mRNA expression level of *CDH2*, *LEP*, *POSTN*, *TIMP1* and *VEGFC* modulates 5-fluorouracil resistance in colon cancer cells

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Abstract. Drug resistance severely affects the clinical efficacy of therapeutic agents in patients with colon cancer. The aim of the present study was to identify genes involved in drug resistance in colon cancer using bioinformatics analysis and to identify the underlying mechanisms *in vitro*. Genes associated with cancer recurrence and chemotherapy resistance were identified using data mining. Immunohistochemistry was performed to analyze the protein expression level of genes of interest in human colon cancer tissues. Reverse transcription-quantitative PCR analysis was performed to analyze the gene expression level in patient samples and in colon cancer cell lines (HCT116 and LoVo). Cell viability was evaluated using the Cell Counting Kit-8 assay in the colon cancer cell lines. Apoptosis was measured using PI staining. The results from the present study revealed 602 genes using both 'cancer recurrence' and 'chemoresistance' terms on the GenCLiP3 website. Gene functional annotation was performed using the Database for Annotation, Visualization and Integrated Discovery then, the protein-protein interaction networks of the 602 genes were analyzed using STRING analysis. Further, in the GEPIA database, 14 genes (*ATM*, *CDH2*, *CDKN2A*, *EPO*, *LEP*, *TGFB1*, *TIMP1*, *PGR*, *VEGFC*, *POSTN*, *BCL6*, *CYP19A1*, *NOTCH3* and *XPA*) were found to be upregulated in colon cancer tissue and were associated with poor prognosis in patients with colon cancer. Further analysis of 33 paired human colon cancer tissues revealed that 8 genes (*ATM*, *CDH2*, *CDKN2A*, *LEP*, *PGR*, *TIMP1*, *POSTN* and *VEGFC*) were significantly upregulated, which was consistent with the results obtained from the earlier analysis and 5 genes (*CDH2*, *LEP*, *POSTN*, *TIMP1* and *VEGFC*) were associated with patient prognosis. Silencing of these 5 genes using small interfering RNAs significantly enhanced the sensitivity of colon cancer cells to the chemotherapeutic agent, 5-fluorouracil (5-FU). Taken together, the results suggested that *CDH2*, *LEP*, *POSTN*, *TIMP1* and *VEGFC* might play a role in chemotherapeutic resistance in colon cancer and represent potential targets for overcoming 5-FU resistance in colon cancer.

Introduction

Chemotherapy, the standard treatment for different types of cancer, plays an important role in improving the survival time of patients with cancer (1,2); however, a large number of patients who receive chemotherapy will develop chemotherapy resistance, leading to tumor recurrence and poor prognosis (3). The pathogenesis of chemotherapeutic resistance in cancer is highly complex and involves numerous biological processes and molecular pathways (4,5).

Chemotherapeutic resistance in tumors has been extensively investigated (6-8). Previous studies have reported that the process of chemotherapeutic resistance may be triggered by adaptive mutations in the tumor (9) or attributed to copy number variation in certain genes (10). For example, the high expression of P-glycoprotein protein caused increased efflux of chemotherapeutic drugs and decreased the sensitivity of tumor cells to these drugs (11). However, few alternative drugs or therapeutic strategies aimed at overcoming drug resistance are available; therefore, an understanding of the specific mechanisms underlying cancer drug resistance would be beneficial to the development of drugs for mitigating this chemotherapeutic resistance.

Colon cancer is the 4th leading cause of cancer-associated death worldwide (12). The most commonly used clinical chemotherapeutic agents for the treatment of colon cancer contain platinum compounds and 5-fluorouracil (5-FU) (13). Patients with colon cancer typically develop chemotherapy resistance (14), which may be an important cause of recurrence and poor prognosis. A satisfactory strategy to overcome colon cancer-related chemotherapeutic resistance remains unavailable

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to date. In recent years, biomedical text mining research, a type of bioinformatics analysis, has been intensively used to identify information in a more accurate and efficient manner; thus, serving as an effective tool to identify differentially expressed genes and to analyze molecular functions (15). A previous study, using biomedical text mining, revealed that several drugs have the potential to be repurposed for colorectal cancer treatment (16). Furthermore, novel microRNA biomarkers for the early diagnosis of colorectal cancer and 5-FU chemotherapeutic resistance were identified (17).

In the present study, data mining was used to identify genes, then Gene Ontology (GO) analysis was used to identify the potential association between gene expression and chemotherapeutic resistance in colon cancer. A deeper understanding of the mechanisms by which these genes perform their biological functions would provide further insights into drug resistance in colon cancer.

Materials and methods

Data mining. From the web-based service platform Human Gene Function and Network Analysis GenCLiP3 (http://ci.smu.edu.cn/genclip3/analysis.php), two gene sets were generated using the search terms, 'cancer recurrence' and 'chemotherapy resistance', respectively. The intersection of the two gene sets was selected and the data was visualized using a Venn diagram online (http://bioinformatics.psb.ugent.be/webtools/Venn/). The intersection of the two gene sets contained 602 genes, which are associated with cancer recurrence and chemotherapy resistance.

Analysis of biological processes and pathways. GO and KEGG pathway enrichment analysis of the genes generated from the intersection of the two gene sets were performed using Database for Annotation, Visualization and Integrated Discovery v6.8 (DAVID 6.8) (https://david.ncifcrf.gov). Among the biological processes, whose values were above the cut-off, those most associated with cancer recurrence and chemotherapy resistance were selected based on available published literature. The pathways associated with other specific diseases were excluded.

Gene expression. The gene expression data (N=602 genes) was obtained from The Cancer Genome Atlas dataset (https://tcga-data.nci.nih.gov/tcga/). EdgR and limma packages were used to calculate the differentially expressed genes between colon cancers and normal group, with the same parameters (|logFC|>1, false discovery rate (FDR) <0.05).

Protein-protein interaction (PPI) networks. PPI networks from the intersection of the associated genes were generated using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) website (http://string-db.org/), which provides an interactive platform for assessing the interactions between proteins. The following parameters were used: i) minimum required interaction score; ii) medium confidence (0.400); and iii) PPI enrichment P-value, 1.75e-05.

Survival analysis using Gene Expression Profiling Interactive Analysis (GEPIA) database. For predicting genes which play a role in drug resistance in colon cancer, the genes previously identified by STRING analysis were further analyzed using the GEPIA database (http://gepia.cancer-pku.cn) to identify the association between gene expression and survival time among patients with colon cancer. Specifically, the candidate genes (N=310) were searched in GEPIA one by one (single gene analysis). Gene expression and its association with survival were recorded.

Clinical samples. A total of 33 patients with colon cancer, who received surgery at Xiang'an Hospital of Xiamen University (Fujian, China) from January 2015 to December 2019 were included in the present study. Tumor and adjacent normal tissues (5 cm distance from the tumor tissue) were collected from patients with colon cancer. All the patients had complete clinicopathological data, were diagnosed with colon cancer using histopathology and had not received any anti-tumor therapy prior to surgery. Patients with other malignancies or comorbidities and those who were pregnant were not included in the study. Among the patients included in the study, there were 19 males and 14 females, aged from 39 to 64 years, with an average age of 63±8.1 years. The postoperative tumor stage was classified according to the American Joint Committee on Cancer/International Union Against Cancer TNM staging system, 7th edition (18), in which there were 5 patients with stage I, 17 patients with stage II, and 11 patients with stage III cancer. The present study was approved by the Ethics Committee of Xiang'an Hospital of Xiamen University (Fujian, China) and all patients provided written informed consent.

Immunohistochemistry (IHC). Colon cancer tissues and adjacent normal samples were fixed in 10% neutral formalin for 48 h at room temperature. Dehydration was done in an ascending alcohol series at 30, 50, 75, 90 and 100% for 10 min at each stage. Subsequently, the samples were embedded in paraffin wax according to standard laboratory procedures. Sections (5 μm) were prepared, mounted on glass slides and dried overnight. The sections were then deparaffinized with xylene three times for 5 min each time, rehydrated through a descending alcohol series at 100, 95, 80, 70 and 50% for 2 min at each stage, and washed with PBS (pH, 7.2-7.4). Antigen retrieval was performed using citrate-EDTA antigen retrieval solution (cat. no. P0086; Beyotime Institute of Biotechnology) in a microwave oven at 100˚C for 15 min. After washing three times with PBS, the sections were permeabilized for 25 min in 0.2% Triton X-100 at room temperature. Sections were washed again with PBS, and blocked with 5% BSA (cat. no. A8010; Beijing Solarbio Science & Technology Co., Ltd.) in PBS for 1 h at room temperature. Primary antibody incubation was subsequently carried out overnight at 4˚C, followed by secondary antibody incubation for 1 h at room temperature. Immunohistochemical reactions were developed using DAB Horseradish Peroxidase Color Development Kit (cat. no. P0203; Beyotime Institute of Biotechnology), and nuclei were counterstained with hematoxylin for 15 sec. The slides were dehydrated using an ascending alcohol series at 80, 95 and 100% for 2 min at each stage, followed by xylene permeabilization twice for 5 min each. Tissue sections were sealed with neutral resins and images were captured using an optical microscope (Nikon Corporation) from at least 10 fields of view

at x200 magnification. The immunohistochemical score was calculated based on the distribution and intensity of staining in the positive cells. The following classification was used: Negative expression (-, score of 0), weakly positive expression (+, score of 1), moderately positive expression (++, score of 2), and strongly positive expression (+++, score of 3). The results were blindly determined by two experienced pathologists. In addition, the percentage of stained cells was scored semi-quantitatively as 1 (0-25%), 2 (26-50%), 3 (51-75%), or 4 (76-100%). Multiplication of the intensity score and percentage score resulted in a score ranging from 0 to 12 for each tissue.

