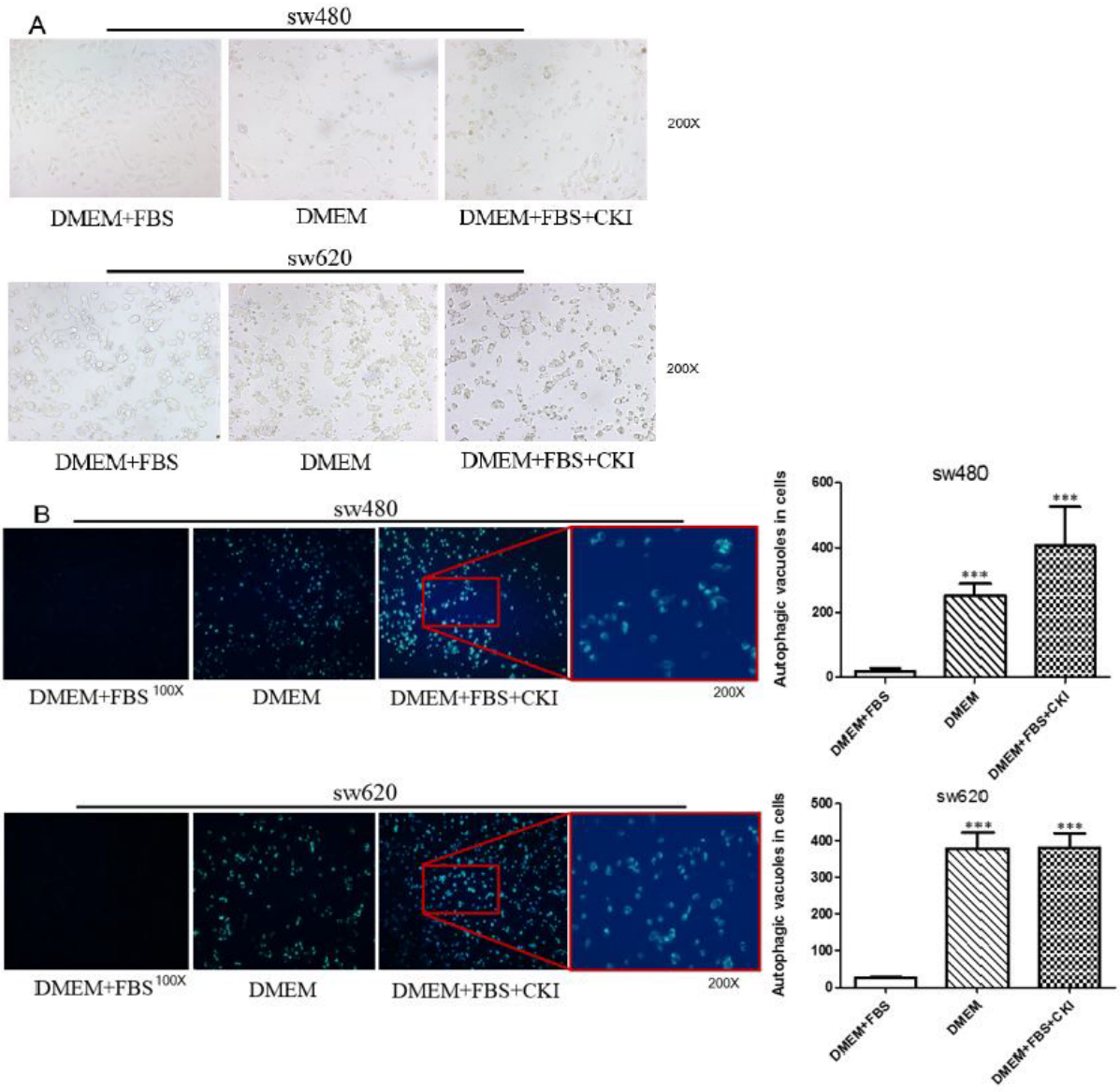

Figure 3

CKI induces autophagy in sw480 and sw620 cells. Sw480 and sw620 cells were incubated in regular DMEM medium containing serum as a negative control and in a serum-depleted medium as a positive control for 24 h. Both control and CKI treated cells were incubated with Autophagy Blue™ working solution for 30 min at 37°C in a 5% CO2 incubator, and then washed four times with wash buffer. A. Cells were imaged with white light under a microscope. B. Cells were imaged under a fluorescence microscope with a DAPI channel. Autophagy is indicated by bright blue dot staining of autophagic vacuoles. Each data point represents the mean± standard deviation (SD) from three independent experiments. ***p<0.001 compared with negative control. CKI, Compound Kushen Injection; FBS, fetal bovine serum

