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Research article

Effects of aspirin on colon cancer using quantitative proteomic analysis

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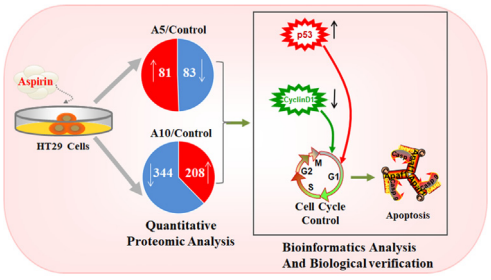
HIGHLIGHTS

- Following exposure to 10 mmol/L of aspirin, 552 proteins were significantly dysregulated.
- Cell cycle-related proteins, such as p53 and cyclin-dependent kinase 1 (CDK1), are the most differentially expressed proteins.
- P53 and CDK1 were upregulated in colon cancer cells after exposure to aspirin.
- Aspirin promotes G1/S arrest of cell cycle in HT29 cells.

GRAPHICAL ABSTRACT



Quantitative Proteomic Analysis of Preventive Potential of Aspirin for Colon Cancer Cell line HT29.Aspirin induces G1 arrest and apoptosis through the p53–Cyclin-dependent kinase 1 pathway as revealed by the protein changes observed in the proteomic analysis.

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ABSTRACT

Background: Colon cancer is one of the most prevalent digestive cancers worldwide. Results of epidemiological, experimental, and clinical studies suggest that aspirin inhibits the development of colon cancer. This study aimed to systematically elucidate the molecular mechanisms by which aspirin prevents colon carcinogenesis.
Methods: We determined the global protein expression profiles of colorectal cancer and aspirin-treated cells using quantitative proteomic analysis. We analyzed the proteomic results using bioinformatics (including differential proteins, protein annotation, Kyoto Encyclopedia of Genes and Genomes [KEGG] pathways, and protein–protein interaction [PPI] network). The viability of the colon cancer cell line and HT29 cells treated with aspirin was determined using the cell counting kit-8 assay. The differentially expressed proteins, such as p53 and cyclin-dependent kinase 1 (CDK1), were quantified using real-time polymerase chain reaction (PCR) and Western blotting. We measured cell cycle distribution and apoptosis in HT29 cells exposed to aspirin using fluorescence-activated cell sorting (FACS).
Results: We found that 552 proteins were significantly dysregulated, of which 208 and 334 were upregulated and downregulated, respectively, in colon cancer cells exposed to 10 mmol/L of aspirin (95% confidence interval [CI]: -1.269 to -0.106, $P < 0.05$). Further gene enrichment analysis revealed that cell cycle-related proteins, such as p53 and CDK1, were significantly differentially expressed. Proteomic analysis showed that after 24 h of aspirin exposure, the level of p53 increased by 2.52-fold and CDK1 was downregulated to half that of the controls in HT29 cells (95% CI: -0.619 to -0.364, $P < 0.05$). Real-time PCR and Western blotting results showed that p53 was upregulated (95%CI: -3.088 to -1.912, $P < 0.001$) and CDK1 was significantly downregulated after aspirin

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exposure in colon cancer cells (95% CI: 0.576 to 1.045, $P < 0.05$). We observed that aspirin promoted G1/S cell cycle arrest in HT29 cells. We confirmed that aspirin induces apoptosis in human HT29 colon cancer cells in a concentration-dependent manner.
Conclusions: These results indicate that aspirin induces G1 arrest and apoptosis in colorectal cancer cells via the p53–CDK1 pathway. Aspirin may be a promising drug candidate for colon cancer prevention.

Introduction

Colorectal cancer is common worldwide, and most colorectal cancers develop from adenomas.[1,2] Colorectal cancer represents a heterogeneous group of neoplasms with varying sets of genetic and epigenetic alterations that are influenced by multiple factors, including medications.[3,4] Aspirin, a nonsteroidal anti-inflammatory drug, is one of the most widely used medications for treating pain, fever, and inflammation.[5,6] A series of epidemiological studies and cardiovascular prevention trials have indicated that low-dose aspirin is associated with a low incidence of colon cancer and mortality in patients with colorectal cancer and the inhibition of metastases.[7] A meta-analysis showed that aspirin decreased the risk of colorectal cancer.[8] Moreover, a large-scale survey of Asian populations indicated that the regular use of aspirin can potentially reduce the incidence and mortality of colorectal cancer.[9]

Although there is substantial evidence that aspirin prevents colon cancer, the underlying molecular mechanisms remain unclear. Aspirin can prevent colorectal cancer by normalizing the expression of the epidermal growth factor receptor (EGFR)[10] and rapidly disrupting EGF and EGF receptor internalization.[11] Aspirin induces autophagy in colorectal cancer cells by inhibiting the mechanistic target of rapamycin (mTOR), a serine/threonine kinase, and activating adenosine monophosphate-activated protein kinase (AMPK).[12] Emerging evidence suggests that aspirin induces senescence of colorectal carcinoma cells by targeting Sirt1 and AMPK.[13,14] Most disruptions in cellular processes, such as those initiated by aspirin exposure, can be studied using high-throughput omics techniques, which can provide for a systematic analysis of the functions of proteins and gene regulatory networks.[15] This study aimed to perform a quantitative proteomic analysis of HT29 cells exposed to aspirin using tandem mass tag (TMT). We found that 552 proteins were dysregulated after 10 mmol/L aspirin treatment. Analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) Cellular Component enrichment of the dysregulated proteins showed that the cell cycle and *p53* signaling pathway obviously changed in response to aspirin, as confirmed by experimental studies and experimental studies also confirmed it. Our findings suggest that aspirin promotes colon cancer cells death by regulating the cell cycle and *p53-CDK1* signaling pathway. Our results provide a novel theoretical basis for the beneficial effects of aspirin against colon cancer.

Methods

Cell culture

Human HT29 colon cancer cells were obtained from the Cell Bank of the Chinese Academy of Medical Sciences (Beijing, China) and cultured in Dulbecco's Modified Eagle Medium (Life Technologies, C11995500BT, Waltham, MA, USA) containing 10% bovine growth serum (HyClone Laboratories, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C and 5% CO_2 atmosphere.

Antibodies

Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sc166545, 1:3000) and the tumor protein, *p53* (DO-1; sc-126, 1:1000), were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Antibodies against cyclin-dependent kinase 1 (*CDK1*) (ab18, 1:1000 dilution) were purchased from Abcam (Cambridge, UK). Secondary horseradish peroxidase-conjugated antibodies against mouse immunoglobulin (Ig) G (ZB2305, 1:5000) were purchased from Zhongshan Golden Bridge Biotechnology. Co., LTD (Guangzhou, China).