The following antibodies were used: Anti-CDH2 (cat. no. 13116; 1:200 dilution; Cell Signaling Technology, Inc.), anti-LEP (cat. no. ab3583; 1:200 dilution; Abcam), anti-POSTN (cat. no. ab219056; 1:500 dilution; Abcam), anti-TIMP1 (cat. no. ab211926; 1:500 dilution; Abcam), anti-VEGFC (cat. no. ab83905; 1:300 dilution; Abcam), anti-rabbit IgG HRP-conjugated antibody (cat. no. 7074; 1:500 dilution; Cell Signaling Technology, Inc.), and anti-mouse IgG HRP-conjugated antibody (cat. no. 7076; 1:500 dilution; Cell Signaling Technology, Inc.).

Cell culture. The human HCT116 and LoVo colon cancer cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences. Both the cell lines were cultured in DMEM, supplemented with 10% FBS, and 1% penicillin-streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.), at 37˚C in a humidified incubator with 5% CO_2.

PI staining. To investigate the sensitivity of the colon cancer cells to 5-FU, apoptosis was analyzed by staining the cells with PI. The colon cancer cells were treated with 1 μg/ml 5-FU for 24, 48 h following transfection with siRNA. Subsequently, PI dye (1 μg/ml) was added and the cells were incubated for 10 min at 37˚C in the dark. The PI fluorescence of the nuclei was observed under a fluorescent microscope at x100 magnification (Leica Microsystems GmbH). PI-positive cells in five randomly selected fields of view were counted for each group.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was extracted from the colon cancer cells and human colon cancer tissues using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The concentration and purity of the RNA was measured according to the optical density (OD) at 260 nm and the 260/280 nm ratio, respectively. Only RNA with a 260/280 ratio between 1.8 and 2.0 was used for the experiments. cDNA was synthesized using a Reverse Transcription kit (Sangon Biotech Co. Ltd.) under the following conditions: 37˚C for 15 min, 85˚C for 5 sec, and 4˚C for 30 min. Subsequently, the following thermocycling conditions were used: Initial denaturation at 95˚C for 3 min; denaturation at 95˚C for 30 sec, annealing at 60˚C for 20 sec for 40 cycles. RT-qPCR was performed using a GoTaq® qPCR Master Mix (Promega Corporation) on an ABI 7500 qPCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative mRNA expression levels of the target genes were calculated using the $2^{-\Delta\Delta Cq}$ method and normalized to that of *GAPDH* (19). All the primer sequences are listed in Table I.

Table I. Sequences of the primers and siRNAs.

A, primer sequences for RT-qPCR

Name	Sequence
GAPDH	F: 5'-GCAAAGTGGAGATTGTTGCCAT-3'
GAPDH	R: 5'-CCTTGACTGTGCCGTTGAATTT-3'
CDH2	F: 5'-TGCGGTACAGTGTAACTGGG-3'
CDH2	R: 5'-GAAACCGGGCTATCTGCTCG-3'
LEP	F: 5'-TGCCTTCCAGAAACGTGATCC-3'
LEP	R: 5'-CTCTGTGGAGTAGCCTGAAGC-3'
POSTN	F: 5'-GCTATTCTGACGCCTCAAAACT-3'
POSTN	R: 5'-AGCCTCATTACTCGGTGCAAA-3'
TIMP1	F: 5'-AGAGTGTCTGCGGATACTTCC-3'
TIMP1	R: 5'-CCAACAGTGTAGGTCTTGGTG-3
VEGFC	F: 5'-GGCTGGCAACATAACAGAGAA-3'
VEGFC	R: 5'-CCCCACATCTATACACACCTCC-3'

B, siRNA sequences used for silencing

Name	Sequence
CDH2	5'-TAAACTTCACATTGAGAAGAG-3'
LEP	5'-TGTGAAATGTCATTGATCCTG-3'
POSTN	5'-ATAATGGTTAATGAAAAGCCC-3'
TIMP1	5'-TCATCTTGATCTCATAACGCT-3'
VEGFC	5'-TAAAGAAGGTGTTTGTCGCGA-3'
Control	5'-TTCTCCGAACGTGTCACGTTT-3'

F, forward; R, reverse; si, small interfering; RT-qPCR, reverse transcription-quantitative PCR.

Western blot analysis. The colon cancer cells were lysed on ice using RIPA lysis buffer with 1% phenylmethylsulfonyl fluoride (Nanjing KeyGen Biotech Co., Ltd.), then centrifuged at 12,000 x g for 15 min at 4˚C. Total protein was collected, then the concentration of the samples was quantified using a BCA assay (Nanjing KeyGen Biotech Co., Ltd.). Next, the proteins (30 μg) were separated using a 10% SDS-PAGE, then transferred to PVDF membranes using wet transfer. After incubation with 5% skimmed milk for 2 h at room temperature, the membranes were incubated with the corresponding primary antibodies overnight at 4˚C. Subsequently, the membranes were washed with TBS-Tween-20, containing 0.1% Tween-20, then incubated with the corresponding secondary horseradish peroxidase-conjugated antibodies at room temperature for 2 h. The western blots were visualized using an enhanced chemiluminescence kit (EMD Millipore). β-actin was used as the loading control. The blots were quantified using ImageJ software (Version 1.8.0; National Institutes of Health).

The following antibodies were used: Anti-CDH2 (cat. no. 14215; 1:1,000 dilution; Cell Signaling Technology, Inc.), anti-LEP (cat. no. ab3583; 1:1,000 dilution; Abcam), anti-POSTN (cat. no. ab219056; 1:1,000 dilution; Abcam), anti-TIMP1 (cat. no. 8946; 1:1,000 dilution; Cell Signaling Technology, Inc.), anti-VEGFC (cat. no. ab83905; 1:1,000

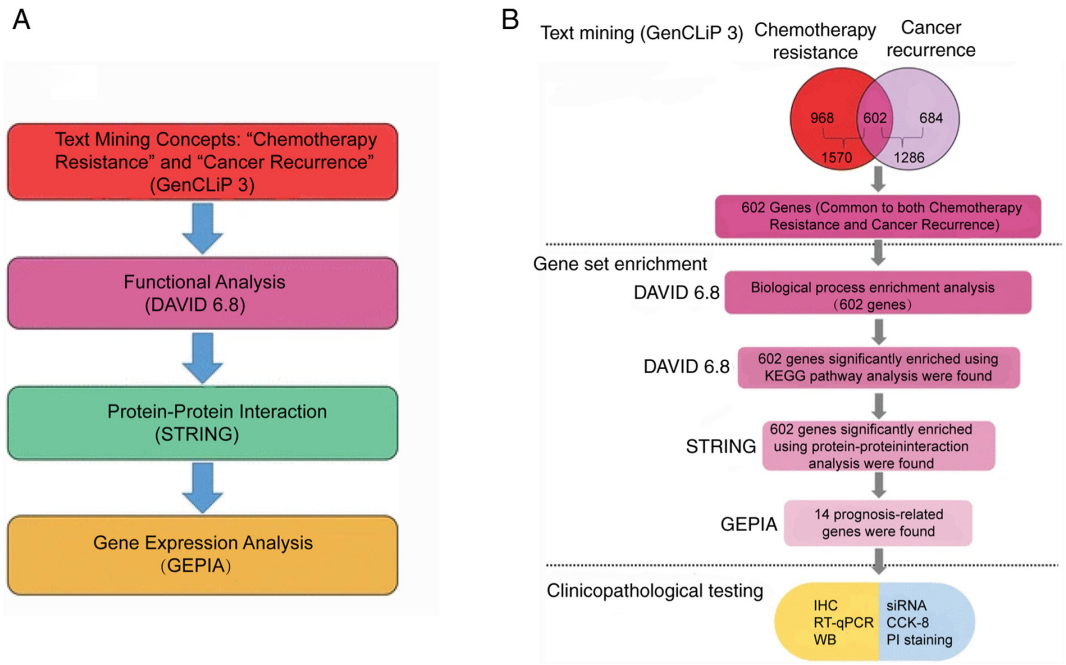

Figure 1. Data mining strategy used in the present study. (A) Online bioinformatics analysis software was used to select the genes of interest, in which GenCLiP3 was used to identify the associated genes using the search terms 'chemotherapy resistance' and 'cancer recurrence'. Gene Ontology analysis was performed using DAVID online tool. Protein-protein interaction was analyzed using the STRING database. The association between the expression level of the candidate genes and the overall survival of patients with colon cancer was analyzed using GEPIA. (B) The workflow used in the present study, where 602 genes were generated and enriched using KEGG pathway and protein-protein interaction analysis. Of these 602 genes, 14 prognosis-related genes were chosen for further investigation. GEPIA, Gene Expression Profiling Interactive Analysis; DAVID, Database for Annotation, Visualization and Integrated Discovery; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; CCK-8, Cell Counting Kit-8; RT-qPCR, reverse transcription-quantitative PCR; IHC, immunohistochemistry; si, small interfering; WB, western blot.