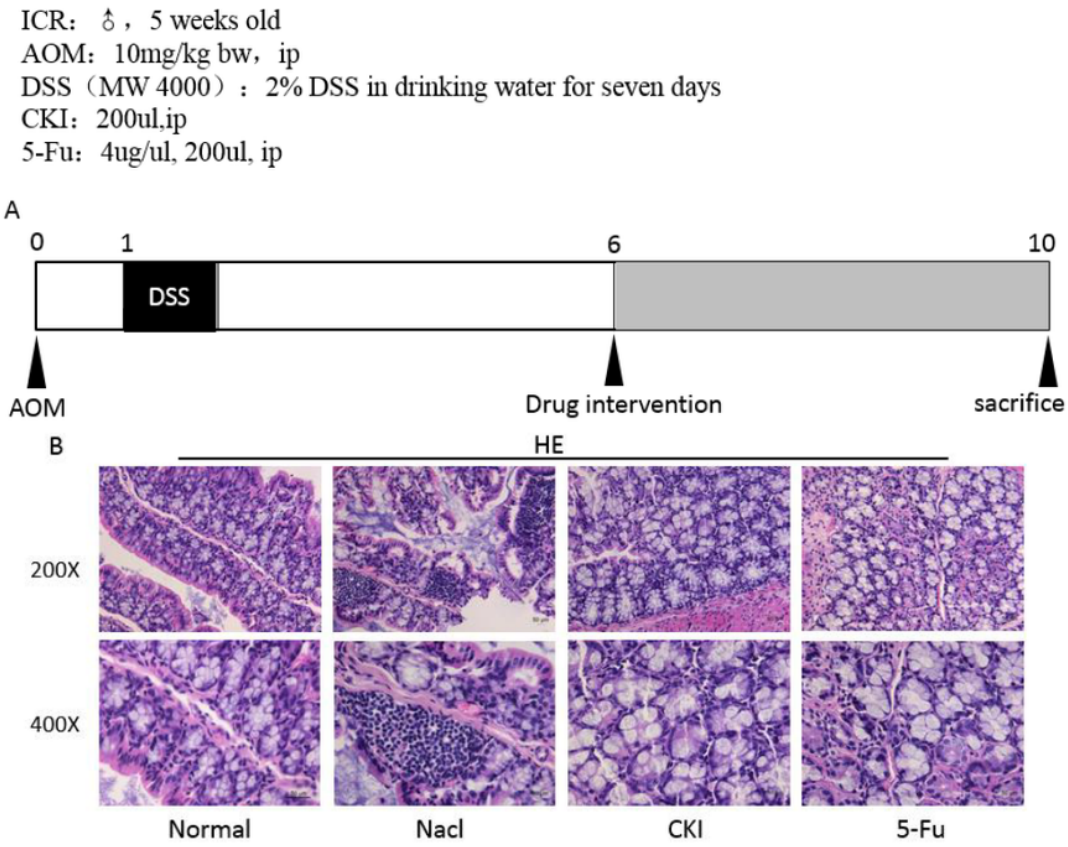

Figure 4

CKI inhibits azoxymethane/dextran sodium sulfate-derived mouse colon tumor progression. A. Schematic drawing of a colitis colon cancer model induced by azoxymethane/dextran sodium sulfate (AOM/DSS) in ICR mice. B. ICR mice were divided into normal (healthy) and colorectal cancer model groups. Mice in the colorectal cancer model groups were given NaCl (control), CKI and 5-Fu, respectively, for intervention. Representative histopathological images of hematoxylin and eosin (HE)staining in non-tumor colon tissues, AOM/DSS-derived colon tumor tissues and drug intervention tissues. CKI, Compound Kushen Injection; 5-Fu, fluorouracil; i.p., intraperitoneal injection. Original magnification, ×200/400.