Real-time polymerase chain reaction

Total ribonucleic acid (RNA) was extracted from the HT29 cells using 1 μg of TRIzol reagent (Invitrogen, 15596026, Waltham, Massachusetts, USA) to synthesize complementary deoxyribonucleic acid (DNA) (Transgen Biotech, Beijing, China). Real-time polymerase chain reaction (PCR) was performed using a quantitative PCR system (Applied Biosystems 7500 Real-time PCR Systems, Thermo Fisher Scientific, Waltham, Massachusetts, USA) under the following conditions: 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The relative expressions of the target genes were normalized to those of the housekeeping gene, *GAPDH*. All real-time PCR experiments were repeated at least thrice, with statistical analyses for each experimental set. Data analyses for the gene expression were performed using the $2^{-\Delta\Delta Ct}$ method. All values in the experiments were expressed as mean ± standard error of the mean (SEM). The primers used in the PCR assays were as follows:

*GAPDH*_F: 5′-GAAGGTGAAGGTCGGAGTC-3′, *GAPDH*_R: 5′-GAAGATGGTGATGGGATTT-3′; *p53*_F: 5′-GGCCCACTTCACCGTACTAA-3′, *p53*_R: 5′-GTGGTTTCAAGGCCAGATGT-3′; *CDK1*_F: 5′-CAGTCTTCAGGATGTGCTTAT-3′, *CDK1*_R:5′-TGACCAGGAGGGATAGAAT-3′.

Western blot

Whole cell extracts of the HT29 cells were prepared as previously described.[16,17]

Cell apoptosis detection

The HT29 cells after corresponding treatment were stained with fluorescein isothiocyanate-conjugated Annexin V and propidium iodide (Beyotime, C1062S, Shanghai, China), and apoptotic cells were detected via fluorescence-activated cell sorting (FACS), as previously described.[18,19]

Cell count kit-8 assay

Logarithmically growing HT29 cells at a concentration of 5×10^4 cells/mL were placed in a 96-well plate (100 μL/well) and incubated with 0, 2.5, 5, 10, and 20 mmol/L of aspirin for 24 h. Thereafter, a total of 10 μL of CCK-8 (Beyotime, C0037, Shanghai, China) was added with further incubation for 4 h. Absorbance was measured in a Microplate Reader (PerkinElmer 2030, Massachusetts, USA) at 450 nm. Three replicates were used for each concentration. All values in the experiments are expressed as the mean ± SEM.

Cell cycle analysis

The HT29 cells were treated with different concentrations of aspirin (0, 5, and 10 mmol/L) for 24 h. Cell cycle analysis was performed as previously described (Cytomics FC 500; Beckman Coulter, California, USA).[16,20] Three replicates were used for each concentration. All experimental values are expressed as the mean ± SEM.

Sample preparation for mass spectrometry

Cell pellets were suspended in iced 200 μL of lysis buffer (4% SDS, 100 mmol/L dithiothreitol, and 150 mmol/L Tris–HCl at pH = 8.0) and boiled for 5 min. The samples were thereafter ultrasonicated and boiled for 5 min. Undissolved cellular debris were removed by centrifugation at 16,000 rpm for 15 min. The supernatant was collected and the proteins were quantified using a BCA Protein Assay Kit (Bio-Rad, Hercules, California, USA).

Protein digestion

The proteins from each sample were digested according to the filter-aided sample preparation procedure described by Wisniewski et al. [21] Finally, the proteins were digested with trypsin (Promega, Madison, USA) for 12 h at 37 °C with a ratio of 50:1 (w/w). The peptide concentration was determined using the OD280 protein calculator by Nanodrop device (Thermo Fisher Scientific).[21]

Tandem mass tag labeling of peptides and fractionation by high-pH reverse-phase

The peptides were labeled with the TMT reagent according to the manufacturer's instructions (Thermo Fisher Scientific). Each aliquot (100 μg of peptide equivalent) was reacted with TMT reagent. The sample was dissolved in 100 μL of 0.05 mmol/L triethylammonium bicarbonate buffer solution, and the TMT reagent was dissolved in 41 μL of anhydrous acetonitrile. The mixture was then incubated at 24 °C for 1 h. Thereafter, 8 μL of 5% hydroxylamine was added to the sample and incubated for 15 min to stop the reaction. The multiplex-labeled samples were pooled and lyophilized and subsequently fractionated using a Pierce High-pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Fifteen fractionations were performed sequentially using appropriate elution solutions according to the manufacturer's instructions. The Peptide content of each fraction was evaporated to dryness and stored at −80 °C for liquid chromatography-mass spectrometry (LC-MS) analysis.

Liquid chromatograph-mass spectrometer analysis

LC-MS analysis was performed using a Q Extractive mass spectrometer coupled to an Easy nLC system (Thermo Fisher Scientific). Peptide from each fraction was loaded onto a C18-reversed phase column (12 cm long, 75 μm ID, 3 μm) in buffer A (2% acetonitrile and 0.1% formic acid) and separated with a linear gradient in buffer B (90% acetonitrile and 0.1% formic acid) at a flow rate of 300 nL/min for 90 min. The linear gradient was set as follows: 0–2 min, 2–5% buffer B; 2–62 min, 5–20% buffer B; 62–80 min, 20–35% buffer B; 80–83 min, 35–90% buffer B; and 83–90 min, buffer B maintained at 90%. MS data were acquired using a data-dependent top 15 method by dynamically choosing the most abundant precursor ions from the survey scan (300–1800 *m/z*) for higher-energy collisional dissociation (HCD) fragmentation. The target value was determined based on predictive automatic gain control (pAGC). The AGC target values of $1e^6$ and a maximum injection time of 50 min were used for full MS, while the target AGC value of $1e^5$ and a maximum injection time of 100 min were used for MS2. The dynamic exclusion duration was 30 s. Survey scans were acquired at a resolution of 70,000 at m/z 200, and the resolution of the HCD spectra was set to 35,000 at m/z 200. The normalized collision energy was 30. The instrument was run in the peptide recognition mode enabled.

Database searching and analysis

The resulting LC-MS/MS raw files were imported into MaxQuant software (Max Planck Institute of Biochemistry, Munich, Germany) (version 1.6.0.16) for data interpretation and protein identification against the database of Uniprot-*Homo sapiens* (Human) [9606]-194324-20201203 (downloaded on December 3, 2020, and including 194,324 protein sequences), which is sourced from the protein database at https://www.uniprot.org/uniprot/?query=homo+sapiens+&sort=score. An initial search was set at a precursor mass window of 6 ppm. The search followed the enzymatic cleavage rule of trypsin/phosphate and allowed for two maximal missed cleavage sites and a mass tolerance of 20 ppm for fragment ions. The modification set was as follows: fixed modification: carbamidomethyl (C), TMT10plex (K), and TMT10plex (N-term); and variable modification: oxidation (M) and acetyl (Protein N-term). A minimum of six amino acids for peptide and ≥1 unique peptides were required per protein. For peptide and protein identification, the false discovery rate (FDR) was set to 1%. The TMT reporter ion intensity was normalized and used for protein quantification.

