

Epithelial-mesenchymal transition and cancer stem cell-like phenotype induced by Twist1 contribute to acquired resistance to irinotecan in colon cancer

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Abstract. Inherent and acquired chemoresistance reduce the effectiveness of irinotecan in the treatment of metastatic colorectal cancer (CRC). However, the molecular mechanisms underlying this resistance process are still unclear. Twist1 is one of the master transcription factors of epithelial-mesenchymal transition (EMT). Our previous study indicated that Twist1 is overexpressed in colon cancer tissues, and demonstrated that Twist1 plays a crucial role in the chemoresistance of CRC. In the present study, we further investigated how Twist1 contribute to acquired resistance to irinotecan in colon cancer. The irinotecan-resistant cells were established by gradual adaptation of increasing irinotecan concentrations in LoVo cells, named LoVo/CPT-11R cells. Results showed that cell viabilities to different anticancer drugs were markedly increased in LoVo/CPT-11R cells compared to LoVo cells. Moreover, LoVo/CPT-11R cells displayed EMT, CSC-like

cellular morphology and relative biomarkers were also significantly increased. In addition, overexpressed Twist1 LoVo cells were established by lentivirus transfection assay, named LoVo/Twist1 cells. Results showed that the LoVo/Twist1 cells perform a distinctly decreased sensitivity to irinotecan, downregulated expression of E-cadherin, upregulated expression of cluster of differentiation 44 (CD44), and a significant enhancement of invasion and migration potential by regulation of MMP2 compared with control cells. In contrast, the inhibition of Twist1 transfected with siRNA could enhance the irinotecan sensitivity in LoVo/CPT-11R cells and down-regulate the expression of vimentin and CD44. Our data provide evidence that EMT and CSC-like phenotype induced by Twist1 contribute to acquire resistance to irinotecan and enhanced migration and invasion in colon cancer.

Introduction

Colorectal cancer (CRC), including colon and rectal cancer, is the third most commonly diagnosed malignancy and the fourth leading cause of cancer-related deaths all over the world. Rapid increases in both CRC incidence and mortality are now observed with the rapid social and economic changes in Eastern Europe, Asia and South America (1). Although improved treatment strategies involving surgery and chemo- and radio-therapy have increased the overall survival rates in the early stages, 40-50% of all patients with CRC present with metastasis either at the time of diagnosis or as recurrent disease upon intended curative therapy (2). Currently, irinotecan (CPT-11) is mainly used in CRC diagnosed patients with metastases, with recorded relapse or progression after application of standard 5-FU based therapy (3,4). However, the response rate to these regimens is only in the range of ~30-55% and the 5-year survival rate is less than 10%. Resistance to chemotherapy is a major limitation to the outcome in CRC (5,6). Irinotecan, a semi-synthetic analog of camptothecin, is a broad-spectrum anticancer drug that specifically target DNA topoisomerase I (Topo I). In humans,

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Abbreviations: CRC, colorectal cancer; EMT, epithelial-mesenchymal transition; CSC, cancer stem cell; CD, cluster of differentiation; MMP, metalloproteinase

Key words: colon cancer, irinotecan, resistance, epithelial-mesenchymal transition, cancer stem cell, Twist1

irinotecan is metabolized by an endogenous carboxylesterase-mediated hydrolyzation into a highly active metabolite, SN-38. The formation of SN-38-Topo I-DNA complex results in lethal double-strand DNA breakage and cell death (7,8). Numerous studies have been done to uncover possible mechanisms for the cellular resistance to this agent, suggesting the following general aspects: i) variable levels of the enzymes involved in the conversion of irinotecan; ii) reduced cellular accumulation from active drug efflux caused by ABC transporters; iii) changed activity of Topo I that decreases levels of the SN-38-TopoI-DNA complex; and iv) alterations in the events downstream from the ternary complex, for example, apoptosis, cell cycle regulation, checkpoints and DNA repair (3,7,9). However, research on the mechanism underlying the resistance to irinotecan is still limited and needs further investigation.

Epithelial-mesenchymal transition (EMT) is a unique process initially characterized in embryonic development in which cells lose epithelial features and gain mesenchymal properties. EMT results in epithelial cells becoming spindle shaped, with loss of cellular polarity similar to mesenchymal cells. These phenotypic changes closely correlate with increased cellular motility, invasion and therapeutic resistance (2,10). Furthermore, cells with different molecular characteristics within the same tumor respond differently to anticancer therapeutics, leading to drug resistance. Cancer cells may also undergo adaptive changes following therapy, exacerbating drug resistance. In epithelial cancers, these adaptive changes may involve, at least in part, EMT and the reverse process (MET) (11). Notably, these properties have also been ascribed to normal stem cells and cancer stem cells. The cancer stem cells (CSC) are specific undifferentiated cancer cells, the tumor-initiating cells, have the ability to self-renew, propagate and differentiate leading to cancer growth and progression (12). Likewise, CSCs display aggressive characteristics including increased invasion, metastatic ability and resistance to therapy and predict poor patient prognosis. In experimental models, CSCs are more resistant than differentiated tumor cells to chemo- and radiotherapy, and they can escape from the effects of conventional cytotoxic treatments (13).

