Quantitative Proteomic Analysis of Preventive Potential of Aspirin for Colon Cancer Cells

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Abstract

Objective: To further systematically understand the molecular mechanisms that aspirin prevents colon carcinogenesis.

Methods: We detected the global protein expression profiles of colorectal cancer along with the treatment of aspirin by a quantitative proteomic approach. We analyzed the proteomic results using bioinformatics including differential proteins, protein annotation, Kegg Pathways and protein-protein interaction network . Cell viability of HT29 cells treated with aspirin was determined by CCK8 assay. The expression of differential proteins p53 and CDK1 were quantified by real-time PCR and Western blot. We measured cell cycle distribution and cell apoptosis of H1299 cells exposed to aspirin by flow cytometric analysis.

Results: We found 552 proteins that were significantly dysregulated after10mM aspirin treatment of colon cancer cells. Further enrichment analysis of the dysregulated proteins suggested that cell cycle-related proteins are the most differential proteins such as p53, CDK1, CyclinB, Chk1, Chk2 and CyclinD. The results of real-time PCR and Western blot showed that p53 and CDK1 obvious upregulation after aspirin exposed to colon cancer cells. We also detected that aspirin promoted the G1/S arrest of cell cycle in HT29 cells. We confirmed aspirin induced cell apoptosis in human HT29 colon cancer cells with concentration-dependent increase.

Conclusions: The result indicated that aspirin induced colorectal cancer cells G1 arrest and cell apoptosis by p53-CDK1 pathway. Accordingly, aspirin may represent a promising preventive candidate for colon cancer.

Introduction

Colorectal cancer is a very common cancer in the world and most of colorectal cancers develop from adenomas[1, 2]. It represents a heterogeneous group of neoplasms with varying sets of genetic and epigenetic alterations, influenced by exposures to multiple factors, including medications[3, 4]. Aspirin, a nonsteroidal anti-inflammatory drug (NSAID), is one of the most widely used medications for treatment of pain, fever and inflammation[5, 6]. A series of epidemiological research and cardiovascular prevention trials have indicated that low-dose aspirin was associated with a lower incidence of colon cancer and lower mortality in colorectal cancer patients, including inhibition of cancer metastases7. A meta-analysis also suggested that aspirin application could decrease the risk of colorectal cancer [8]. A large-scale survey study with Asian populations also indicated that regular aspirin use had the potential to reduce colorectal cancer incidence and mortality[9].

Although evidence that aspirin prevents colon cancer is remarkable, the underlying molecular mechanism remains indistinct. Indeed, studies have shown that aspirin can prevent colorectal cancer by normalizing EGFR expression[10]. Aspirin induces autophagy in colorectal cancer cells by inhibiting mTOR signaling and activating AMP-activated protein kinase[11]. Emerging evidence suggested that aspirin induced

senescence of colorectal carcinoma cells by targeting sirt1 and AMPK[12, 13]. Most perturbation of cellular process, such as those initiated by exposure to aspirin, can now be researched by high-throughput omics techniques, which can provide a synthetical and systematic view of the functioning of proteins and gene regulatory networks[14, 15]. In the present study, we employed the strategy of tandem mass tagged (TMT) to execute quantitative proteomics analysis of HT29 cells exposed to aspirin. We found that 552 proteins were dysregulated after 10mM aspirin treatment. Analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology Cellular Component enrichment of the dysregulated proteins showed that cell cycle and p53 signaling pathway obviously changed in response to aspirin and experimental studies also confirmed it. Our findings suggest that aspirin promotes colon cancer cells death by regulation of cell cycle and p53-CDK1 signaling pathway .Our results provide a novel theoretical basis for the beneficial effects of aspirin against colon cancer.

Materials And Methods

1 Cell culture

Human HT29 colon cancer cells were obtained from the Cell Bank of Chinese Academy of Medical Sciences (Beijing, China) and were cultured in DMEM (Dulbecco's Modified Eagle Medium) containing 10% bovine growth serum (HyClone Laboratories, Inc.) at 37℃ under 5% CO_2 atmosphere.

2 Real-time PCR

Total RNA was extracted from HT29 cells with Trizol reagent (Invitrogen), and 1 μg was used to synthesize cDNA (Tiangen Biotech). Real-time PCR was performed on a quantitative PCR system (Applied Biosystems 7500 Real-Time PCR Systems, Thermo-Fisher Scientific, Waltham, MA, USA) under the following conditions: 95°C for 5 min followed by 40 cycles of 95°C for 15s and 60°C for 30s. The relative expression levels of the target genes were normalized to that of the housekeeping gene β-actin. All the qRT-PCR experiments were repeated at least three times with statistical analyses for each individual 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 mean (SEM). The primers used in PCR assays were as follows:

GAPDH_F: 5'GAAGGTGAAGGTCGGAGTC3', GAPDH_R: 5' GAAGATGGTGATGGGATTT3';

p53_F: 5'GGCCCACTTCACCGTACTAA3'□p53_R: 5'GTGGTTTCAAGGCCAGATGT3'□CDK1_F: 5'CAGTCTTCAGGATGTGCTTAT3'; CDK1_R:5'TGACCAGGAGGGATAGAAT3'; p21_F: 5'GGACAGCAGAGGAAGACCAT3', p21_R:5'GAGTGGTAGAAATCTGTCATGCT3'.

3 Western blot

Whole cell extracts (WCE) of HT29 cells were prepared as described[16] .

4 Cell Apoptosis detection

HT29 cells after corresponding treatment were stained by FITC-conjugated Annexin V and PI, and apoptotic cells were detected by FACS as previously described[17].

5 CCK-8 assay

The logarithmically growing HT29 cells at concentration of 5×10^4 cells/mL were plated in 96-well plate (100μl/well). After incubating with various concentrations of aspirin at 0, 2.5, 5, 10 and 20 mg/ml for 24 h, a total of 10 μl of CCK-8 was added for 4h incubation. Absorbance was measured in a Microplate Reader (Perkin Elmer 2030, USA) at 450 nm. There were three repetitions per concentration. All values in the experiments were expressed as mean ± SEM.

6. Cell cycle analysis

HT29 cells were treated with aspirin at different concentrations (0, 5, 10mM) for 24 hours. Cell cycle analysis was carried out as described (Cytomics FC 500, Beckman coulter, USA) [16]. There were three repetitions per concentration. All values in the experiments were expressed as mean ± SEM.