dilution; Abcam), anti-β-actin (cat. no. sc-47778; 1:3,000 dilution; Santa Cruz Biotechnology, Inc.), anti-rabbit IgG HRP-linked antibody (cat. no. 7074; 1:5,000 dilution; Cell Signaling Technology, Inc.) and anti-mouse IgG HRP-linked antibody (cat. no. 7076; 1:5,000 dilution; Cell Signaling Technology, Inc.).

Small interfering (si)RNA transfection. All the siRNA sequences and the disordered sequence were synthesized by Sangon Biotech Co., Ltd. and are listed in Table I. Scrambled siRNA sequences were used as negative control. The colon cancer cells were cultured in complete DMEM until the cell density reached 60%, and transfected with the different siRNAs at a final concentration of 10 nM, and control siRNA at a final concentration of 10 nM using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After transfection for 48 h at 37˚C, transfection efficiency was evaluated by RT-qPCR.

CCK-8 assay. The colon cancer cells in the logarithmic growth phase were digested with 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.), resuspended, then seeded into 96-well plates, at a density of $1x10^3$ cells per well. Next, 100 μl complete medium was added. The cells were cultured for 12 h at 37˚C in a humidified incubator with 5% CO_2. Then, the cells were treated with different concentrations of 5-FU (0, 0.5, 1, 2 and 4 μg/ml) for 48 h. Subsequently, 10% CCK-8 reagent (Beijing Solarbio Science and Technology Co., Ltd.) was added to each well according to the manufacturer's instructions, followed by incubation for 2 h at 37˚C. The OD at 450 nm was measured using a microplate reader (BioTek China). Cell-free wells with CCK-8 reagent served as a negative control.

Statistical analysis. Statistical analysis was performed using SPSS v19.0 (IBM Corporation). The differences between the two independent groups were analyzed using an unpaired Student's t-test, while the differences between paired samples was analyzed using a Wilcoxon signed-rank test. Parametric data was presented as the mean ± SD, while non-parametric data was presented as the median ± interquartile range. In addition, the mRNA expression levels of the target genes in 33 tumor and adjacent normal tissues were analyzed using a paired Student's t-test and presented as the mean ± SD. Kaplan-Meier survival curves and statistics (Log-rank) were used to analyze survival time in the 33 paired samples. $P<0.05$ was considered to indicate statistically significant difference. Each experiment was repeated independently, at least three times.

Results

Data mining strategies. To increase the number of candidate genes associated with cancer recurrence that are currently known from previous studies, search terms were used in the GenCLiP3 database, to preliminary screen genes. The workflow used is shown in Fig. 1A. A total of 1,286 genes were associated with cancer recurrence and 1,570 genes were associated with chemoresistance, while a total of 602 genes were shared by both lists (Fig. 1B). The list of these genes and their expression levels are shown in Tables SI and SII.

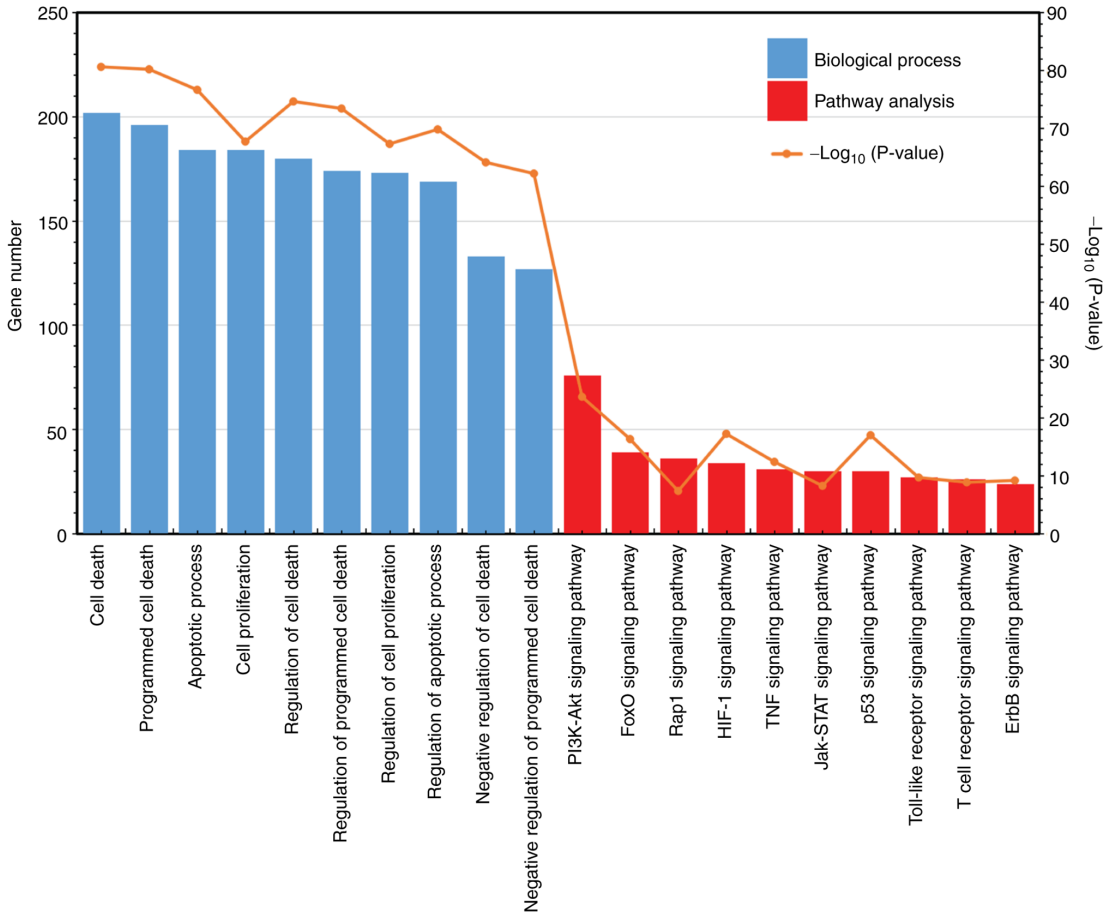

Figure 2. Gene Ontology enrichment analysis of the 602 genes of interest. The top 10 significantly associated genes for biological process and pathway analysis are shown.

GO enrichment analysis of gene sets. The 602 genes were validated by performing GO analysis, including biological processes alone. Results demonstrated that the top ten GO terms were selected and are shown in Fig. 2. The top 3 enriched biological processes were 'cell death' ($P=2.60x10^{-81}$), 'programmed cell death' ($P=6.78x10^{-81}$) and 'apoptotic process' ($P=2.39x10^{-77}$), containing 202, 196 and 184 genes, respectively. Additional highly enriched biological processes included 'regulation of programmed cell death', 'regulation of apoptotic process' and 'regulation of cell proliferation'.

Furthermore, these 602 genes were significantly enriched using pathway analysis. KEGG pathway enrichment analyses were performed using DAVID 6.8. Ten pathways, which were significantly enriched were also selected. Among these, the top two pathways were 'PI3K-Akt signaling pathway' ($P=2.50x10^{-24}$) and 'FoxO signaling pathway' ($P=5.02x10^{-17}$), containing 76 and 39 genes, respectively. Other pathways were the 'Rap1 signaling pathway' (N=36, $P=4.64x10^{-8}$), 'HIF-1 signaling pathway' (N=34, $P=6.22x10^{-18}$), 'TNF signaling pathway' (N=31, $P=4.30x10^{-13}$), 'Jak-STAT signaling pathway' (N=30, $P=5.95x10^{-9}$), 'p53 signaling pathway' (N=30, $P=1.07x10^{-17}$), 'Toll-like receptor signaling pathway' (N=27, $P=2.17x10^{-10}$), 'T cell receptor signaling pathway' (N= 26, $P=1.48x10^{-9}$), and 'ErbB signaling pathway' (N=24, $P=7.10x10^{-10}$).