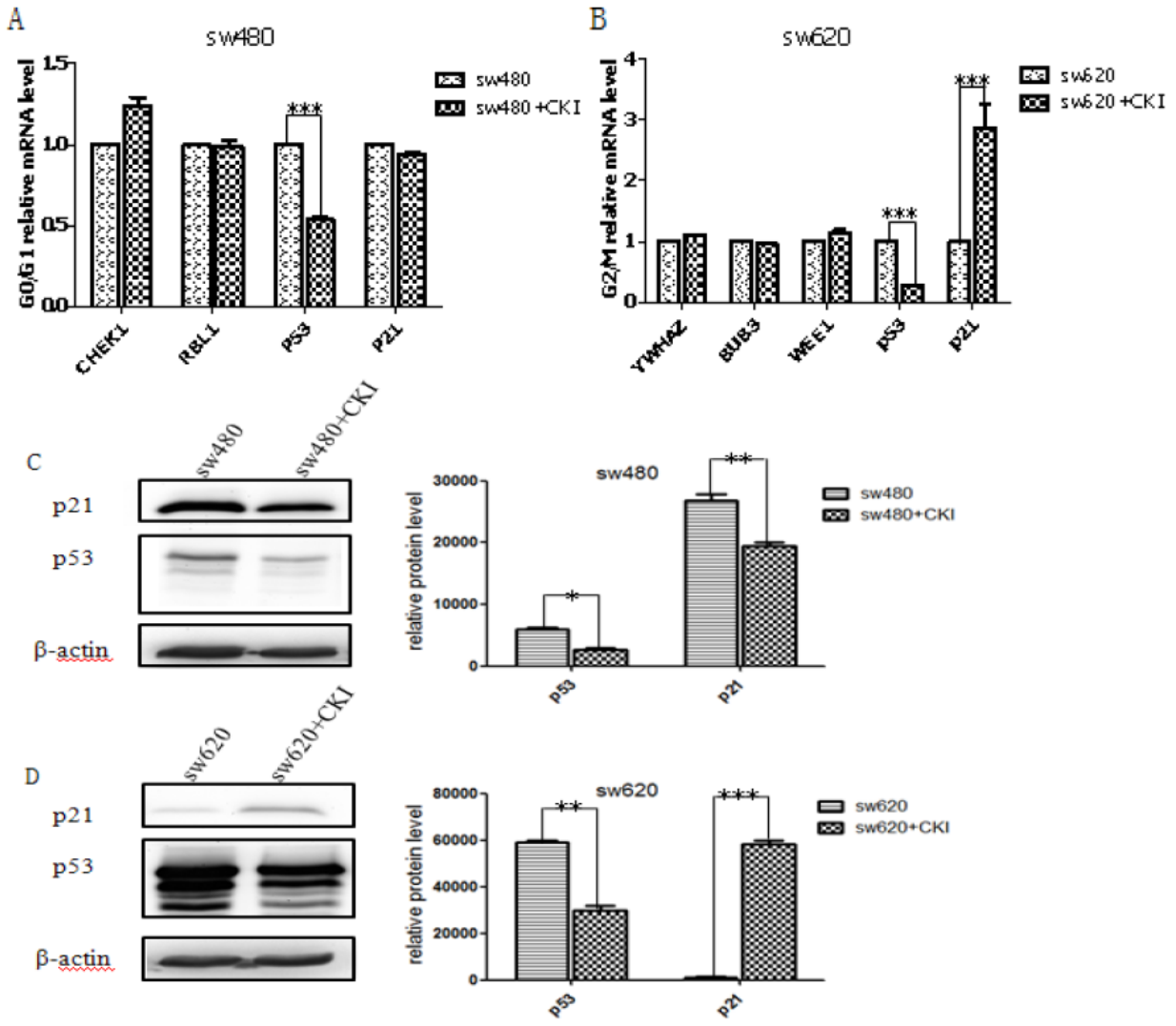

Figure 5

CKI alters the expression of cell cycle regulatory factors in sw480 and sw620 cells. A. The mRNA abundances of CHEK1, RBL1, p53, and p21 were analyzed by reverse transcription quantitative PCR (RT-qPCR) in sw480 cells, treated with CKI, relative to untreated control. B. The mRNA abundances of YWHAZ, BUB3, WEE1, p53, and p21 were analyzed by RT-qPCR in sw620 cells. C. Western blot analysis of p53 and p21 in sw480 and CKI treated sw480 cells. D. Immunoblot for p21 and p53 derived from sw620 and CKI treated sw620 cells, with β-actin serving as a loading control. All western blot bands were quantified using custom ImageJ script. The results are shown as the mean± standard deviation (SD) from three independent experiments. *p<0.05, **p<0.01, ***p<0.001 compared with untreated group. CKI, Compound Kushen Injection.

Supplementary Files

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