7 Sample Preparation of Mass Spectrometry (MS)

7.1 Sample preparation

Cell pellets were suspended on ice in 200µl lysis buffer (4% SDS, 100 mM DTT, 150 mM Tris-HCl pH 8.0) and boiling for 5min. The samples were further ultrasonicated and boiling again for another 5 min. Undissolved cellular debris were removed by centrifugation at 16000 rpm for 15min. The supernatant were collected and quantified with a BCA Protein Assay Kit (Bio-Rad, USA).

7.2 Protein Digestion

Protein from each samplewas digested according to the FASP procedure described by Wisniewski, Zougman et al[18]. Finally, the protein was digested with trypsin (Promega) overnight at 37 °C with ratio 50:1 (w/w), The peptide concentration was determined with OD280 by Nanodrop device[18].

7.3 TMT Labeling of peptides and Frationation by HPRP

Peptides were labeled with TMT reagents according to the manufacturer's instructions (Thermo Fisher Scientific). Each aliquot (100 µg of peptide equivalent) was reacted with one tube of TMT reagent, respectively. After the sample was dissolved in 100 µl of 0.05M TEAB solution, pH 8.5, the TMT reagent was dissolved in 41 µl of anhydrous acetonitrile. The mixture was incubated at room temperature for 1 h. Then 8µl of 5% hydroxylamine to the sample and incubate for 15 minutes to quench the reaction. The Multiplex labeled samples were pooled together and lyophilized.Multiplex labeled samples were fractionated using Pierce High-pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific)

following manufacturer's instructions[19]. Fifteen fractionation were performed by stepwise using the appropriate elution solutions according to manufacturer's instructions. Peptide content of each fraction was evaporated to dryness and stored at −80 °C for LC-MS analysis.

7.4 LC-MS/MS Analysis

LC- MS analysis was performed on a Q Exactive mass spectrometer that was coupled to Easy nLC (Thermo Fisher Scientific). Peptide from each fraction was loaded onto a the C18-reversed phase column (12cm long, 75μm ID, 3μm) in buffer A (2% acetonitrile and 0.1% Formic acid) and separated with a linear gradient of buffer B (90% acetonitrile and 0.1% Formic acid) at a flow rate of 300 nL/min over 90 min. The linear gradient was set as follows: 0-2 min, linear gradient from 2% to 5% buffer B; 2–62 min, linear gradient from 5% to 20% buffer B; 62-80 min, linear gradient from 20% to 35% buffer B; 80–83 min, linear gradient from 35% to 90% buffer B; 83-90 min, buffer B maintained at 90%. MS data was acquired using a data-dependent top15 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 m/z) for HCD fragmentation. Determination of the target value is based on predictive Automatic Gain Control (pAGC). The AGC target values of $1e^6$, and maximum injection time 50 ms were for full MS, and a target AGC value of $1e^5$, maximum injection time 100 ms for MS2. Dynamic exclusion duration was 30s. Survey scans were acquired at a resolution of 70,000 at m/z 200 and resolution for HCD spectra was set to 35,000 at m/z 200. Normalized collision energy was 30. The instrument was run with peptide recognition mode enabled.

7.5 Database Searching and Analysis

The resulting LC-MS/MS raw files were imported into MaxQuant software (version 1.6.0.16) for data interpretation and protein identification against the database Uniprot-Homo sapiens (Human) [9606]-194324-20201203 (downloaded on 03/12/2020, and including 194324 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 an enzymatic cleavage rule of Trypsin/P and allowed maximal two missed cleavage sites and a mass tolerance of 20ppm for fragment ions. The modification set was as following: fixed modification: Carbamidomethyl (C), TMT10plex(K), TMT10plex(N-term), Variable modification Oxidation(M) and Acetyl (Protein N-term). The minimum 6 amino acids for peptide, ≥1 unique peptides were required per protein. For peptide and protein identification, false discovery rate (FDR) was set to 1%. TMT reporter ion intensity was normalized and used for protein quantification.

7.6 Bioinformatics analysis

Analysis of bioinformatics data were carried out with Perseus software[20], Microsoft Excel and R statistical computing software. Differentially significant expressed proteins were screened with the cutoff of a ratio fold-change of >1.20 or <0.83 and P-values < 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[21], Kyoto Encyclopedia of Genes and Genomes (KEGG)[22], and Gene Ontology (GO)[23]. GO and KEGG enrichment analyses were carried out with the Fisher's exact test, and FDR correction for multiple testing was also performed. GO terms were grouped into three categories: biological process (BP), molecular function (MF), and cellular component (CC). Construction of protein–protein interaction (PPI) networks were also conducted by using the STRING database with the cytoscape software[24].

7.7 Statistical analysis

Data were shown as mean ± SEM. The Student's two-tailed *t* test was used for comparisons of two groups. Multiple comparisons were analyzed with a one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis. Probabilities of $p < 0.05$ were considered significant.

Results

Cell 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, 20mM) for 24 h. Cell viability was measured by CCK8 cytotoxicity assay and the result (Figure1A) exhibited that the ratio of cell death increases with increasing aspirin concentration in the medium. At 2.5mM, 5mM, 10mM and 20mM of aspirin concentration, cell mortality was 6%, 12.5%, 50% and 75%, respectively. Therefore, we chose the two intermediate concentrations of 5 and 10 mM for the subsequent proteomics study to explore the underlying mechanism of aspirin against colorectal cancer.

In order to investigate the mechanism of aspirin on HT29 cells, the cells were treated with 5mM and 10mM concentrations of aspirin for 24 h. HT29 cells were collected , after purification, trypsin digestion and TMT labeling, samples were applied for mass spectrometry analysis. The results indicated that the samples treated with 5mM aspirin revealed 6890 confident proteins and cells exposed to10mM aspirin showed a total 6926 proteins. The number of proteins in the three comparisons with *p*-value<0.05 and fold change greater than 1.5 were considered to be statistically significantly regulated. 81 up-regulated proteins and 83 down-regulated proteins were collected between untreated or treated by 5mM aspirin (Figure 1B), and 208 up-regulated and 344 down-regulated proteins were differentially expressed between untreated or treated by 10mM aspirin(Figure 1C).

Pearson's correlation analysis was used to evaluate the correlation of expression level between the control and aspirin-treated experiments. The result showed when cells were treated with 5 mM and 10 mM aspirin, the logarithm of the ratio of treated/control proteins were 0.734 and 0.903 respectively , indicating that a good deal of protein levels changed obviously and rather accordantly in the repeated experiments(Figure 1D,E). The data and correlation analyses showed that the quantitation values in our TMT experiments are assured and can be employed for further analysis.