Bioinformatics analysis

Bioinformatics data were analyzed using Perseus software (Max Planck Institute for Astronomy, MPIA Munich, Germany),[22] Microsoft Excel (version: excel 2016, Washington, USA), and R statistical computing software (verison: 4.3.1). Differentially expressed proteins were screened with a cutoff of a fold-change ratio of >1.20 or <0.83 and $P < 0.05$. Expression data were grouped together by hierarchical clustering according to the protein level. To annotate the sequences, information was extracted from UniProtKB/Swiss-Prot,[23] Kyoto Encyclopedia of Genes and Genomes (KEGG),[24] and Gene Ontology (GO).[25] KEGG enrichment analyses were performed using Fisher's exact test. FDR correction for multiple testing was also performed. GO terms were grouped into three categories: biological processes (BP), molecular functions (MF), and cellular components (CC). Protein–protein interaction (PPI) networks were constructed using the STRING database and Cytoscape software (Beijing, China).[26]

Statistical analysis

Data are presented as mean ± SEM. Student's two-tailed *t*-test was used to compare the two groups. Multiple comparisons were performed using a one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* analysis. Two-sided $P < 0.05$ was considered significant.

Results

Death of HT29 cells induced by aspirin exposure and proteomic data of aspirin-treated HT29 cells

Human HT29 colon cancer cells were treated with aspirin at four different concentrations (2.5, 5, 10, and 20 mmol/L) for 24 h. Cell viability was measured using the CCK8 cytotoxicity assay, and the result [Figure 1A] showed that the ratio of cell death increased with increasing aspirin concentration in the medium. At 2.5, 5, 10, and 20 mmol/L of aspirin, cell mortality was 6%, 12.5%, 50%, and 75%, respectively. Therefore, we chose two intermediate concentrations, 5 and 10 mmol/L, for subsequent proteomic studies to explore the underlying mechanism of aspirin action against colorectal cancer.

To investigate the mechanism underlying aspirin action in the HT29 cells, the cells were treated with 5 or 10 mmol/L of aspirin for 24 h. The HT29 cells were collected and underwent purification, trypsin digestion, and TMT labeling. The samples were analyzed using mass spectrometry. The results indicated that the samples treated with 5 and 10 mmol/L aspirin expressed 6890 and 6926 proteins, respectively. The proteins in the three comparisons with *P*-value <0.05 and fold change greater than 1.2 were considered to be statistically significantly regulated. Overall, 81 upregulated proteins and 83 downregulated proteins were identified between untreated cells or cells treated with 5 mmol/L of aspirin [Figure 1B], and 208 upregulated and 344 downregulated proteins were differentially expressed by untreated cells and cells treated with 10 mmol/L of aspirin [Figure 1C].