Twist is a basic helix-loop-helix transcription factor, which is one of the master regulators of EMT process, including Twist1 and Twist2 (14). Our previous study indicated that Twist1 is overexpressed in colon cancer tissue, its positive expression is related to histological grade, TNM stage, recurrence and poor overall survival and is a significant independent prognostic indicator in CRC patients (15). Different studies have also reported that high expression of Twist1 is closely associated with more aggressive behavior of breast (16), hepatocellular (17), pancreatic (18) and esophageal squamous cell cancer (19). In addition, Twist is critical for the maintenance of EMT associated CSC-like characteristics (20,21). Moreover, Twist1 is also responsible for paclitaxel resistance in breast cancer cells (16), cisplatin resistance in lung cancer cells (22) and 5-FU resistance in colon cancer cells (23). However, the relationship Twist1, CSC and resistance to irinotecan in colon cancer is still ambiguous. A better understanding of these underlying resistance mechanisms is a major concern for the future development of new Topo I inhibitors and the identification of biomarkers that could be used to predict tumor response to these drugs clinically.

Materials and methods

Cell culture and establishment of irinotecan-resistant LoVo cell subline. Colon cancer LoVo cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The LoVo cells were cultured as monolayers in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, São Paulo, Brazil) and 1% penicillin/streptomycin (Beijing Solarbio Science and Technology, Co. Ltd., China). Cells were grown at 37°C in a humidified atmosphere containing 5% CO₂. Irinotecan-resistant LoVo cell subline, referred to as LoVo/CPT-11R cells, were established by gradual adaptation of the original cell line to increasing irinotecan concentrations over a period of 10 months. The concentrations increased as follows: 1 µg/ml → 3 µg/ml → 5 µg/ml → 10 µg/ml → 20 µg/ml → 30 µg/ml → 40 µg/ml → 50 µg/ml → 60 µg/ml → 70 µg/ml. Cells were maintained at a particular irinotecan concentration for ~10 passages or until they displayed, more or less, standard growth and survival after subculture. Cells were passaged twice a week if appropriate. Otherwise, the medium was changed twice a week until the cell culture reached conditions allowing passaging. Prior to the subsequent experiments, the cells were maintained in drug-free growth medium for at least 3 weeks.

Reagents and antibodies. Irinotecan was purchased from Jiangsu Hengrui Medicine, Co., Ltd. (Jiangsu, China), 5-fluorouracil, and cisplatin were purchased from Sigma-Aldrich (St. Louis, MO, USA), dissolved in phosphate-buffered saline (PBS), curcumin (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich), aliquoted and stored at 4°C according to the manufacturer's instructions. EMT antibody sampler kit was purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal antibody against Twist1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against respectively β-actin, MMP2, MMP9, CD44 and CD133 were purchased from Proteintech Group, Inc. (Rosemont, IL, USA). Rabbit polyclonal antibody against P-gp and the secondary antibodies, goat anti-rabbit IgG or goat anti-mouse IgG, were purchased from Abcam (Cambridge, MA, USA).

Cell viability assay. Cell viability assay was performed using the Cell Counting kit-8 assay (CCK-8; Dojindo Laboratories, Kumamoto, Japan). Briefly, cells (8×10³/well) were seeded onto a 96-well plate in 100 µl RPMI-1640 medium supplemented with 10% FBS at 37°C in 5% CO₂. After 24 h, drugs diluted with the culture medium were added to each well. The concentrations of the drug (irinotecan, 5-fluorouracil, cisplatin or curcumin) were 5, 10, 20 and 40 µg/ml, respectively. Following drug treatment for 24 h, the culture medium in the 96-wells was replaced with fresh medium containing 10% CCK-8 reagent, after 2 h, the absorbance at 450 nm was measured and the values were corrected by subtracting the absorbance of blank wells that did not contain cells.

Cellular morphology and immunofluorescence assay. Cells grown on coverslips were washed three times with PBS. They were fixed for 10 min with cold paraformaldehyde and washed

3 times with PBS. The coverslips were then blocked in a solution of PBS, 10% bovine serum, and 0.1% Tween-20 for 1 h. The cells were incubated with primary antibody (E-cadherin, 1:100; vimentin, 1:100; CD44, 1:100) for 1 h, then washed twice with PBS, and incubated with secondary antibodies diluted in PBS (Alexa Fluor 594 (red/green)-conjugated anti-rabbit IgG antibody (Santa Cruz Biotechnology) diluted 1:100 in blocking buffer). After incubating on coverslips for 1 h at 37°C the coverslips were washed as described above and mounted on slides in medium containing DAPI. Cells were observed and images were captured through an epifluorescence microscope (Olympus BX51; Olympus, Tokyo, Japan).