We compared the protein expression profiles of treated with and without aspirin by dividing the intensities from different channels. The hierarchical cluster analysis of the ratios indicated that all replicates clustered showing lots of the quantification data was replicable. Many changes were observed in untreated vs. treated by 5mM vs. 10mM aspirin comparisons within HT29 cells (Figure 2A, B, C). This is consistent with the pie chart. Based on the combined analysis result, an arbitrary cutoff of 1.5-fold and statistical significance $p<0.05$ was used to define proteins as differential expressed in any two comparisons. The volcano plots show the significant variation of protein expressions between untreated or treated by 5mM or 10mM aspirin (Figure 2D, E, F).

Protein annotation and Kegg Pathways Analysis

Gene Ontology (GO) is aim to identify the functional enrichment of gene sets. We used GO to classify the three functions of the target proteins and the important GO processes in each function are presented in Figure 3A. The three functions are biological process (Figure 3B), molecular function(Figure 3C) and cellular component(Figure 3D) respectively.

KEGG (Kyoto Encyclopedia of Genes and Genomes) databases were used to analyze the cell signaling pathways of the differential proteins. The associated pathways are shown in Figure 4. The top pathway was the cell cycle signaling pathway (Figure 4).

Differential proteins enrichment pathways and protein-protein interaction network analysis

P53 signaling pathway was also detected which is closely associated with cell cycle (Figure 5A).

We then predicted the potential interactions of the identified proteins or differentially expressed proteins as well as of other proteins that interacted directly with them. The entire PPI network that was expressed as nodes and links, obtained effective protein information from kinds of view and contributing integrated information that could only be acquired by analysis of proteome. The results indicated that cell cycle signaling pathway was the most important signal transduction pathway of the entire system through intergroup analysis (Figure 5B).

P53 and CDK1 levels show the greatest elevation after aspirin exposure

KEGG pathway analysis indicated that cell cycle was highly dysregulated (Figure 4). Cell cycle dysregulation is an important inducement of infinite proliferation of cancer cells[25]. Therefore, blocking the cell cycle is regarded effective to prevent cell proliferation. CDK1 (Cyclin-dependent kinase 1) was reported as a key regulator in the S phase. P53 signaling pathway got our attention because it played an important role in both cell cycle arrest and apoptosis. CDK1 was decreased by 0.5 fold and p53 was increased by 2.5 fold in HT29 cells treated with 10mM aspirin when compared to HT29 cells untreated with aspirin, which were detected by MS. Our real-time PCR experiment confirmed that the expression of CDK1 was decreased and the level of p53 was increased after HT29 cells treated with 10mM aspirin (Figure 6 A, B). To further verify the differential expression of cell cycle related protein (p53 and CDK1)

after HT29 cells treated with aspirin, we selected 5 mM and 10 mM aspirin to treat HT29 cells. The expression levels of p53 and CDK1 were determined using western blot. The results showed that aspirin induced upregulation of p53 and downregulation of CDK1 with increased concentration of aspirin (Figure 6 C). This is consistent with that of real-time PCR.

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

In the proteomic enrichment results, “mitotic cell cycle” processes were enriched. To check how the cell cycle was affected, we calculated the distribution of cell cycle phase of HT29 cells treated with aspirin and untreated with aspirin by the resultant DNA content histogram using flow cytometric analysis. The proportion of S phase was significantly decreased after HT29 cells treated with aspirin. All these changes were of statistical significance, indicating that aspirin promoted the G1/S arrest of cell cycle in HT29 cells(Figure 7A).

To further explore whether aspirin promotes cell apoptosis after aspirin treatment, Annexin V and PI assays were executed to detect the rate of apoptosis. In the experiment, we selected 2.5, 5, and 10 mM of aspirin to treat HT29 cells. The apoptosis rates were 17.51%, 20.12% and 50.12%, respectively (Figure 7B).

Discussion

Colon cancer is a malignant tumor with relatively high incidence and mortality, which is very harmful to human's health[26, 27]. Currently, there is no effective specific treatment for colon cancer. Thus, it’s very important to effectively decrease the risk of colon cancer for public health and economics[28, 29]. Aspirin has been found to have a good preventive effect on colon cancer[7–9], however, the mechanisms remain to be further explored. In this study we found that aspirin treatment induced the cell apoptosis of colorectal cancer cells and cell cycle dysregulation.

We determined that the upregulation of p53 and downregulation of CDK1 leads to the G1/S arrest of cell cycle in colorectal cancer cells exposed to aspirin. To detect how aspirin affects colorectal cancer cells at the protein level, we employed proteomics coupled with TMT method to profile the proteome changes in HT29 cells after aspirin treatment. The results indicated that p53 and CDK1 were the two most differential proteins following the treatment of HT29 cells with 10mM aspirin. p53 is a transcriptional regulator that functions as a tumor suppressor in the nucleus[30, 31],which induces the target genes p21WAF1/CIP1 and BAX for cell cycle arrest and cell death[16, 32].

Cell proliferation is mediated by several signaling molecules and checkpoints (CDKs) that regulate cell division[33, 34]. CDK1 mediates the role of cell cycle progression into G2 and M phase[35, 36].In the present study, we confirmed increases in p53 mRNA and protein levels. The expressions of CDK1 in mRNA and protein levels were decreased. The results showed that p53 and CDK1 were the important protein in the cellular response of HT29 cells exposed to aspirin. Therefore, both p53 and CDK1 may be regarded as

markers for HT29 cells responding to aspirin. From the flow cytometry detection results, aspirin promoted the G1/S arrest of cell cycle in HT29 cells. From the proteomic results,p53 was increased by 2.52 fold and the level of CDK1 was decreased by 0.5 fold in HT29 cells treated with 10mM aspirin. Both KEGG and GO pathway analyses consistently showed that the most enriched pathway for differential proteins is regulation of cell cycle. In the meantime, the result of proteomics displayed that microtubule-associated protein that is related to the cytoskeleton was upregulated. Studies have found that cytoskeletal structures are related to the invasion and metastasis of tumor cells[37, 38]. It indicated that aspirin treatment may inhibit the invasion and metastasis of colon cells. Enrichment of KEGG pathway displayed that PI3K-Akt signaling pathway that regulated cell proliferation and promoted cell apoptosis[39, 40] was also changed.