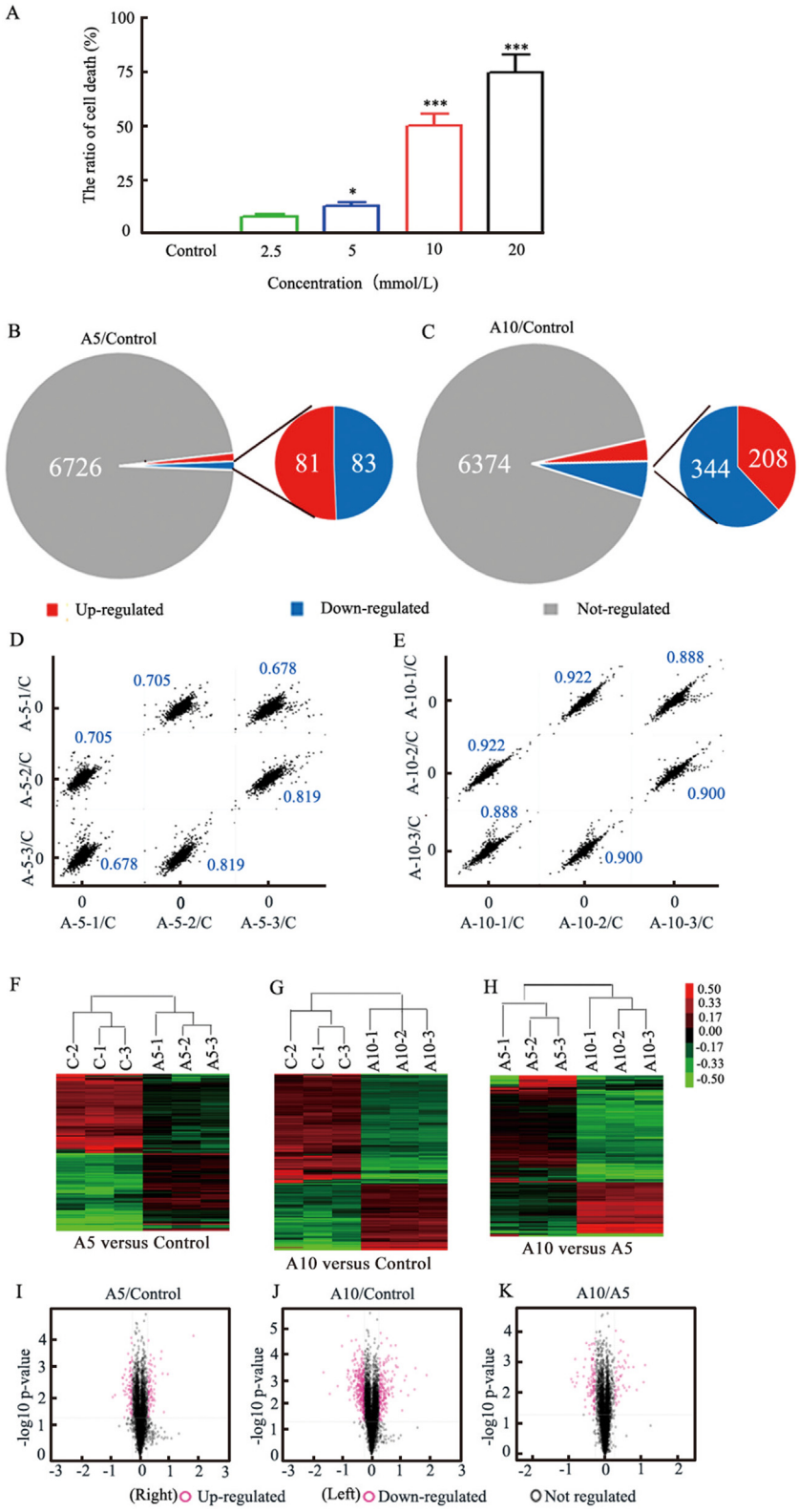


Figure 1. Effects of aspirin on the proliferation of HT29 cells and comprehensive quantitative proteomics data. (A) The survival rate of HT29 cells was determined by CCK8 assay. *P < 0.05, ***P < 0.001, 5 mmol/L versus control, 10 mmol/L versus control, and 20 mmol/L versus control. Independent experiment was performed at least three times. In total, 6890 proteins were identified in the HT29 cells treated with 5 mmol/L aspirin, and (B) 6926 proteins were obtained in the HT29 cells treated with 10 mmol/L aspirin (C) considering the regulated and non-regulated ones. When P < 0.05 and the 1.2-fold cutoff was used to define the obviously changed proteins, 164 (B) and 552 (C) proteins were differentially regulated between HT29 cells untreated with aspirin and HT29 cells treated with 5 mmol/L or 10 mmol/L aspirin (D and E). The reproducibility of quantification of the duplicated experiments was revealed by the protein ratio correlations analyzed by *Pearson's* correlation. (F, G, and H) Heatmap showing the logarithm expression intensities from green (low expression) to red (high expression) in control and HT29 cells treated with aspirin at 5 and 10 mmol/L for 24 h. Each column and row represent a sample and protein, respectively. C, A5, and A10 refer to three replicates. (I, J, and K) Volcano plots showed the distributions of proteins between HT29 cells untreated with aspirin