STRING-based analysis of PPI. PPI analysis of the 602 genes was performed using STRING software. The 602 genes were found to be significantly enriched using PPI analysis, whereby 4 patterns with strong interactions were generated, with pattern 1 containing 109 genes (PPI enrichment $P<1.0x10^{-12}$; Fig. 3), pattern 2 producing 69 genes (PPI enrichment $P<1.0x10^{-8}$; Fig. S1), pattern 3 producing 73 genes (PPI enrichment $P<1.0x10^{-6}$; Fig. S2) and pattern 4 producing 59 genes (PPI enrichment $P<1.0x10^{-5}$; Fig. S3). These data suggested that the genes in these patterns formed a tight interaction network.

Candidate genes are associated with prognosis in patients with colon cancer. After STRING PPI analysis, all the aforementioned candidate genes (N=310) were searched using the GEPIA database to identify the genes that were significantly associated with survival time in patients with colon cancer. The results demonstrated that 14 of 310 prognosis-related genes were highly expressed, and a high expression level of *ATM*, *CDH2*, *CDKN2A*, *EPO*, *LEP*, *TGFB1*, *TIMP1*, *PGR*, *VEGFC*, *POSTN*, *BCL6*, *CYP19A1*, *NOTCH3* and *XPA* was significantly associated with poor prognosis in patients with colon cancer (Fig. 4).

Gene expression in clinical samples. To further confirm the biological functions of these genes (*ATM*, *CDH2*, *CDKN2A*, *EPO*, *LEP*, *TGFB1*, *TIMP1*, *PGR*, *VEGFC*, *POSTN*, *BCL6*, *CYP19A1*, *NOTCH3* and *XPA*), experiments were performed to verify their expression level in colon cancer. A total of

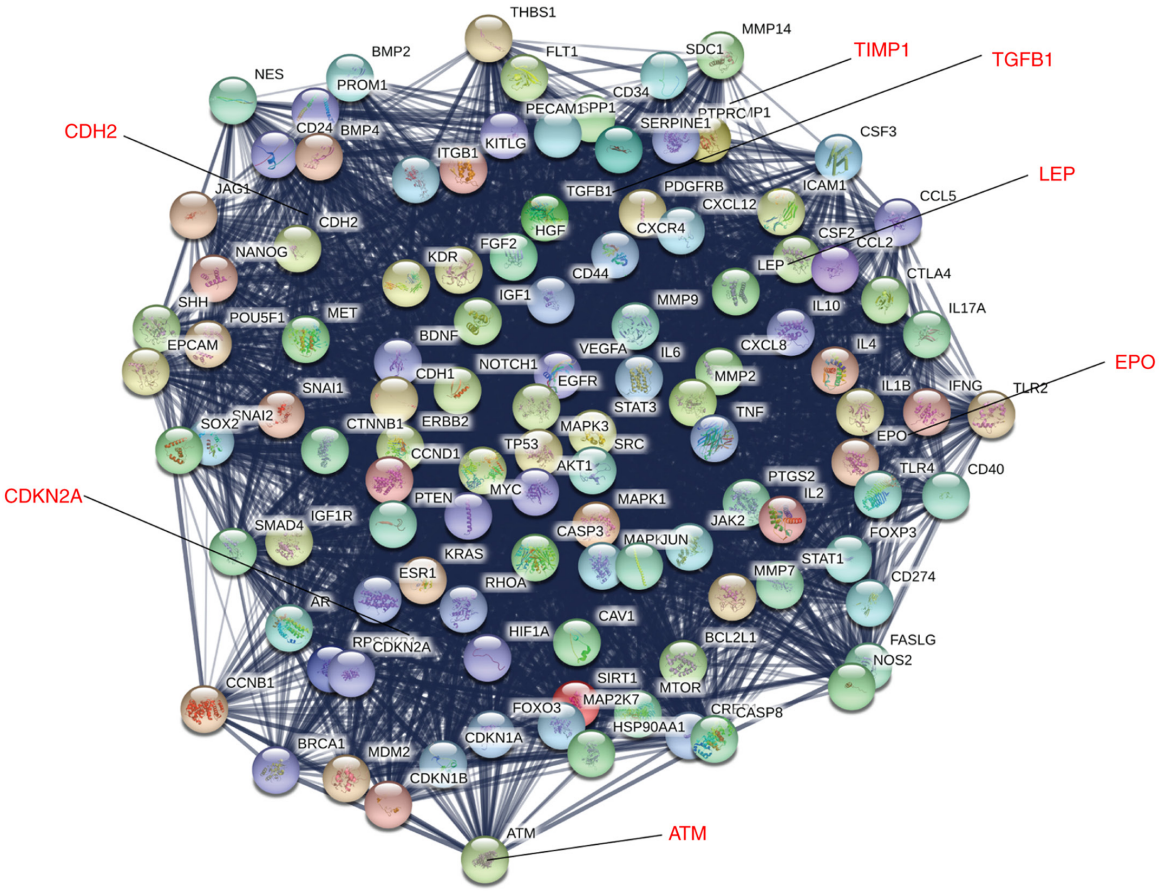

Figure 3. PPI network of the significantly enriched genes. PPI analysis of all the significantly enriched genes (PPI enrichment P-value: $<1.0x10^{-12}$), with the interactions with high confidence scores presented as nodes (90% confidence intervals). The genes selected for subsequent experiments are marked in red. PPI, protein-protein interaction.

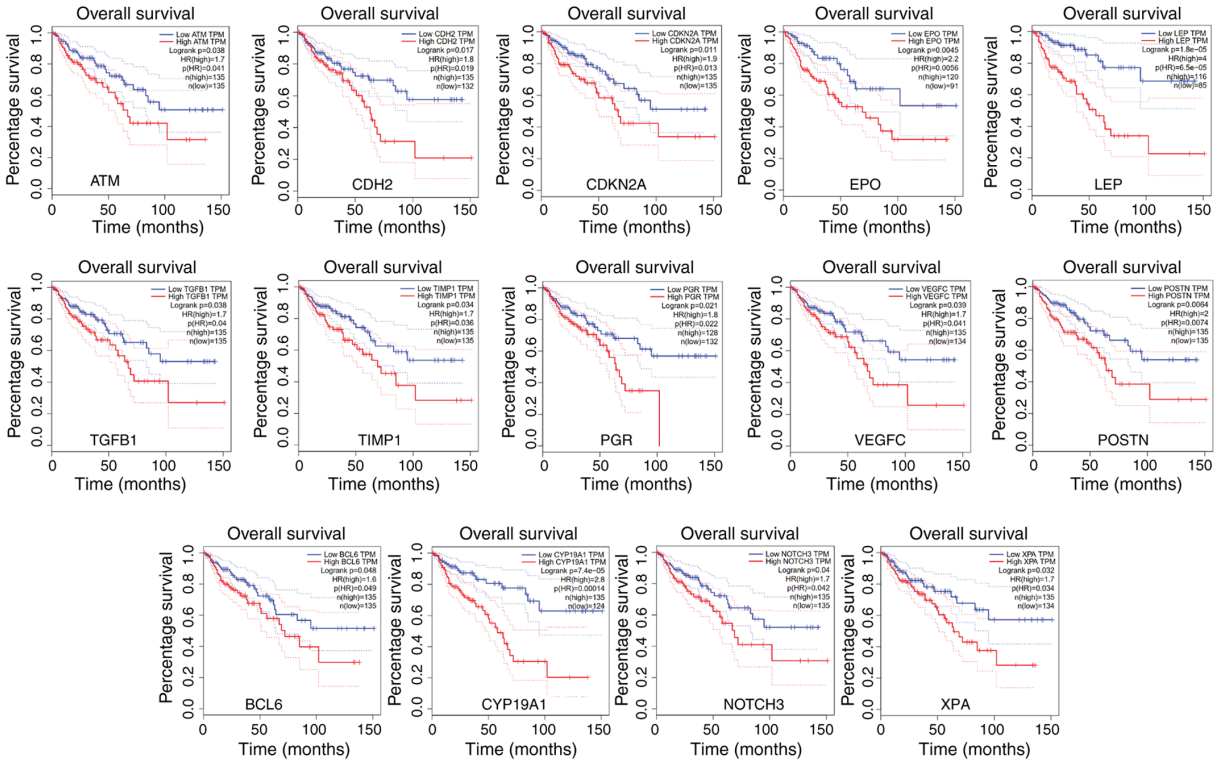

Figure 4. Overall survival analysis was performed using the GEPIA database. The survival time of patients with colon cancer and high expression level of *ATM*, *CDH2*, *CDKN2A*, *EPO*, *LEP*, *TGFB1*, *TIMP1*, *PGR*, *VEGFC*, *POSTN*, *BCL6*, *CYP19A1*, *NOTCH3* and *XPA* was significantly reduced using data from the GEPIA database. GEPIA, Gene Expression Profiling Interactive Analysis