In summary, aspirin promoted the G1/S arrest of cell cycle by upregulating the levels of p53 and downregulating the expression of CDK1, caused cell apoptosis. The proteomic profiling of HT29 cells exposed to aspirin has revealed several pathways and proteins include but not limit to cell cycle. We have mainly investigated the mechanism of aspirin treatment on colon cancer cells in vitro by proteomics methods. We will build animal models of colon cancer to further verify the results obtained in cell experiment in the further study. P53 will be overexpressioned and knocked down in vivo and in vitro to further explore the regulation mechanism in the next step of our experiment.

We expect such a proteome profiling using the model cell of colon cancer will be helpful in understanding the cellular impact of aspirin. Our results provide theoretical basis for aspirin to be used as a preventive drug for colon cancer.

Declarations

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Author Contributions:

Methodology, Wei Han and Rong Wang; Validation, Yu Ji; Formal Analysis, Yan Zhang, Haitao Sun and Yu Ji; Investigation, Yan Zhang, Haitao Sun and Yu Ji; Writing -Original Draft, Yan Zhang and Haitao Sun; Writing Review & Editing, Wei Han and Rong Wang. All authors had full ac cess to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict Of Interest

The authors declare no conflict of interest. None of the authors has a commercial interest in the material presented in this work.

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Figures

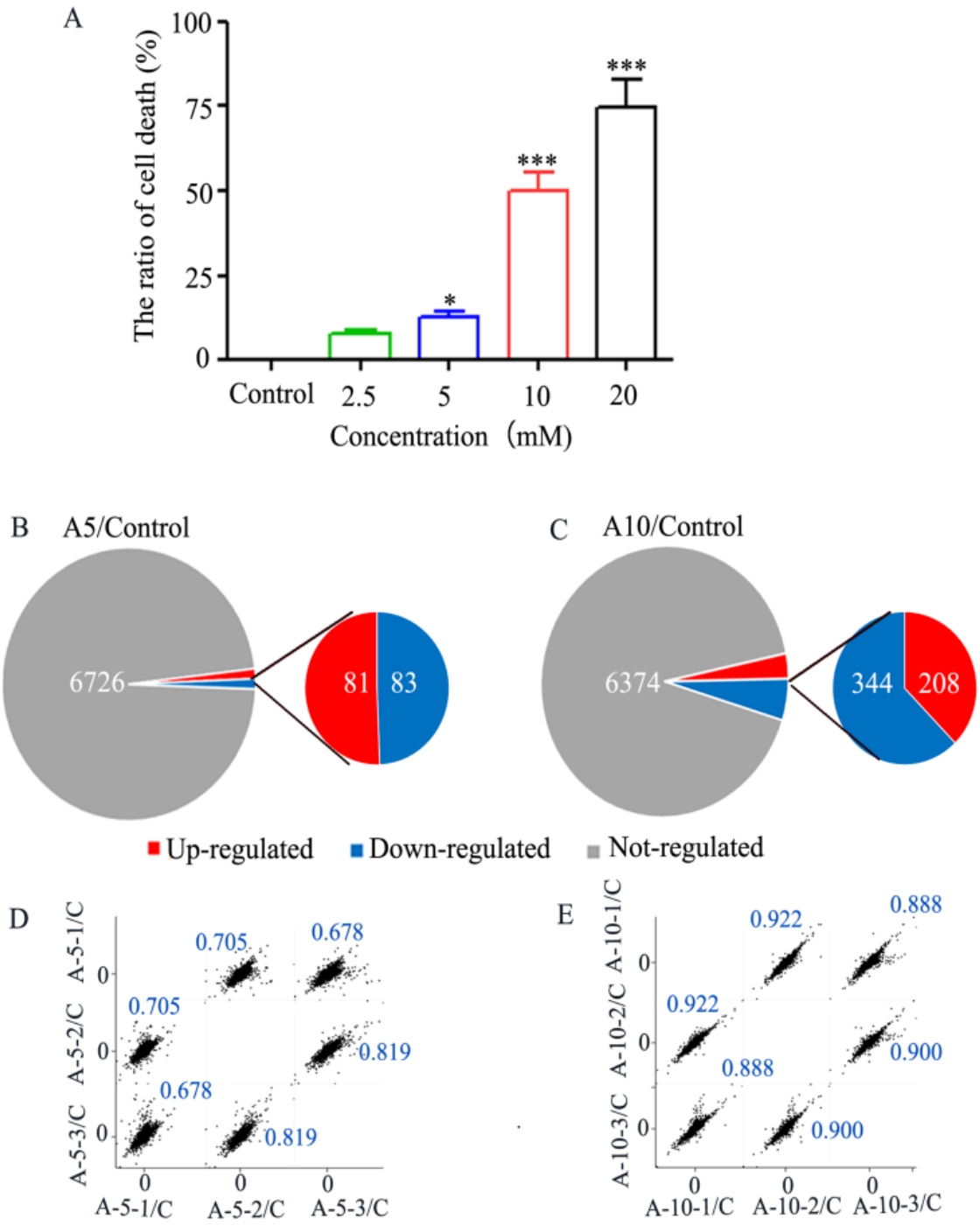

Figure 1

Effects of aspirin on proliferation of HT29 cells and comprehensive quantitative proteomics data. (A)The survival rate of HT29 cells was determined by CCK-8 assay. Data are represented as mean ± SEM. * p<0.05, *** p <0.001, 5mM versus Control, 10mM versus Control, 20mM versus Control. Independent experiment was performed at least three times. In total, 6890 proteins were identified in HT29 cells treated with 5mM aspirin(B) and 6926 proteins were obtained in HT29 cells treated with 10mM aspirin (C)

(considering the regulated and not regulated ones). When p<0.05 and 1.5-fold cutoff is 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 5mM or 10mM aspirin (D,E). The reproducibility of quantification of the duplicated experiments is revealed by the protein ratio correlations and analyzed by Pearson's correlation.