and HT29 cells treated with 5 mmol/L or 10 mmol/L aspirin. The horizontal line corresponds to a 2-fold (log2 scaled) changes up or down, and the vertical line represents a P-value of <0.05 (-log10 scaled). The pink points on the plot represent the differentially expressed proteins with a one-fold change up or down in proteins with statistical significance (P < 0.05).

Pearson's correlation analysis was used to evaluate the correlation between the expression of the protein in the control and aspirin-treated groups. The result showed that for the HT29 cells treated with 5 and 10 mmol/L aspirin, the logarithm of the ratio of proteins expressed by treated and control cells were 0.734 and 0.903 respectively, indicating significant changes in protein levels and rather according to the repeated experiments [Figures 1D and E]. The data and correlation analyses showed that the quantitative values obtained from the TMT experiments were valid and could be used for further analyses.

We compared the protein expression profiles of cells treated with aspirin and those of controls by dividing the intensities obtained from the different channels. Hierarchical cluster analysis of the ratios indicated that all replicates occurred in clusters, suggesting that many of the quantification data were replicable. Many changes were observed in the

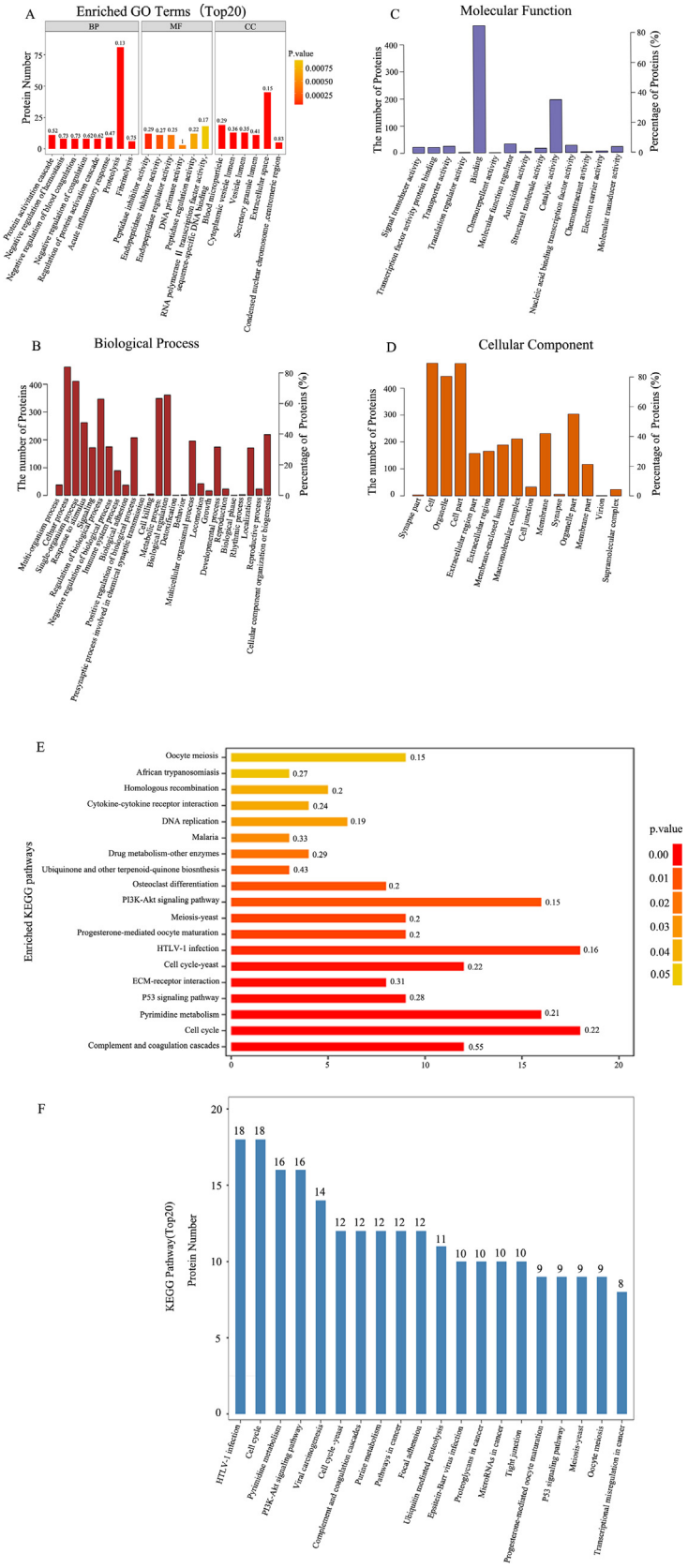
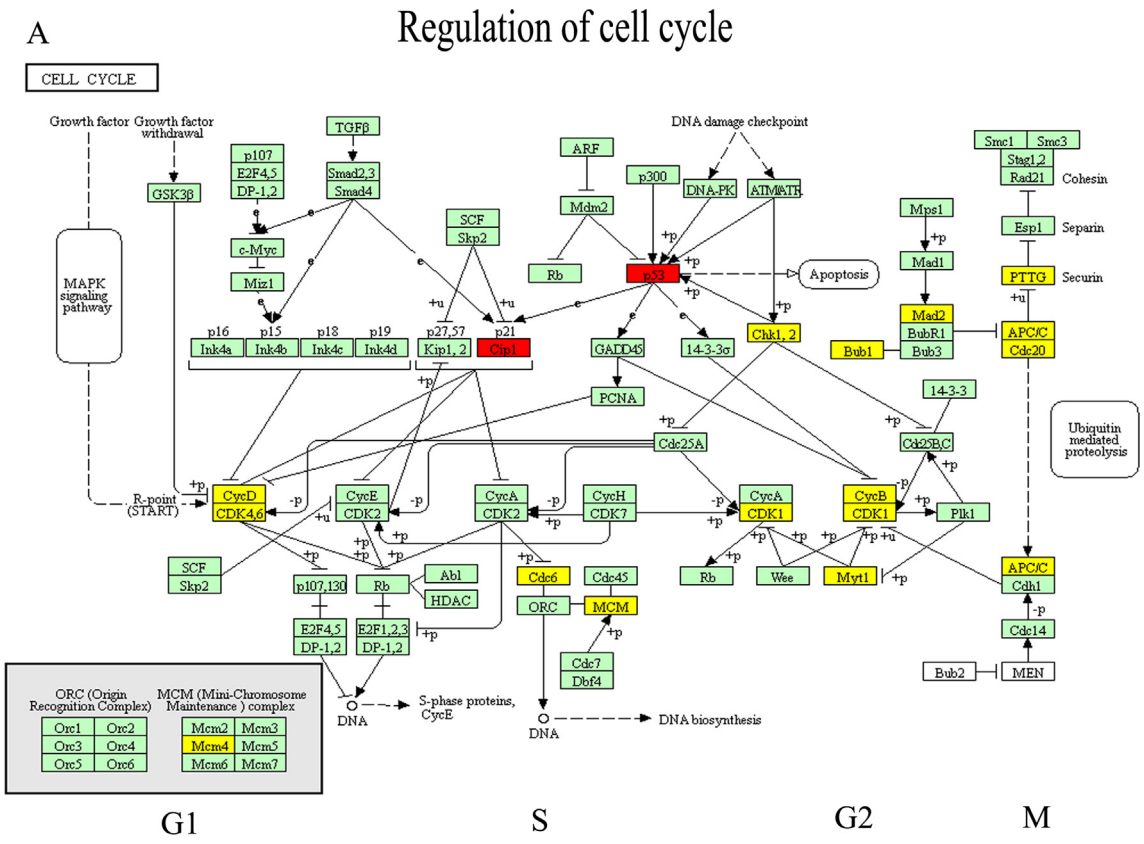