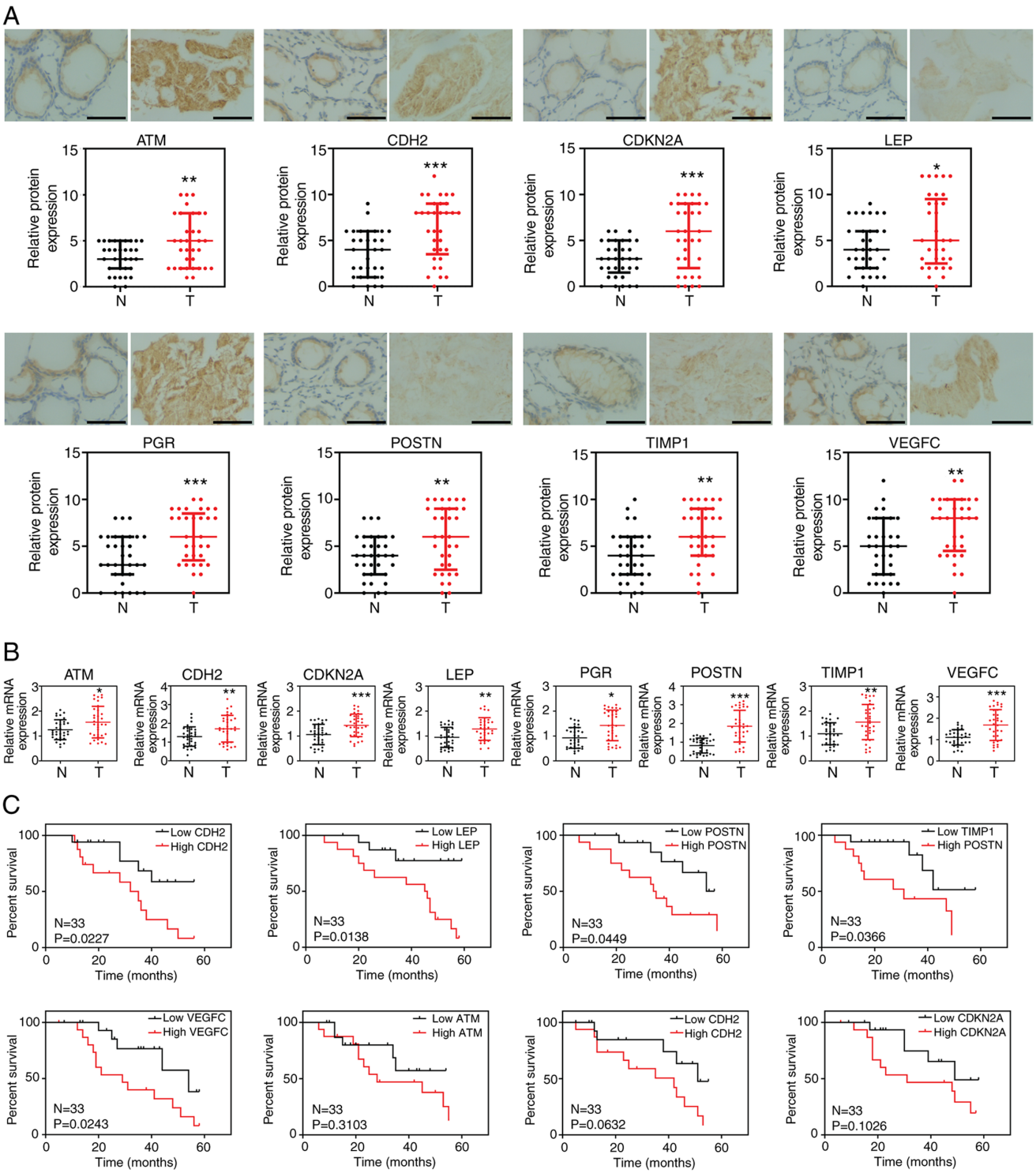

Figure 5. Validation of protein and gene expression level in human colon cancer clinical samples. (A) Representative images and statistical analysis of the expression of the target genes using IHC in 33 paired colon cancer samples. Scale bar, 200 μm. The data was analyzed using a Wilcoxon signed-rank test and expressed as the median ± interquartile range. (B) The mRNA expression levels of target genes were detected using reverse transcription-quantitative PCR in 33 paired colon cancer tissues. The data was analyzed using a paired Student's t-test and expressed as mean ± SD. (C) Survival analysis between the expression level of target genes and patient survival time in 33 paired colon cancer tissues. Each assay was performed independently from 3 repeats. *P<0.05. **P<0.01. ***P<0.001. N, normal adjacent tissue; T, tumor tissue; IHC, immunohistochemistry.

33 tumor and adjacent normal tissues were collected from patients with colon cancer and the expression levels were analyzed using IHC and RT-qPCR. The results confirmed that the protein and mRNA expression of *ATM*, *CDH2*, *CDKN2A*, *LEP*, *PGR*, *TIMP1*, *POSTN* and *VEGFC* were significantly increased in colon cancer tissues compared with that in the normal adjacent tissues (Fig. 5A and B), while there were no significant differences in the expression level of *BCL6*, *EPO*, *CYP19A1*, *TGFB1*, *NOTCH3* and *XPA* (Fig. S4). Notably, the high expression level of *CDH2*, *LEP*, *POSTN*, *TIMP1* and *VEGFC* in colon cancer tissues was significantly associated with poor prognosis in patients with colon cancer. However,

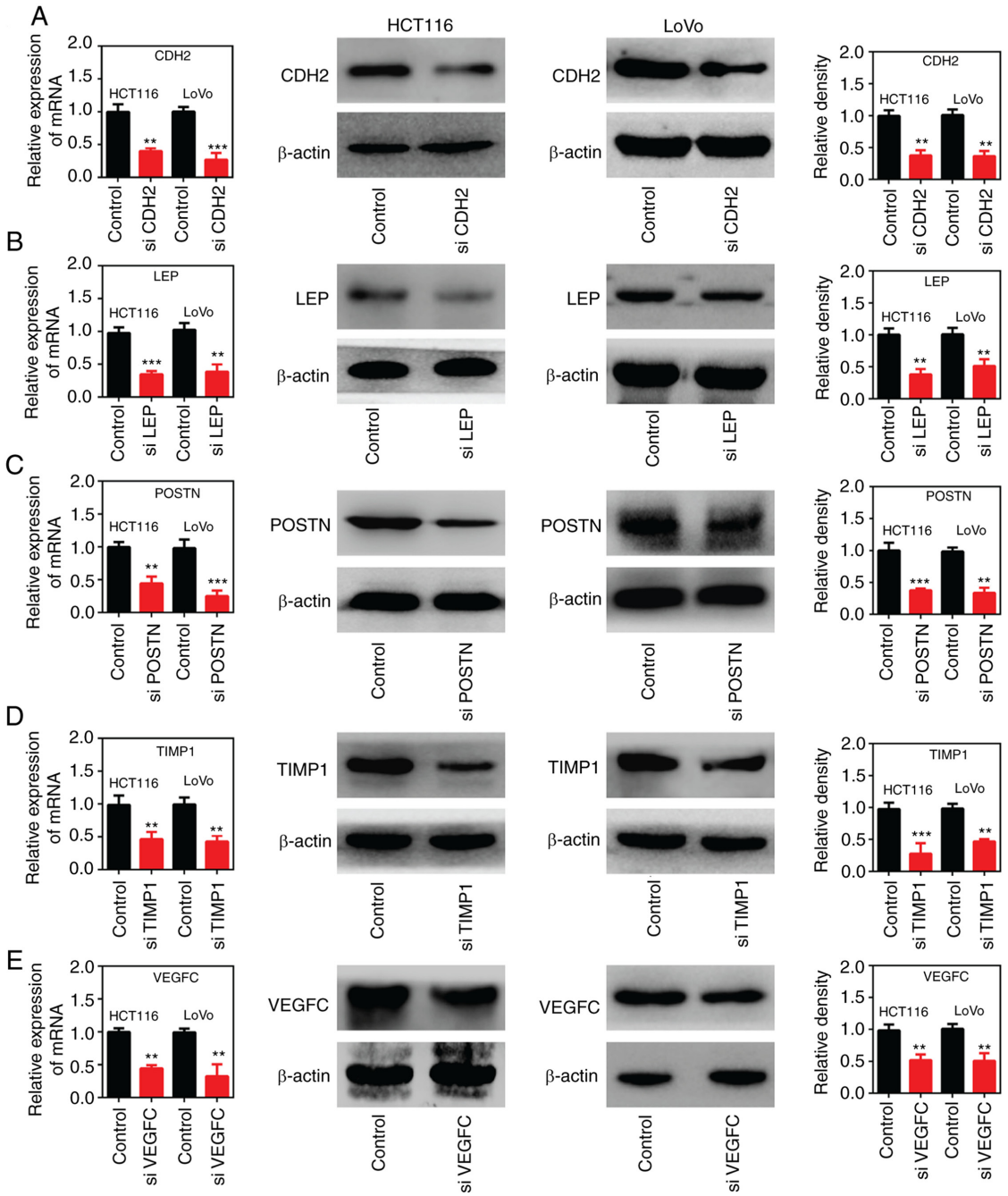

Figure 6. Protein and mRNA expression levels of *CDH2*, *LEP*, *POSTN*, *TIMP1* and *VEGFC* in colon cancer cells following transfection with target siRNAs. After silencing of (A) *CDH2*, (B) *LEP*, (C) *POSTN*, (D) *TIMP1* and (E) *VEGFC* in the colon cancer cells using siRNAs, the mRNA and protein expression levels were detected using reverse transcription-quantitative PCR and western blot analysis. The data was analyzed using an unpaired Student's t-test and expressed as the mean ± SD. Each assay was performed in independently 3 times. **P<0.01. ***P<0.001. si, small interfering.

no association was found between *ATM*, *PGR* or *CDKN2A* and patient survival (Fig. 5C).