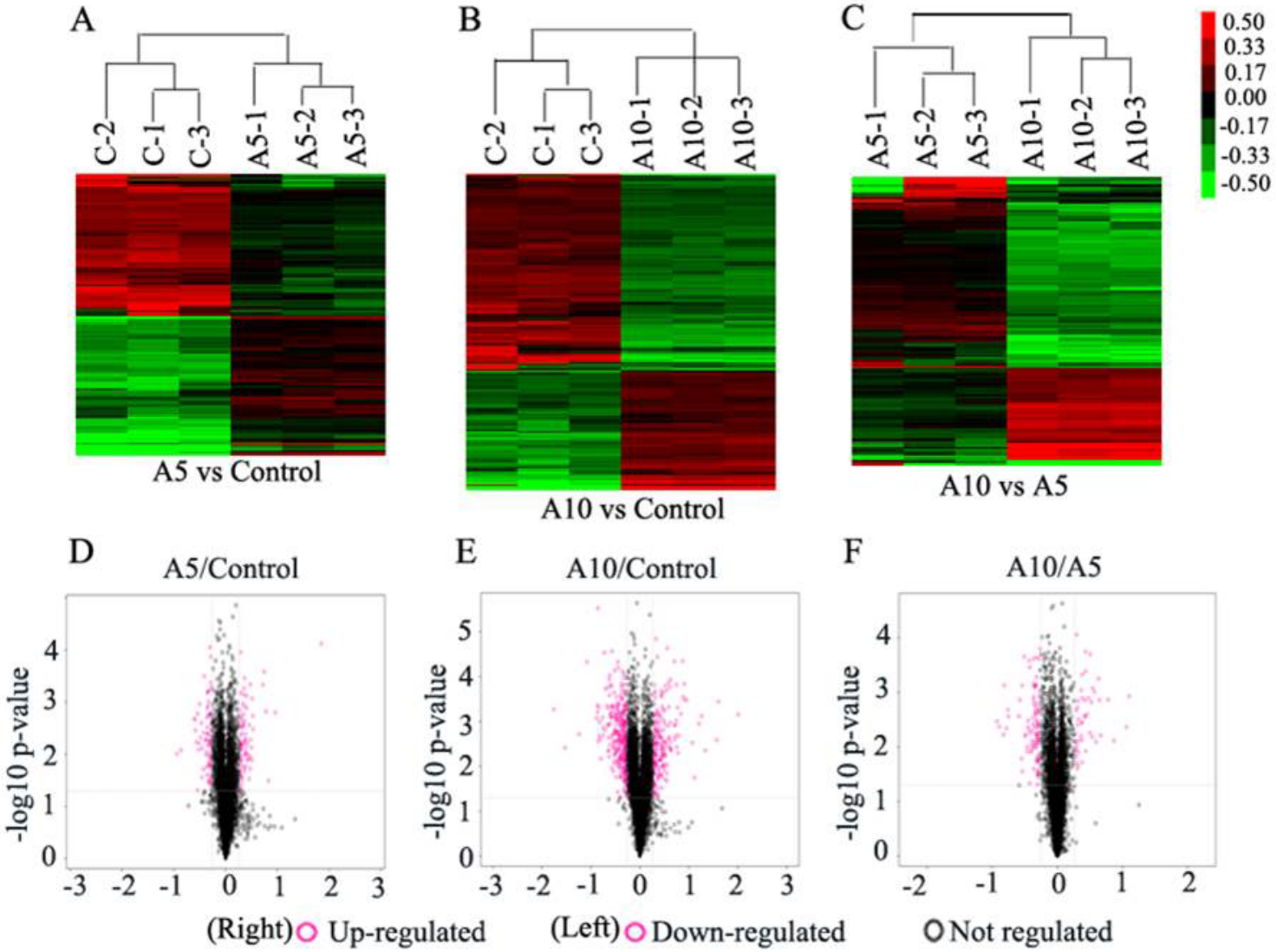

Figure 2

Cluster analysis of expressed protein comparisons in HT29 cells treated with aspirin at 0, 5, 10mM for 24 h. (A,B,C)Heat map showed logarithm of expression intensities from green (low expression) to red (high expression). Each column represents a sample, and each row represents a protein. C, A5 and A10 refer to three replicates. (D, E, F) Volcano plots show the distributions of proteins between HT29 cells untreated with aspirin and HT29 cells treated with 5mM or 10 mM aspirin. The horizontal line corresponds to a 2 fold (log2 scaled) change up or down, and the vertical line represents a p-value of less than 0.05 (-log10 scaled). The pink points on the plot represent the differentially expressed proteins with a 1-fold change up or down in proteins with statistical significance ($p < 0.05$).