Figure 2. The GO annotations and KEGG database analysis of the cell signaling pathway annotations for differential proteins detected in HT29 cells exposed to 10 mmol/L aspirin for 24 h. The GO analysis categorized the differential proteins into different groups (A) MF (B), BP (C), and CC (D). The protein numbers of each GO category are shown on the Y-axis. (E) The vertical axis represents the enriched pathways analyzed by the KEGG database, and the horizontal axis represents the *P* pathway values. $P < 0.05$. (F) The vertical and horizontal axes represent the number of proteins and the enriched pathways, respectively analyzed by the KEGG database. BP: Biological process; CC: Cellular component; ECM: Extracellular matrix; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; MF: Molecular function. HTLV-1: Human T-cell leukemia virus type 1; P13K-Akt: Phosphoinositide-3-kinase–protein kinase B/Akt.



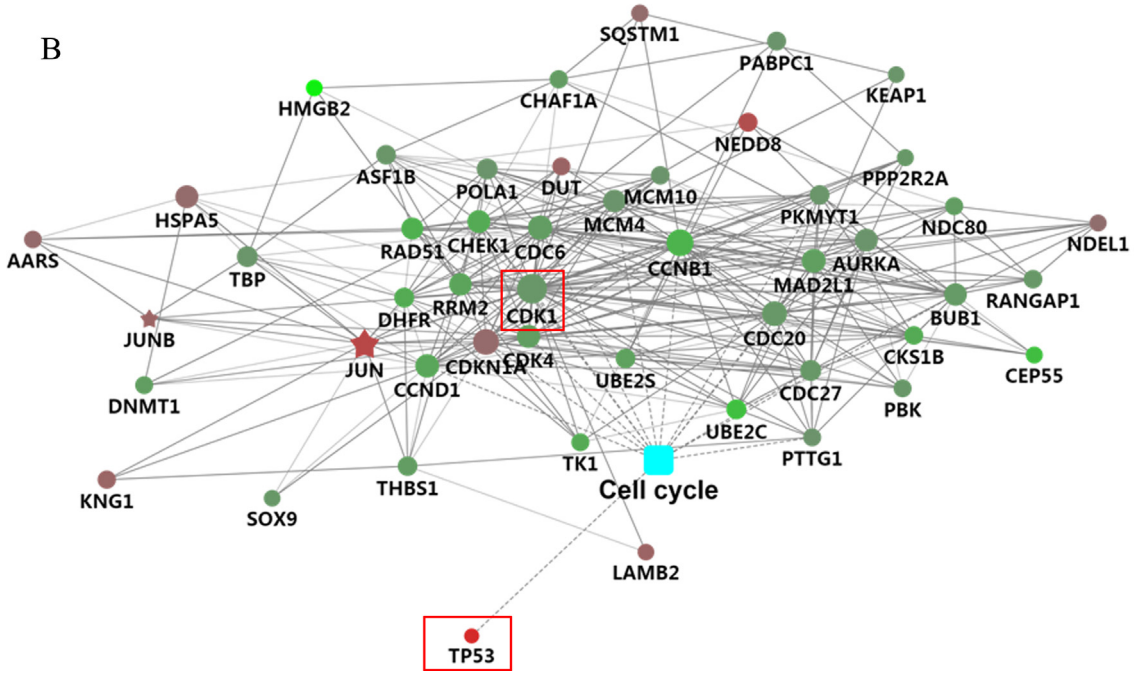


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Figure 3. Bioinformatics analysis results of the cell cycle regulation of associated protein networks and PPI networks analysis in HT29 cells treated with 10 mmol/L of aspirin. (A) Mapping of dysregulated proteins in HT29 cells treated with 10 mmol/L aspirin to "regulation of cell cycle" pathway. Each box shows a gene of a certain protein, within which was the symbol of the gene. Arrows indicated the process of the pathway. Red and yellow: differential proteins. (B) PPI analysis related to cell cycle regulation. Cell cycle-related proteins as important node proteins. AARS: Alanyl-tRNA synthetase; APC: Adenomatous polyposis coli protein; ARF: Tumor suppressor ARF; ASF1B: Anti-silencing function protein 1 homolog B; ATM: Serine-protein kinase ATM; ATR: Serine/threonine-protein kinase ATR; AURKA: Aurora kinase A; Bub1,2,3: Mitotic checkpoint serine/threonine-protein kinase Bub1,2,3; BubR1: Mitotic spindle checkpoint protein BubR1; CCNB1: G2/mitotic-specific cyclin-B1; CCND1: G1/S-specific cyclin-D1; CDC: Cell division cycle protein;CDC6: Cell division control protein 6 homolog; Cdc: APC/C activator protein, Cell division cycle protein 20; Cdc25a: M-phase inducer phosphatase 1; CdcD: Cell division cycle protein 48; CEP55: Centrosomal protein of 55 kDa; CHEK1: Serine/threonine-protein kinase; CycE: G1/S-specific cyclin-E; Cdh1: APC/C activator protein Cdh1; CDK: Cyclin-dependent kinase; CHAF1A: Chromatin assembly factor 1 subunit A; Chk1,2: Cell cycle checkpoint kinase, Checkpoint kinase-1,2; CKS1B: Cyclin-dependent kinases regulatory subunit 1; c-Myc: Myc proto-oncogene protein;14-3-3σ: 14-3-3 protein zeta; Dbf4: Dumbbell forming protein 4; DNA-PK: DNA-dependent protein kinase; DNMT1: DNA (cytosine-5)-methyltransferase 1; DHFR: Dihydrofolate reductase; DP-1,2: Transcription factor Dp-1 and Dp-2; DUT: Deoxyuridine 5'-triphosphate nucleotidohydrolase; E2F4,5: Transcription factor E2F4 and E2F5; GADD45: DNA damage-inducible transcript 1 protein; GSK3β: Glycogen synthase kinase-3 beta; HDAC: Histone deacetylase; HMGB2: High mobility group protein B2; HSPA5: Endoplasmic reticulum chaperone BiP; Jun: Transcription factor Jun; JunB: Transcription factor JunB; KEAP1:Kelch-like ECH-associated protein 1; KNG1: Kininogen-1; NDC80: Kinetochore protein NDC80 homolog; LAMB2: Laminin subunit beta-2; Mad1,2: Spindle assembly checkpoint component MAD1,2; MAD2L1: Mitotic spindle assembly checkpoint protein MAD2A; MCM: Mini-chromosome maintenance; Mdm2: E3 ubiquitin-protein ligase Mdm2; Miz1: Myc-interacting zinc finger protein 1; Mps1: Mitogen-activated protein kinase MPS1; MEN: Menin; Myt1: Myelin transcription factor 1; NDEL1: Nuclear distribution protein nude-like 1; NEDD8: Neddylin, Ubiquitin-like protein Nedd8; ORC: Origin recognition complex; PABPC1: Polyadenylate-binding protein 1; p16(Ink4a): Cyclin-dependent kinase 4 inhibitor A; p15(Ink4b): Cyclin-dependent kinase inhibitor p15; p18(Ink4c): Cyclin-dependent kinase 4 inhibitor C; Esp1: Exocrine gland-secreted peptide 1; p19(Ink4d): Cyclin-dependent kinase 4 inhibitor D; p21(Cip1): Cyclin-dependent kinase inhibitor 1; p27(Kip1): Cyclin-dependent kinase inhibitor p27; p57(Kip2): Cyclin-dependent kinase inhibitor p57; p107: Retinoblastoma-like protein 1107 kDa retinoblastoma-associated protein; p300: Histone acetyltransferase p300; PBK: Lymphokine-activated killer T-cell-originated protein kinase; PCNA: Proliferating cell nuclear antigen; POLA1: DNA polymerase alpha catalytic subunit; PPI: Protein-protein interaction; PTTG: Pituitary tumor-transforming gene 1 protein; p130: 130 kDa retinoblastoma-associated protein; PIK1: Pelle-like serine/threonine-protein kinase pik-1; PKMYT1: Membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase; PPP2R2A: Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform; Rad21: Double-strand-break repair protein rad21 homolog;RAD51: DNA repair protein RAD51 homolog 1; RANGAP1: Ran GTPase-activating protein 1; Rb: Retinoblastoma-associated protein; R-point: Restriction point ; RRM2: Ribonucleoside-diphosphate reductase subunit M2; SCF: Stem cell factor; Skp2: S-phase kinase-associated protein 2; Smad2,3,4: Mothers against decapentaplegic homolog2,3,4; Smc1,3: Structural maintenance of chromosomes protein 1,3; SOX9: Transcription factor SOX-9; Stag1,2: Stromal antigen 1,2; TBP: TATA-box-binding protein; TGFβ: Transforming growth factor beta proprotein; THBS1: Thrombospondin-1; TK1: Thymidine kinase, cytosolic; UBE2C: Ubiquitin-conjugating enzyme E2 C; UBE2S: Ubiquitin-conjugating enzyme E2 S; Wee: Wee1-like protein kinase..

untreated cells and cells treated with 5 or 10 mmol/L of aspirin within HT29 cells [Figures 1F, G, and H]. Based on the combined analysis result, an arbitrary cutoff of 1.2-fold and statistical significance $P < 0.05$ was used to define proteins that are differentially expressed using any two comparisons. Volcano plots showed a significant variation in protein expression between untreated cells and cells treated with 5 or 10 mmol/L aspirin [Figures 1I, J, and K].

Protein annotation and Kyoto Encyclopedia of Genes and Genomes pathway analysis

The important GO processes for each function are illustrated in Figure 2A. GO analysis was performed to identify the functional enrichment of the gene sets. We classified the functions of the target proteins into three: BP [Figure 2B], MF [Figure 2C], and CC [Figure 2D].

The KEGG database was used to analyze the cell signaling pathways of the differentially expressed proteins. The top pathway was the cell cycle signaling pathway [Figures 2E and F].

Differential proteins enrichment pathways and protein–protein interaction network analysis

The *p53* signaling pathway, which is closely associated with the cell cycle, was detected [Figure 3A]. We predicted the potential interactions between the identified proteins or differentially expressed proteins and those of other proteins. The entire PPI network, expressed as nodes and links, obtained effective protein information from various viewpoints and contributed integrated information that could only be acquired through proteomic analysis. The results of the intergroup analysis indicated that the cell cycle signaling pathway was the most important signal transduction pathway in the entire system [Figure 3B].

The p53 and cyclin-dependent kinase 1 levels show the greatest elevation after aspirin exposure

KEGG pathway analysis indicated that the cell cycle was highly dysregulated. Cell cycle dysregulation is an important inducer of the infinite proliferation of cancer cells.[27] Therefore, blocking the cell cycle is considered to be effective in preventing cell proliferation. *CDK1* is a key regulator of the S phase. The *p53* signaling pathway has received attention because it plays an important role in both cell cycle arrest and apoptosis. According to MS results, *CDK1* was decreased to 50% and *p53* was increased by 2.52-fold in HT29 cells treated with 10 mmol/L aspirin compared to untreated HT29 cells. Our real-time PCR experiments confirmed that the expression of *CDK1* decreased and that of *p53* increased in HT29 cells treated with 10 mmol/L aspirin [Figure 4A]. To further verify the differential expression of cell cycle-related proteins (*p53* and *CDK1*) after HT29 cells were treated with aspirin, 5 mmol/L and 10 mmol/L of aspirin were used. The expression levels of *p53* and *CDK1* were determined by Western blotting. The results showed that aspirin induced the upregulation of *p53* and downregulation of *CDK1* in a concentration-dependent manner [Figure 4B]. This is consistent with the results of real-time PCR.

Aspirin promoted the G1/S arrest of the cell cycle and cell apoptosis in HT29 cells

In the proteomic enrichment results, "mitotic cell cycle" processes were enriched. To determine how the cell cycle was affected, we calculated the distribution of the cell cycle phase of HT29 cells treated with aspirin and those untreated by the resultant DNA content histogram using flow cytometric analysis. The proportion of cells in the S phase significantly decreased after HT29 cells were treated with aspirin. All these changes were statistically significant, indicating that aspirin promoted G1/S cell cycle arrest in HT29 cells [Figure 5A].

To further explore whether aspirin promotes cell apoptosis after aspirin treatment, Annexin V and PI assays were performed to determine the rate of apoptosis. The rate of apoptosis observed for 2.5, 5, and 10 mmol/L of aspirin were 17.51%, 20.12%, and 50.12%, respectively [Figure 5B].