Expression levels of CDH2, LEP, POSTN, TIMP1 and VEGFC genes were significantly decreased in colon cancer cells following transfection with siRNA. In the present study, siRNA sequences were designed to target *CDH2*, *LEP*, *POSTN*, *TIMP1* and *VEGFC* mRNAs. Subsequently, the cell lines, HCT116 and LoVo were transfected with the different siRNAs and the expression levels were evaluated using RT-qPCR and western blot analysis. The results revealed that the expression levels of these genes were significantly decreased following transfection with the different siRNAs compared with that in the cells transfected with siNC (Fig. 6).

Expression levels of CDH2, LEP, POSTN, TIMP1 and VEGFC are associated with the sensitivity of colon cancer cells to 5-FU resistance. Following silencing of *CDH2*, *LEP*, *POSTN*, *TIMP1* and *VEGFC*, the sensitivity of the colon cancer cells to 5-FU was determined using a CCK-8 assay. The results revealed that *CDH2*, *LEP*, *POSTN*, *TIMP1* and *VEGFC* knockdown in colon cancer cells significantly enhanced their sensitivity to 5-FU. Furthermore, following treatment with different concentrations of 5-FU, cell viability and the IC_{50} values were significantly

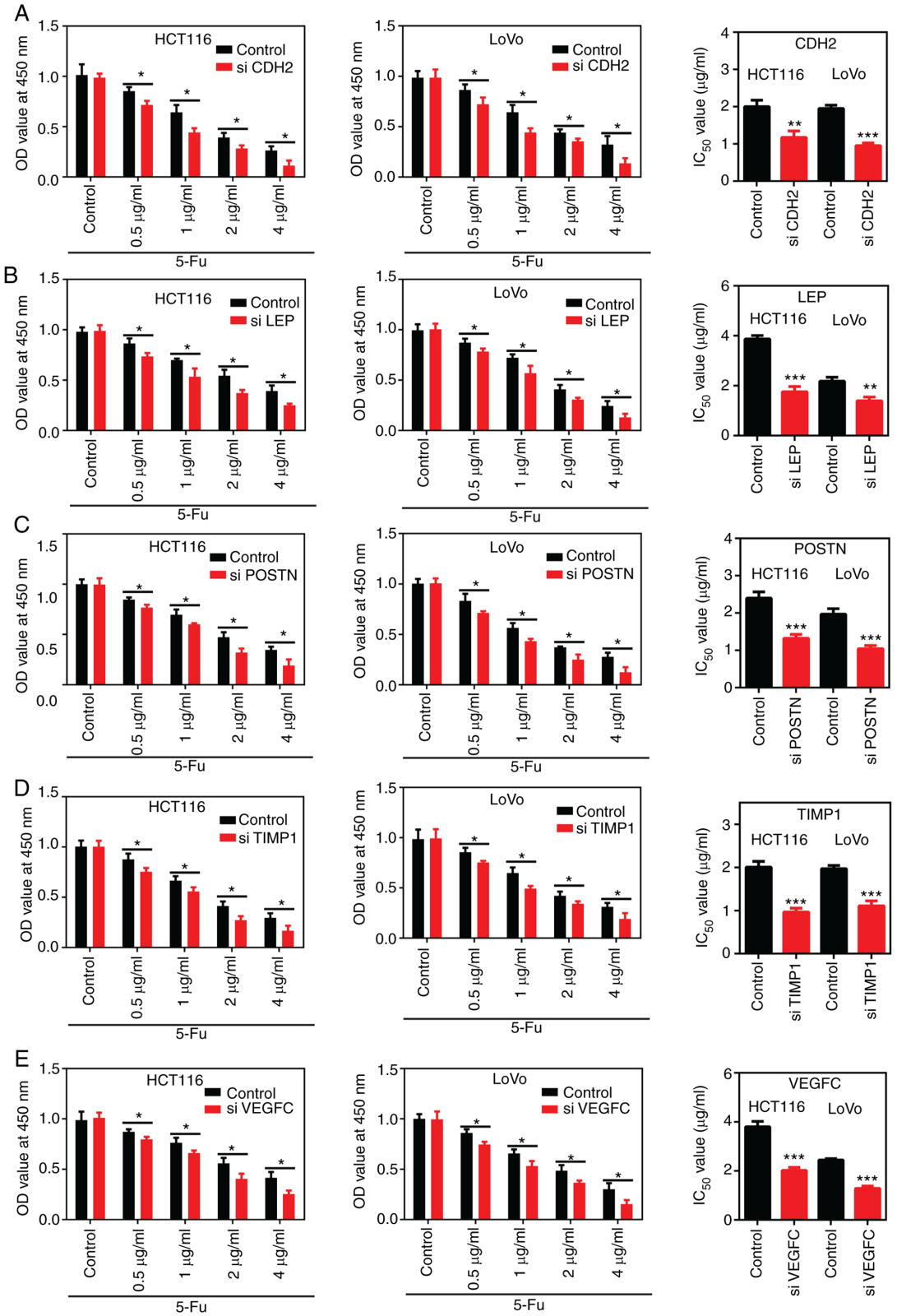

Figure 7. Silencing of *CDH2*, *LEP*, *POSTN*, *TIMP1* and *VEGFC* enhanced the chemosensitivity of the colon cancer cells. After different concentrations (0.5, 1, 2 and 4 μg/ml) of 5-FU treatment, cell viability and the IC_{50} values of 5-FU were monitored using a Cell Counting Kit-8 assay in the HCT116 and LoVo cells following silencing of (A) *CDH2*, (B) *LEP*, (C) *POSTN*, (D) *TIMP1* and (E) *VEGFC*. The data was analyzed using an unpaired Student's t-test and expressed as the mean ± SD. Each assay was performed independently 3 times. *P<0.05; **P<0.01; ***P<0.001. OD, optical density; si, small interfering; 5-FU, 5-fluorouracil.

decreased in gene-silenced colon cancer cells compared with that in the cells transfected with siNC (Fig. 7).

In addition, following silencing of *CDH2*, *LEP*, *POSTN*, *TIMP1* and *VEGFC* using siRNA in the HCT116 or LoVo cells and treatment with 5-FU (1 μg/ml) for 24 h, there was a significant increase in the number of PI-positive cells compared with that in cells transfected with siNC (Fig. 8). Taken together, these results indicated that siRNA-mediated silencing of