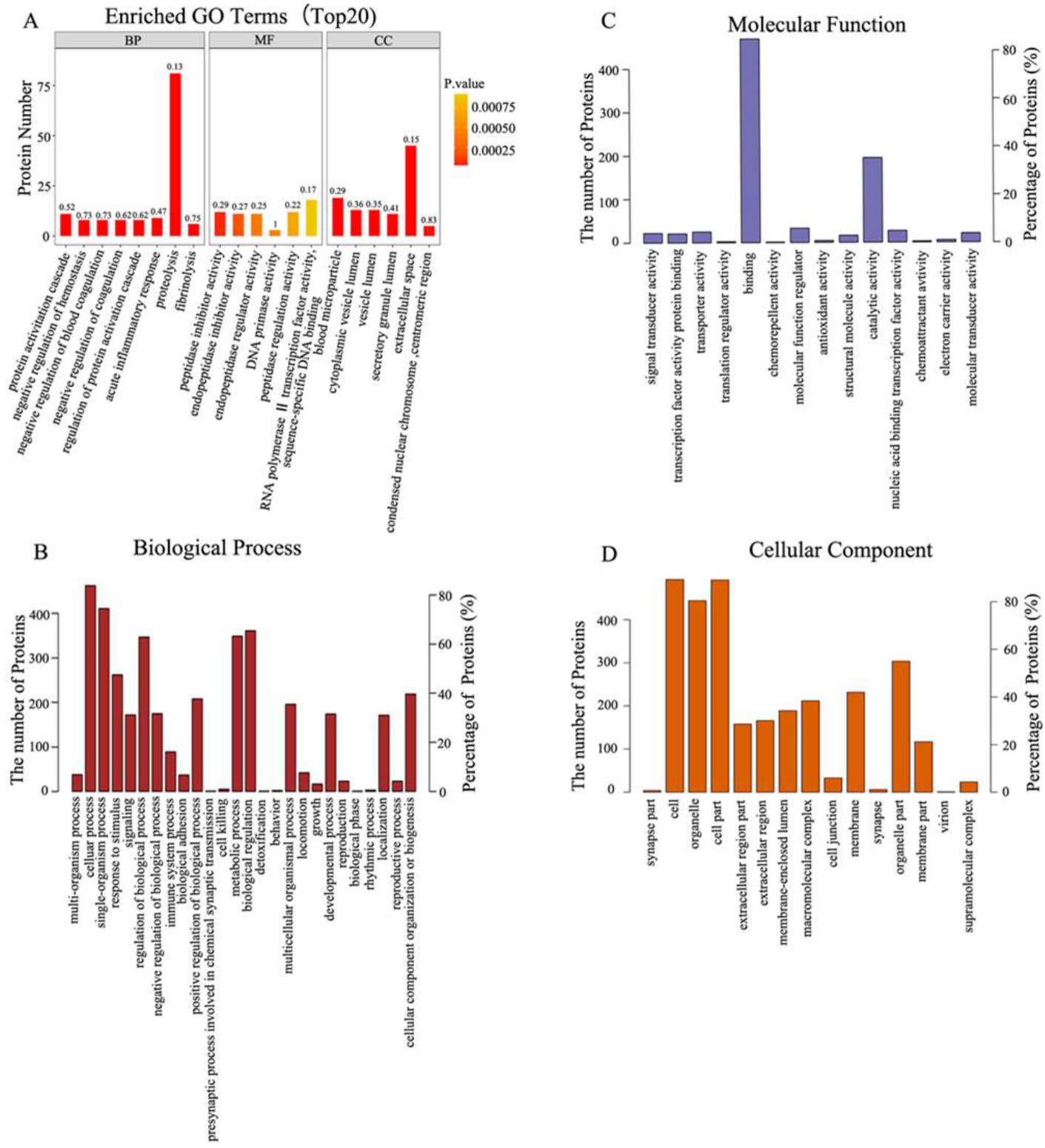

Figure 3

The GO annotations for differential proteins. The GO analysis categorized the differential proteins into different groups under the theme of molecular function (MF, B), biological process (BP, C) and cellular component (CC, D). The protein numbers of each GO category are showed in Y-axis.

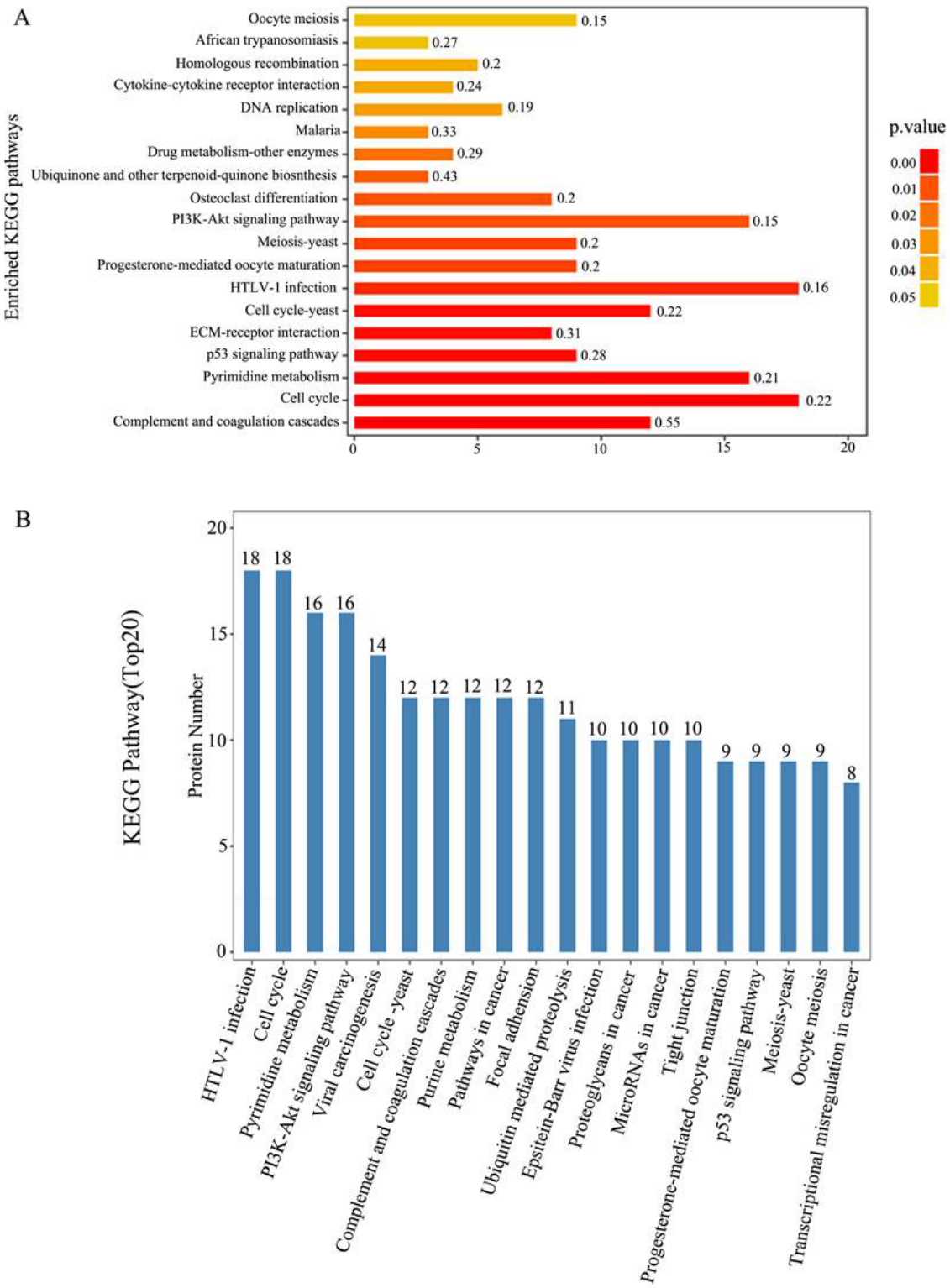

Figure 4

(A, B) The KEGG database analyses of the cell signaling pathway annotations for differential proteins. The vertical axis represents the enriched pathways, and the horizontal axis represents the p pathway values. Numbers near the histograms represent p values ($p<0.05$).