Discussion

Colon cancer is a malignant tumor with relatively high incidence and mortality.[27,28] There are currently no effective treatments for colon

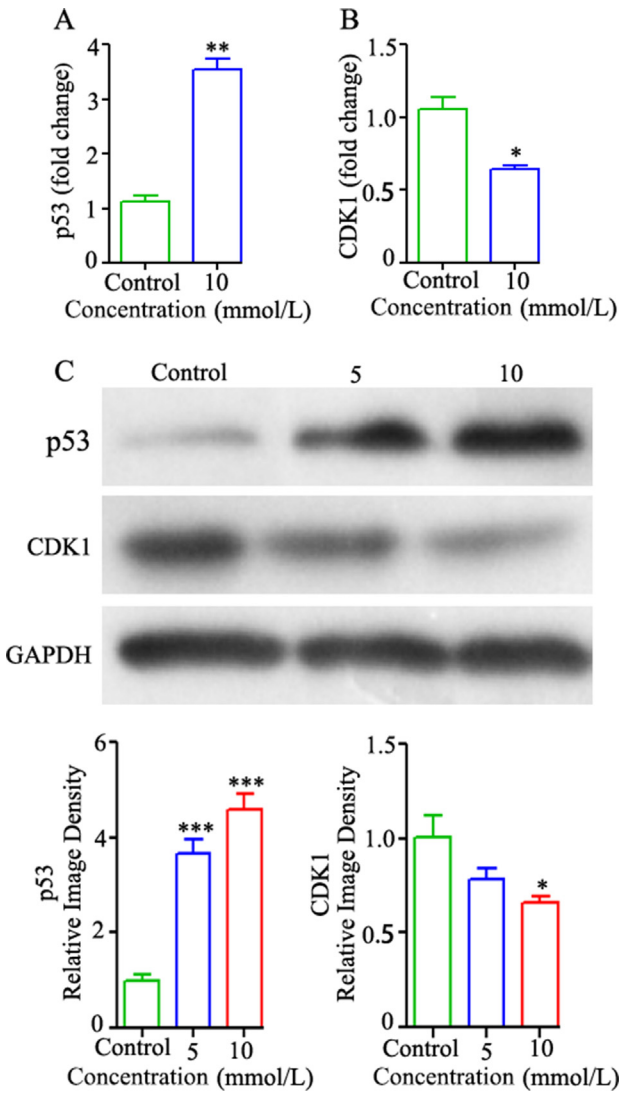

Figure 4. Validation of proteins and mRNAs changes in *p53* and cyclin-dependent kinases 1 (*CDK1*) in HT29 cells exposed to aspirin. (A) Real-time PCR analysis of *p53* and *CDK1* mRNA expression in HT29 cells treated with 10 mmol/L of aspirin. Each bar represents the mean value ± SEM from at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, 10 mmol/L versus control. (B) Western blot shows the *p53* and *CDK1* protein expression in HT29 cells treated with 5 mmol/L and 10 mmol/L of aspirin. All values were expressed as mean ± SEM of three repeats. The *t*-test was performed with respect to the control and $^{*}P < 0.05$, $^{***}P < 0.001$, 10 mmol/L versus control, and 5 mmol/L versus control. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. PCR: Polymerase chain reaction; SEM: Standard error of the mean.

cancer. Thus, it is important to effectively decrease the risk of colon cancer among the public and its associated economic challenges.[29,30] Epidemiological studies confirmed that low-dose aspirin is associated with a low incidence of colon cancer and mortality in patients with colorectal cancer. Kelvin et al. investigated the use of aspirin for the prevention of different cancers among the Chinese population.[9] The population-based cohort study included 612,509 participants that were followed up for >14 years to assess the relationship between aspirin use and the most common cancers. Their study found that the long-term use of low-dose aspirin was associated with a decrease in the risk of cancer in the colorectum, esophagus, liver, stomach, lung, pancreas, and blood. A meta-analysis also suggested that aspirin reduces the risk of colorectal cancer. A systematic review and meta-analysis of all observational studies on aspirin and cancers of the digestive tract published in March 2019 was performed, which indicated that regular use of aspirin was related to a decreased risk of colorectal cancer (relative risk [RR] = 0.73, 95% confidence interval [CI] = 0.69–0.78, 45 studies).[8] However, the underlying mechanisms remain unknown. In this study, we found that aspirin treatment induced apoptosis of colorectal cancer cells and cell cycle dysregulation in HT29 cells. We determined that the upregulation of *p53* and downregulation of *CDK1* led to G1/S cell cycle arrest in colorectal cancer cells exposed to aspirin. To detect the effect of aspirin on cancer cells at the protein level, we employed proteomics coupled with the TMT method to profile proteomic changes in HT29 cells after aspirin treatment. The results indicated that *p53* and *CDK1* were the two most differentially expressed proteins following the treatment of HT29 cells with 10 mmol/L aspirin. *P53* is a transcriptional regulator that functions as a tumor suppressor in the nucleus[31,32] and induces the target genes *p21*WAF1/CIP1 and *BAX* for cell cycle arrest and cell death.[16,33]

Cell proliferation is mediated by several signaling molecules and checkpoints that regulate cell division.[34,35] *CDK1* mediates cell cycle progression in the G2 and M phase.[36,37] In this study, we confirmed the increase in *p53* messenger RNA (mRNA) and protein levels. *CDK1* expressions in mRNA and protein levels were decreased. These results showed that *p53* and *CDK1* are important proteins in the cellular response of HT29 cells to aspirin. Therefore, both *p53* and *CDK1* may be regarded as biomarkers of the response of HT29 cells to aspirin. Flow cytometry revealed that aspirin promoted G1/S cell cycle arrest in HT29 cells.

The study by Santini et al. showed that the expression of *p53* and *p21*WAF-l/cipl by HT29 cells was not significantly altered after 4 days under serum starvation conditions with aspirin and sodium salicylate of approximately 1 mmol/L.[38] They detected a reduction of approximately one-third of *p53* and a 3-fold induction of *p21*WAF-l/cipl at 10 mmol/L of sodium salicylate.[38] However, Ai et al. found that the expression of *p53* increased gradually when HT29 cells were treated with different concentrations (0.1 mmol/L, 0.25 mmol/L, 0.5, 1, 2 mmol/L, and 2.5 mmol/L) of aspirin for 24 h.[39] These effects may be related to the duration of action of aspirin on HT29 cells. In our proteomic results, the *p53* level increased by 2.52-fold, whereas the *CDK1* level decreased to 50% in HT29 cells treated with 10 mmol/L of aspirin for 24 h. Verification experimental results of *p53* and *CDK1* expression are shown in Figure 4 A and B. Related BP and signaling pathways were obtained according to GO and KEGG enrichment analyses, in which the cell cycle signaling pathway was implicated. This indicates that aspirin treatment may inhibit the invasion and metastasis of colon cancer cells. Enrichment of the KEGG pathway revealed that the phosphatidylinositol 3 kinase-protein kinase B (*PI3K-Akt*) signaling pathway, which regulates cell proliferation and promotes cell apoptosis, was altered.