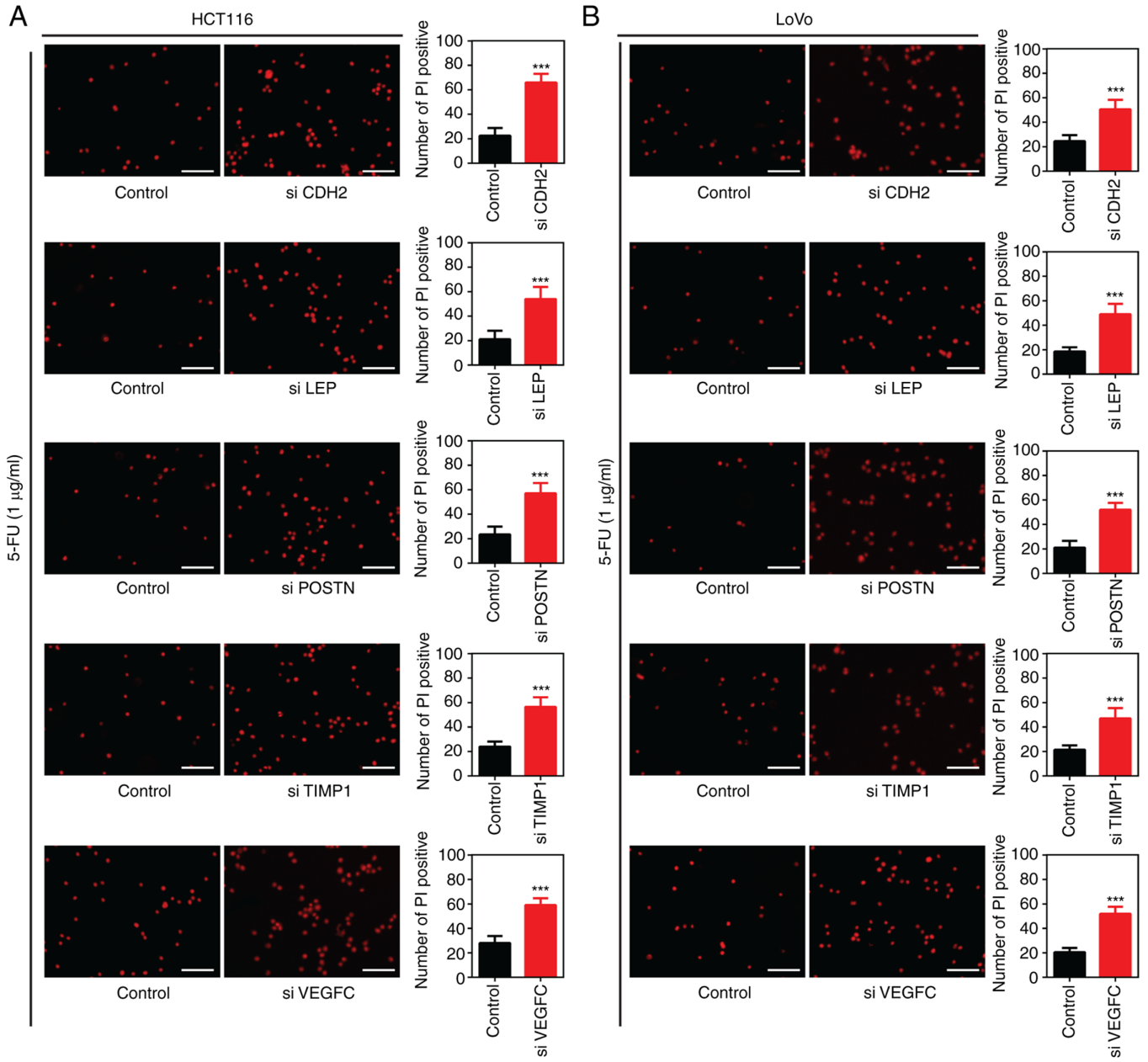

Figure 8. siRNA-mediated silencing of *CDH2*, *LEP*, *POSTN*, *TIMP1* and *VEGFC* increased the proportion of PI-positive colon cancer cells. After 5-FU treatment (1 μg/ml) and silencing of *CDH2*, *LEP*, *POSTN*, *TIMP1* or *VEGFC*, PI-positive (A) HCT116 and (B) LoVo cells were measured. The data was analyzed using an unpaired Student's t-test and expressed as the mean ± SD. Each assay was performed independent 3 times. ***P<0.001. si, small interfering; 5-FU, 5-fluorouracil.

CDH2, *LEP*, *POSTN*, *TIMP1* and *VEGFC* enhanced the sensitivity of the colon cancer cell lines to 5-FU.

Discussion

In present study, an online public database was searched, using the terms 'cancer recurrence' and 'chemoresistance' and two gene sets were obtained, which included 602 shared genes. Following GO analysis, the 602 genes were mainly associated with cell death, cell proliferation and apoptosis, which are believed to play important roles in the development of drug resistance (14). Besides, KEGG pathway enrichment analyses showed the top two pathways, of which 'PI3K-Akt signaling pathway', 'FoxO signaling pathway', 'HIF-1 signaling pathway', 'p53 signaling pathway' and 'TNF signaling pathway' have been reported in previous studies to be significantly associated with drug resistance in tumors (20-25). STRING PPI analysis was subsequently performed and a total of 4 patterns with strong interactions were generated. Furthermore, the candidate genes associated with prognosis in patients with colon cancer were analyzed using the GEPIA database and the highly expressed genes, *ATM*, *CDH2*, *CDKN2A*, *EPO*, *LEP*, *TGFB1*, *TIMP1*, *PGR*, *VEGFC*, *POSTN*, *BCL6*, *CYP19A1*, *NOTCH3* and *XPA* were associated with poor prognosis in patients with colon cancer. To confirm that the genes identified were associated with chemotherapeutic resistance in colon cancer cell lines, siRNAs were transfected into the cells, and cell viability and apoptosis was analyzed following treatment with 5-FU. The results showed that *CDH2*, *LEP*, *POSTN*, *TIMP1* and *VEGFC* were significantly increased in the human

colon cancer cells and that their post-transcriptional silencing enhanced the sensitivity of colon cancer cells to 5-FU.

Cancer drug resistance severely limits the effectiveness of chemotherapy in patients with cancer and has been shown to be an important cause of treatment failure and tumor recurrence in a large cohort of patients (8). Colon cancer is also one of the most likely tumors to develop chemotherapeutic resistance in clinical practice (26). There have been numerous studies on chemotherapeutic resistance in colon cancer. It is currently hypothesized that the expression of genes, such as *ABCB1* (27,28), *ATR* (29) and *ATM* (30) promoted the development of cancer drug resistance in tumors by enhancing the efflux of chemotherapeutic drugs or increasing the level of DNA damage repair. No effective drug candidates, which overcome drug resistance in colon cancer, have been identified so far; however, understanding the mechanisms by which drug resistance occurs will benefit the development of potential therapeutic agents. In the present study, text mining strategies based on public databases provided a useful tool to further understand the mechanisms underlying drug resistance in colon cancer.

CDH2, also known as N-cadherin (31), was increased in epithelial cells during carcinogenesis and is a marker of epidermal-mesenchymal transformation (32). Investigation in colon cancer cells has suggested that *CDH2* mRNA expression was associated with drug resistance in colon cancer (33).

VEGFC belongs to the vascular endothelial growth factor family, which is highly expressed in oxaliplatin-resistant colorectal cancer cells compared with that in the parental cells (34). In addition, *LEP*, *TIMP1* and *POSTN* have been newly discovered to be associated with drug resistance in the present study. *LEP* plays an important role in regulating metabolism (35). A previous study in triple-negative breast cancer (TNBC) cells has shown that leptin signaling increased the mRNA expression of chemoresistance-related genes, including *ABCB1*, which contributed to chemotherapy failure, whereas inhibition of the leptin receptor re-sensitized the TNBC cells to chemotherapeutics (36).

TIMP1, also known as tissue inhibitor of metalloproteinases-1, is a multifunctional protein that promotes cell proliferation and exhibits anti-apoptotic functions (37,38). A recent study reported that TIMP1 knockdown using short hairpin RNA in gemcitabine (GEM)-resistant pancreatic ductal adenocarcinoma cells enhanced GEM sensitivity and reversed chemoresistance by inducing cell apoptosis (39). *POSTN* expresses extracellular matrix periostin, which is involved in the activation of the PI3K/Akt signaling pathway (40,41) and was found to be an independent negative prognostic factor in non-small cell lung carcinoma (42). In addition, *POSTN* protein expression has been confirmed to be positively associated with cancer drug resistance (43). The present study has identified the genes associated with drug resistance in colon cancer; however, the specific mechanisms by which these candidate genes exert their biological effects on drug resistance require further investigation. Furthermore, a limitation of the current study is that the association between the candidate genes, chemotherapy drugs and prognosis of patients is not definite.

In summary, identification of genes associated with cancer drug resistance was achieved using bioinformatics tools, which were validated using functional experiments. The identified genes included *CDH2*, *LEP*, *POSTN*, *TIMP1* and *VEGFC*, and therapeutic targeting of these genes may have considerable clinical benefits in overcoming chemotherapeutic resistance.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WL and RX conceived the study. TL, RX, CL, XCh and XCa performed the experiments, data analysis and prepared the first draft of the manuscript. WL supervised the study and revised the manuscript. WL and RX confirm the authenticity of all the raw data. All authors approved the final version and agreed to publish the manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Xiang'an Hospital of Xiamen University. All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that there have no competing interests.