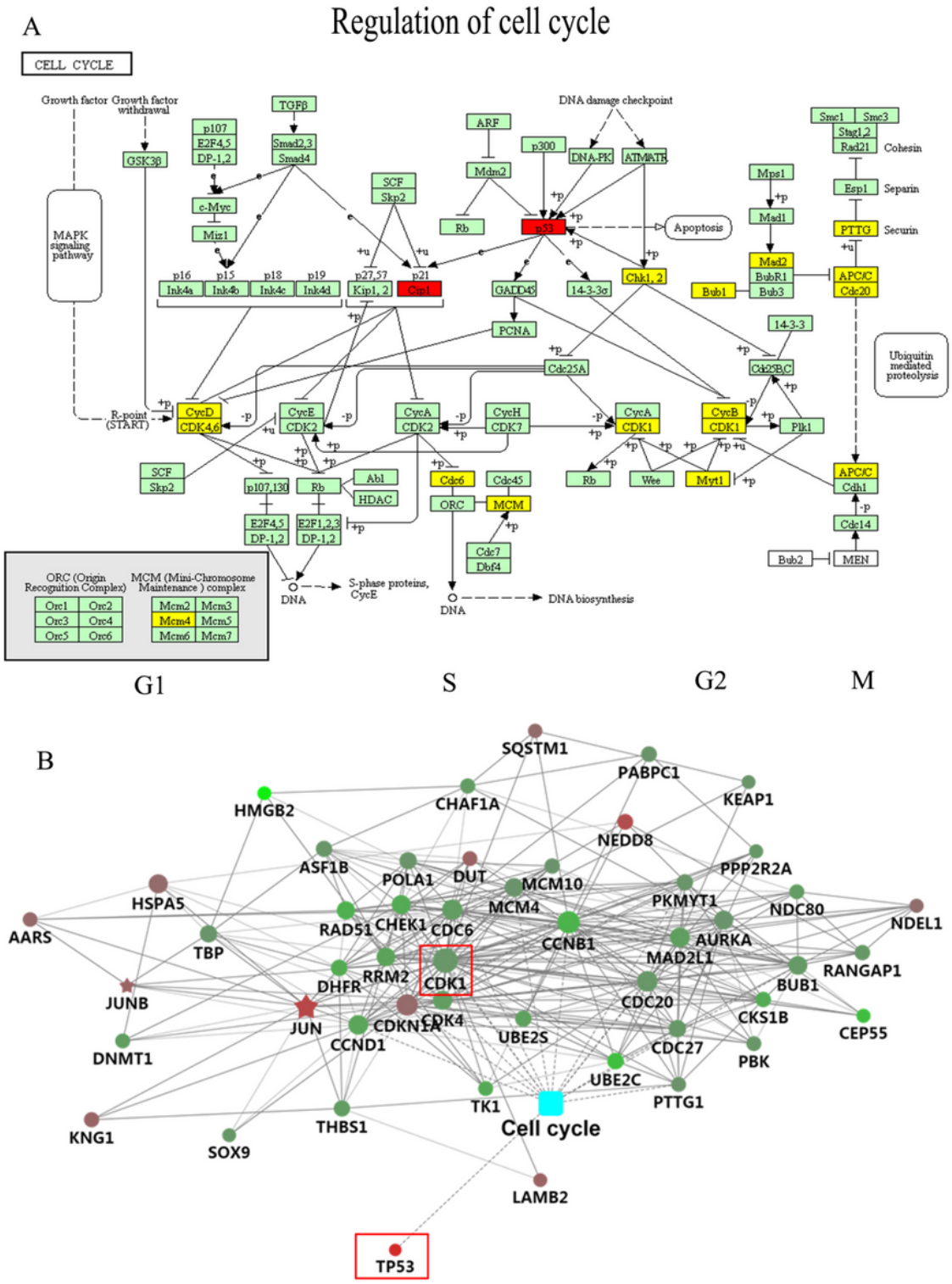

Figure 5

A. Mapping of dysregulated proteins in HT29 cells treated with 10mM aspirin to “Regulation of cell cycle” pathway. Each box shows a gene of certain protein, within which is the symbol of the gene. Arrows indicate the process of the pathway. Red and Yellow: differential proteins.