Summarily, aspirin promotes G1/S cell cycle arrest by upregulating *p53* expression and downregulating *CDK1* expression via cell apoptosis. Proteomic profiling of HT29 cells exposed to aspirin revealed several pathways and proteins, including, but not limited to, the cell cycle [Supplemental Table 1]. Furthermore, we investigated the mechanism of aspirin treatment in colon cancer cells *in vitro* using proteomics methods.

This study had some limitations. First, our study was only conducted in cell models. We will build animal models of colon cancer to further verify the results obtained from cell experiments in future studies. Second, our research on the mechanism of action of aspirin in colon cancer mainly involved the cell cycle pathway, which is still relatively superficial. In future experiments, we will include the detection of other pathways in proteome screening and further explore the underlying mechanisms. Third, clinical trials on drug use are important. In the future, we will conduct a population-based cohort study to analyze the

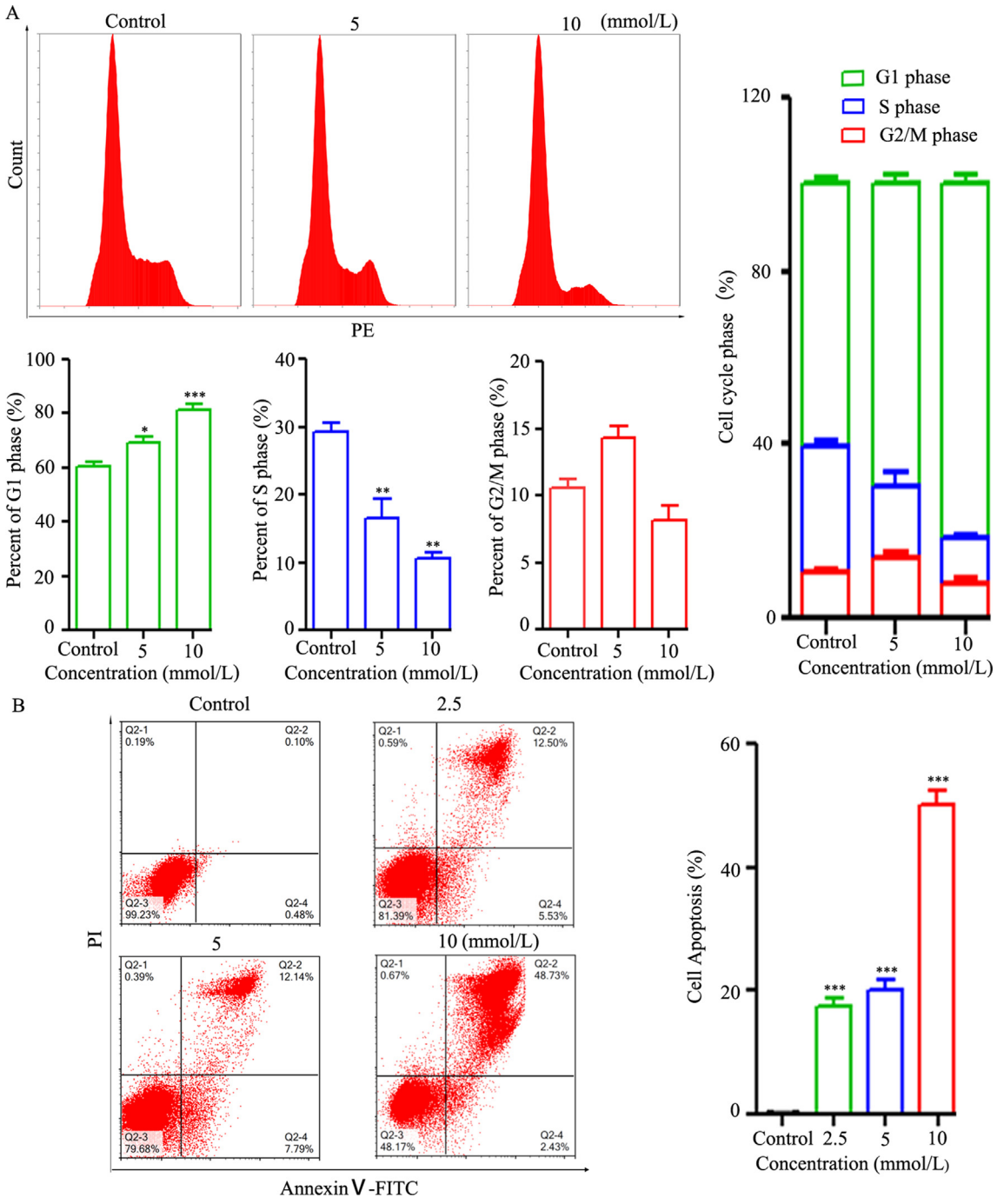

Figure 5. The changes in cell cycle and apoptosis of HT29 cells exposed to 10 mmol/L aspirin for 24 h. (A) Flow cytometric analysis of cell cycle distribution of HT29 cells exposed to 5 mmol/L and 10 mmol/L of aspirin. Cell cycle analysis was performed as described in the materials and methods. Each column represents the effect of control, 5 mmol/L, and 10 mmol/L aspirin on the percentage of phase in the cell cycle as indicated at the bottom of the histogram. Each bar represented the mean value ± SEM from at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, 10 mmol/L versus control, 5 mmol/L versus control. (B) Flow cytometry analysis of HT29 cells treated with aspirin for 24 h. HT29 cells were stained using Annexin V and propidium iodide before flow cytometry analysis. The rate of cell apoptosis was statistically analyzed on the basis of the flow cytometer results. Data were represented as mean ± SEM. Independent experiment was performed at least three times, $^{***}P < 0.001$, 10 mmol/L versus control, 5 mmol/L versus control, and 2.5 mmol/L versus control. FITC: Fluoresceine isothiocyanate; SEM: Standard error of the mean.

preventive effects of aspirin and to verify its mechanism of action in patients with colon cancer.

In conclusion, we expect that proteomic profiling using colon cancer model cells will be helpful for understanding the cellular effects of aspirin. Our results provide a theoretical basis for the use of aspirin as a preventive drug against colon cancer.

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Authors contribution

Wei Han and Rong Wang: Methodology; Yu Ji: Validation; Yan Zhang, Haitao Sun, and Yu Ji: Formal analysis; Yan Zhang, Haitao Sun, and Yu Ji: Investigation; Yan Zhang and Haitao Sun: Writing original draft; Wei Han and Rong Wang: Writing review & editing. All authors had full access to all the data in the study and take responsibility for the integrity of the data and accuracy of the data analysis.

Ethics statement

None.

Data availability statement

The data that supported the funding of this study are available upon request from the corresponding author and are not publicly available because of privacy or ethical restrictions.

Conflict of interest

The authors declare that they have no competing interest with Jiye Hou at Shanghai Bioprofile Technology Company Ltd.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cpt.2023.06.003.

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