References

1. Holohan C, Van Schaeybroeck S, Longley DB and Johnston PG: Cancer drug resistance: An evolving paradigm. Nat Rev Cancer 13: 714-726, 2013.
2. Vasan N, Baselga J and Hyman DM: A view on drug resistance in cancer. Nature 575: 299-309, 2019.
3. Zhu H, Luo H, Zhang W, Shen Z, Hu X and Zhu X: Molecular mechanisms of cisplatin resistance in cervical cancer. Drug Des Devel Ther 10: 1885-1895, 2016.
4. Nikolaou M, Pavlopoulou A, Georgakilas AG and Kyrodimos E: The challenge of drug resistance in cancer treatment: A current overview. Clin Exp Metastasis 35: 309-318, 2018.
5. Panda M and Biswal BK: Cell signaling and cancer: A mechanistic insight into drug resistance. Mol Biol Rep 46: 5645-5659, 2019.
6. Salgia R and Kulkarni P: The genetic/non-genetic duality of drug 'resistance' in cancer. Trends Cancer 4: 110-118, 2018.
7. Kartal-Yandim M, Adan-Gokbulut A and Baran Y: Molecular mechanisms of drug resistance and its reversal in cancer. Crit Rev Biotechnol 36: 716-726, 2016.
8. Chatterjee N and Bivona TG: Polytherapy and Targeted Cancer Drug Resistance. Trends Cancer 5: 170-182, 2019.
9. Hirose M, Hosoi E, Hamano S and Jalili A: Multidrug resistance in hematological malignancy. J Med Invest 50: 126-135, 2003.

10. Hackl H, Astanina K and Wieser R: Molecular and genetic alterations associated with therapy resistance and relapse of acute myeloid leukemia. J Hematol Oncol 10: 51, 2017.
11. Waghray D and Zhang Q: Inhibit or evade multidrug resistance P-glycoprotein in cancer treatment. J Med Chem 61: 5108-5121, 2018.
12. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F: Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136: E359-E386, 2015.
13. Wu C: Systemic therapy for colon cancer. Surg Oncol Clin N Am 27: 235-242, 2018.
14. Hu T, Li Z, Gao CY and Cho CH: Mechanisms of drug resistance in colon cancer and its therapeutic strategies. World J Gastroenterol 22: 6876-6889, 2016.
15. Han B, Feng D, Yu X, Zhang Y, Liu Y and Zhou L: Identification and interaction analysis of molecular markers in colorectal cancer by integrated bioinformatics analysis. Med Sci Monit 24: 6059-6069, 2018.
16. Irham LM, Wong HS, Chou WH, Adikusuma W, Mugiyanto E, Huang WC and Chang WC: Integration of genetic variants and gene network for drug repurposing in colorectal cancer. Pharmacol Res 161: 105203, 2020.
17. Zhang X, Zhang H, Shen B and Sun XF: Novel MicroRNA biomarkers for colorectal cancer early diagnosis and 5-fluorouracil chemotherapy resistance but not prognosis: A atudy from databases to AI-assisted verifications. Cancers (Basel) 12: E341, 2020.
18. Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL and Trotti A (eds): AJCC Cancer Staging Manual. 7th edition. Springer, New York, NY, 2010.
19. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
20. Li Q, Lai Z, Yan Z, Peng J, Jin Y, Wei L and Lin J: Hedyotis diffusa Willd inhibits proliferation and induces apoptosis of 5 FU resistant colorectal cancer cells by regulating the PI3K/AKT signaling pathway. Mol Med Rep 17: 358-365, 2018.
21. Li Q, Wei L, Lin S, Chen Y, Lin J and Peng J: Synergistic effect of kaempferol and 5 fluorouracil on the growth of colorectal cancer cells by regulating the PI3K/Akt signaling pathway. Mol Med Rep 20: 728-734, 2019.
22. Fallah J and Rini BI: HIF Inhibitors: Status of current clinical Development. Curr Oncol Rep 21: 6, 2019.
23. Feng X, Liu H, Zhang Z, Gu Y, Qiu H and He Z: Annexin A2 contributes to cisplatin resistance by activation of JNK-p53 pathway in non-small cell lung cancer cells. J Exp Clin Cancer Res 36: 123, 2017.
24. Zhao F and Lam EW: Role of the forkhead transcription factor FOXO-FOXM1 axis in cancer and drug resistance. Front Med 6: 376-380, 2012.
25. Lai M, Liu G, Li R, Bai H, Zhao J, Xiao P and Mei J: Hsa_circ_0079662 induces the resistance mechanism of the chemotherapy drug oxaliplatin through the TNF-α pathway in human colon cancer. J Cell Mol Med 24: 5021-5027, 2020.
26. Kozovska Z, Gabrisova V and Kucerova L: Colon cancer: cancer stem cells markers, drug resistance and treatment. Biomed Pharmacother 68: 911-916, 2014.
27. Yuan Z, Shi X, Qiu Y, Jia T, Yuan X, Zou Y, Liu C, Yu H, Yuan Y, He X, *et al*: Reversal of P-gp-mediated multidrug resistance in colon cancer by cinobufagin. Oncol Rep 37: 1815-1825, 2017.
28. Hu T, To KK, Wang L, Zhang L, Lu L, Shen J, Chan RL, Li M, Yeung JH and Cho CH: Reversal of P-glycoprotein (P-gp) mediated multidrug resistance in colon cancer cells by cryptotanshinone and dihydrotanshinone of *Salvia miltiorrhiza*. Phytomedicine 21: 1264-1272, 2014.
29. Abu-Sanad A, Wang Y, Hasheminasab F, Panasci J, Noë A, Rosca L, Davidson D, Amrein L, Sharif-Askari B, Aloyz R, *et al*: Simultaneous inhibition of ATR and PARP sensitizes colon cancer cell lines to irinotecan. Front Pharmacol 6: 147, 2015.
30. Zhou Y, Wan G, Spizzo R, Ivan C, Mathur R, Hu X, Ye X, Lu J, Fan F, Xia L, *et al*: miR-203 induces oxaliplatin resistance in colorectal cancer cells by negatively regulating ATM kinase. Mol Oncol 8: 83-92, 2014.
31. Alimperti S and Andreadis ST: CDH2 and CDH11 act as regulators of stem cell fate decisions. Stem Cell Res (Amst) 14: 270-282, 2015.
32. Loh CY, Chai JY, Tang TF, Wong WF, Sethi G, Shanmugam MK, Chong PP and Looi CY: The E-cadherin and N-cadherin switch in epithelial-to-mesenchymal transition: Signaling, therapeutic implications, and challenges. Cells 8: E1118, 2019.
33. Serova M, Astorgues-Xerri L, Bieche I, Albert S, Vidaud M, Benhadji KA, Emami S, Vidaud D, Hammel P, Theou-Anton N, *et al*: Epithelial-to-mesenchymal transition and oncogenic Ras expression in resistance to the protein kinase Cbeta inhibitor enzastaurin in colon cancer cells. Mol Cancer Ther 9: 1308-1317, 2010.
34. Tomida C, Yamagishi N, Nagano H, Uchida T, Ohno A, Hirasaka K, Nikawa T and Teshima-Kondo S: VEGF pathway-targeting drugs induce evasive adaptation by activation of neuropilin-1/cMet in colon cancer cells. Int J Oncol 52: 1350-1362, 2018.
35. Zhang Y and Chua S Jr: Leptin function and regulation. Compr Physiol 8: 351-369, 2017.
36. Lipsey CC, Harbuzariu A, Robey RW, Huff LM, Gottesman MM and Gonzalez-Perez RR: Leptin signaling affects survival and chemoresistance of estrogen receptor negative breast cancer. Int J Mol Sci 21: E3794, 2020.
37. Lao G, Ren M, Wang X, Zhang J, Huang Y, Liu D, Luo H, Yang C and Yan L: Human tissue inhibitor of metalloproteinases-1 improved wound healing in diabetes through its anti-apoptotic effect. Exp Dermatol 28: 528-535, 2019.
38. Lee JH, Choi JW and Kim YS: Serum TIMP-1 predicts survival outcomes of invasive breast carcinoma patients: A meta-analysis. Arch Med Res 42: 463-468, 2011.
39. Tan Y, Li X, Tian Z, Chen S, Zou J, Lian G, Chen S, Huang K and Chen Y: TIMP1 down-regulation enhances gemcitabine sensitivity and reverses chemoresistance in pancreatic cancer. Biochem Pharmacol 189: 114085, 2021.
40. Liu C, Feng X, Wang B, Wang X, Wang C, Yu M, Cao G and Wang H: Bone marrow mesenchymal stem cells promote head and neck cancer progression through Periostin-mediated phosphoinositide 3-kinase/Akt/mammalian target of rapamycin. Cancer Sci 109: 688-698, 2018.
41. Xiao ZM, Wang XY and Wang AM: Periostin induces chemoresistance in colon cancer cells through activation of the PI3K/Akt/survivin pathway. Biotechnol Appl Biochem 62: 401-406, 2015.
42. Ratajczak-Wielgomas K, Kmiecik A, Grzegrzołka J, Piotrowska A, Gomulkiewicz A, Partynska A, Pawelczyk K, Nowinska K, Podhorska-Okolow M and Dziegiel P: Prognostic significance of stromal periostin expression in non-small cell lung cancer. Int J Mol Sci 21: E7025, 2020.
43. Park SY, Piao Y, Jeong KJ, Dong J and de Groot JF: Periostin (POSTN) regulates tumor resistance to antiangiogenic therapy in glioma models. Mol Cancer Ther 15: 2187-2197, 2016.