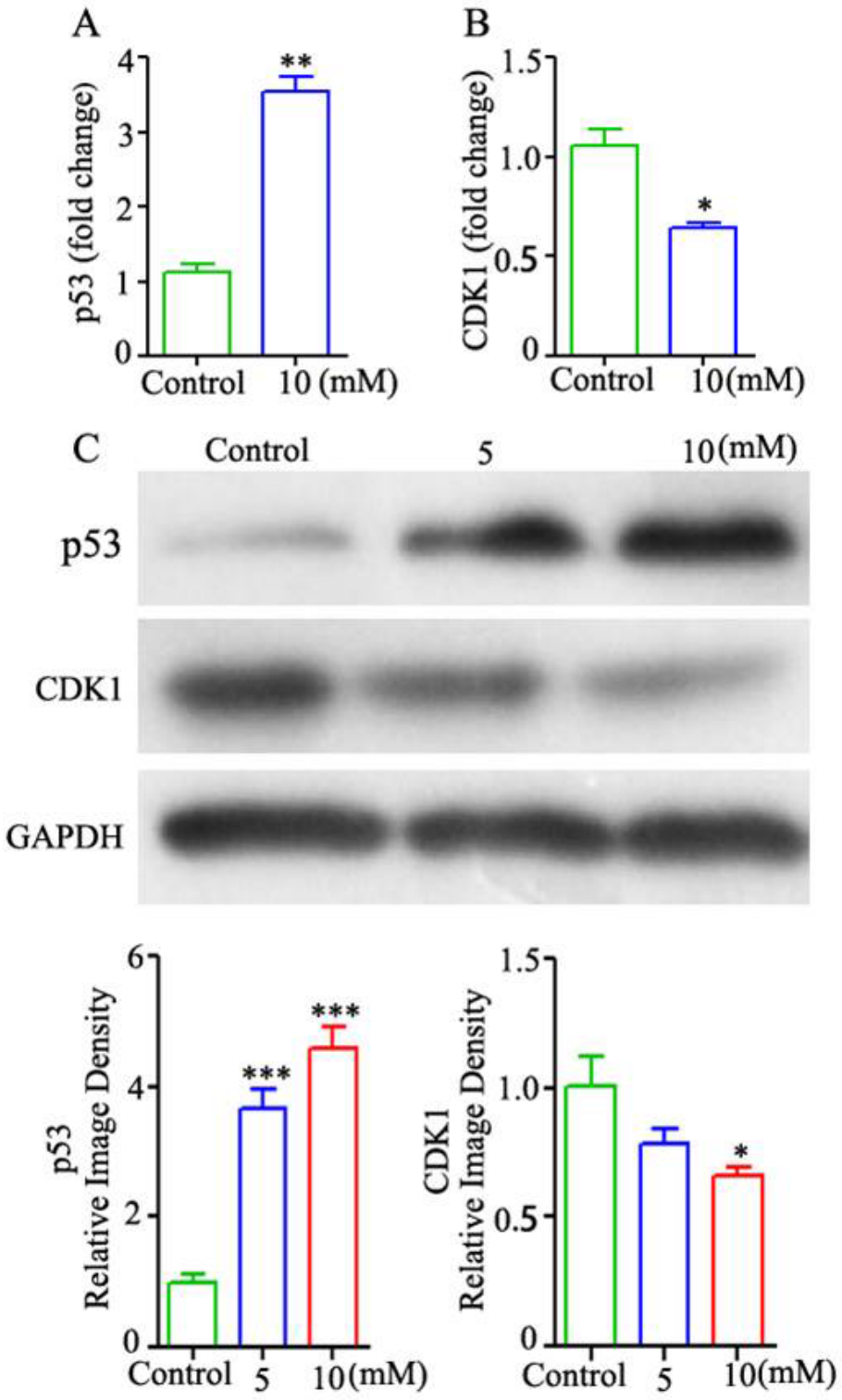

Figure 6

Validation of proteins and mRNAs changes of p53 and CDK1 in HT29 cells exposed to aspirin. (A,B) Real-time PCR analysis of p53 and CDK1 mRNA expression in H1299 cells treated with 10mM aspirin. Each bar represents mean value±S.D. from at least three independent experiments. ** p <0.01, * p<0.05, 10mM versus Control.(C) Western blot shows the p53 and CDK1 protein expression in H1299 cells treated with

5mM and 10mM aspirin. All values are expressed as mean ± SD of three repeats. t-test was performed with respect to the control and * p<0.05, *** p <0.001, 10mM versus Control, 5mM versus Control.

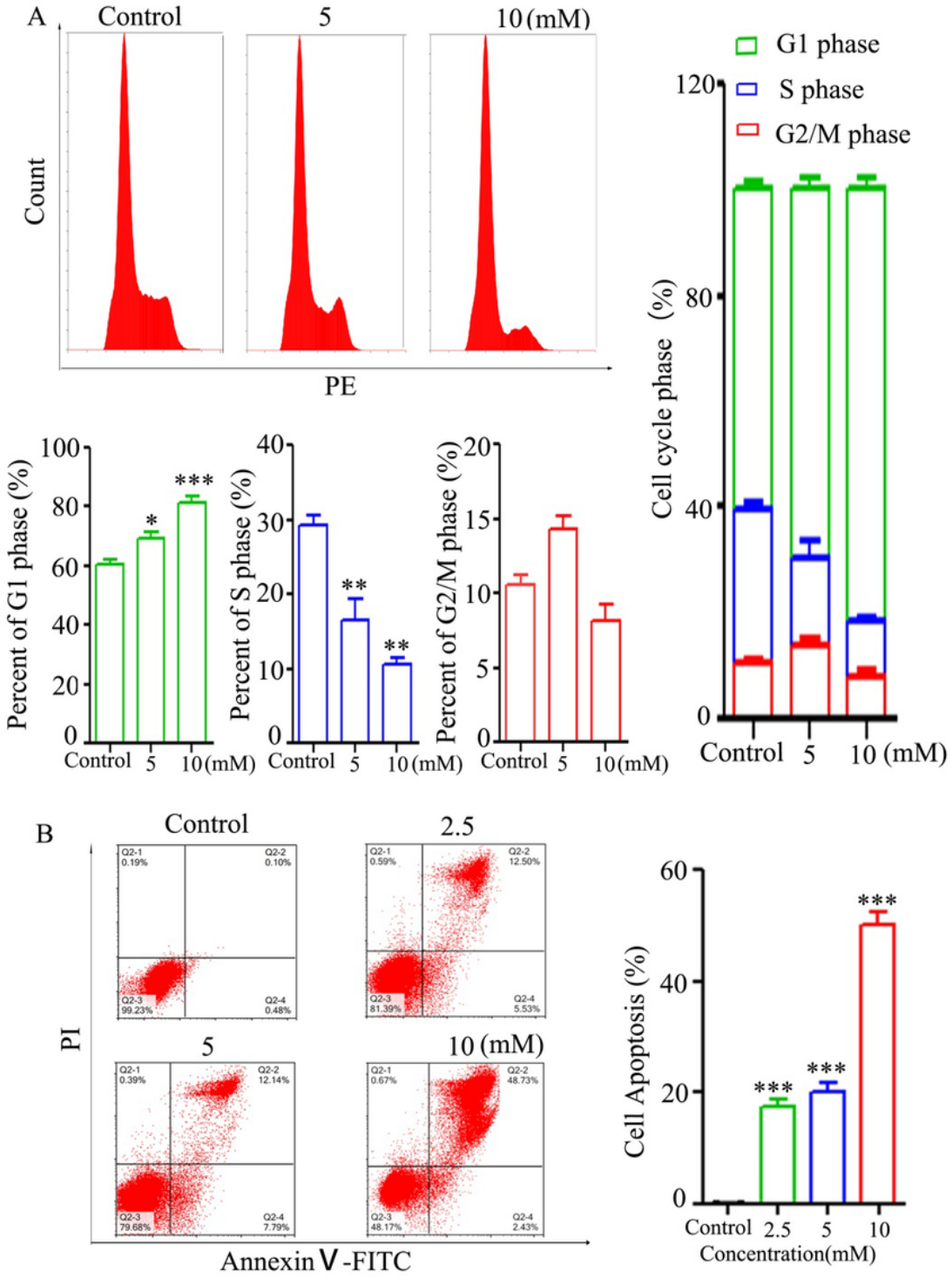

Figure 7

(A) Flow cytometric analysis of cell cycle distribution of H1299 cells exposed to 5mM and 10mM aspirin. Cell cycle analysis was performed as described in materials and methods. Each column represents the effect of Control, 5mM, 10 mM aspirin on the percentage of phase in the cell cycle as indicated at the

bottom of the histogram. Each bar represents mean value± S.D. from at least three independent experiments. * p<0.05, ** p<0.01, *** p <0.001, 10mM versus Control, 5mM versus Control. (B) Flow cytometry analysis of HT 29 cells treated with aspirin for 24h. HT 29 cells were stained using Annexin V and PI before flow cytometry analysis. The rate of cell apoptosis was statistically analyzed on the basis of the flow cytometer results. Data are represented as mean ± SEM. independent experiment was performed at least three times, *** p <0.001, 10mM versus Control, 5mM versus Control, 2.5mM versus Control.