Lentiviruses transfection assay. Lentiviruses overexpressing human Twist1 and empty vector were built by Shanghai Genechem, Co., Ltd. (Shanghai, China). For lentiviral transfection of LoVo cells, 100 multiplicity of infection Twist1 or empty vector lentiviruses were added to a well containing 5×10^4 cells, medium and 8 $\mu\text{g}/\text{ml}$ polybrene. After 24 h of incubation, transfected cells were selected with 2 $\mu\text{g}/\text{ml}$ puromycin (Sigma-Aldrich). Empty vector lentivirus was used as a control. Selected cells were maintained in growth medium with 0.5 $\mu\text{g}/\text{ml}$ puromycin.

Inhibition of Twist1 expression by RNAi. Twist1-targeted small interfering RNA (si-Twist1) (target DNA sequence is 5'-GG UACAUCGACUCCUCUATT-3') and no-target control siRNA (NC) were purchased from Guangzhou RiboBio, Co., Ltd. (Guangzhou, China). Cells (2×10^5) were seeded in 6-well plates in triplicate, after overnight incubation, the cells were transfected with NC or si-Twist1. The siRNA (50 nmol) and Lipofectamine 3000 (Invitrogen, Waltham, MA, USA) were mixed in 100 μl of minimal essential medium (Opti-MEM; Gibco) for 15 min, and the siRNA/Lipofectamine 3000 mixture was added into the serum-free culture medium. Six hours later, the medium was replaced with complete medium, and then the cells were cultured for an additional 48 h prior to the detection of Twist1 expression knockdown by RT-PCR.

Migration and invasion assay. Cell migration and invasion were assessed with Boyden chambers or modified Boyden chambers according to the protocol of the manufacturer (Becton-Dickinson Labware, Bedford, MA, USA). Briefly, 100 μl RPMI-1640 medium without FBS included 100,000 cells was placed on an 8.0- μm pore size membrane insert in 24-well plates, and 600 μl RPMI-1640 medium with 10% FBS was placed in the bottom wells. After 24 h at 37°C, cells that did not migrate were removed from the top side of the inserts with a cotton swab. After fixed with 4% paraformaldehyde for 10 min, cells that had migrated to the underside of the inserts were stained with 0.1% crystal violet solution and the cells on each insert were counted using a microscope (Olympus BX51; Olympus). Cells were counted in five random fields per insert. The invasion assay was done in a similar fashion except the 8.0- μm pore size membrane inserts were coated with Matrigel. Results were expressed as cells migrated per field.

Quantitative real-time PCR assay. Total mRNA of the cells was extracted using the Total RNA kit (TransGen Biotech, Inc., Beijing, China). First step cDNA synthesis was generated from

500 ng total mRNA. RT-PCR amplification was performed in triplicate on LightCycler® 480 II (Roche, Berlin, Germany). The primers used in each reaction were synthesized in Sangon Biotech, Co., Ltd. (Shanghai, China). Expression levels for each target gene were normalized to β -actin gene. Results were calculated using the comparative threshold cycle ($\Delta\Delta\text{CT}$) method. Data are presented as the mean \pm standard deviation (SD) from three independent experiments.

Western blotting assay. Cells were harvested with a plastic scraper and washed twice with cold PBS. Cells were then lysed with ice-cold protein extract solution RIPA (Beyotime Institute of Biotechnology, Shanghai, China) and protein concentration was quantified using the BCA procedure (Beyotime Institute of Biotechnology). Equal amounts (30 μg) of protein samples were separated by SDS-PAGE using 12% or 10% polyacrylamide gel and then transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% skim milk TBST (Tris-buffered saline Tween-20) buffer for 1 h at room temperature. The membranes were incubated with primary antibodies including EMT antibody sampler kit (1:1,000), anti-Twist1, anti-MMP1, anti-MMP9, anti-CD44, anti-CD133 (1:500); anti-P-gp, anti- β -actin (1:3,000) at 4°C overnight. After washed with TBST, the blot was incubated with the appropriate horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:5,000). The antigen was detected by WesternBright ECL HRP substrate kit (Millipore) in gel image analysis system (Kodak, Rochester, NY, USA). The intensities of the protein bands were analyzed by Molecular Imaging software. β -actin protein was used as the internal control.

Statistical analysis. Quantitative data are expressed as the mean \pm SD of three independent experiments. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) test followed by Dunnett's test. P-values are two-sided, and a value of 0.05 was considered to be statistically significant. All statistical calculations were performed using SPSS software (version 19.0; SPSS, Inc., Chicago, IL, USA). Graphs were prepared using GraphPad Prism 5.

Results

Cross-resistance to anticancer drugs and increased expression of ABCB1 in irinotecan-resistant cells. Via long-term culturing of original irinotecan-sensitive LoVo cells by gradual adaptation of increasing irinotecan concentrations, we successfully established irinotecan-resistant variant LoVo/CPT-11R cells. The resistant cells were capable of long-term survival and proliferation in media containing 70 $\mu\text{g}/\text{ml}$ irinotecan. In order to investigate whether the LoVo/CPT-11R cells acquired cross-resistance to other anticancer drugs, their sensitivities to various anticancer drugs used to treat CRC, including 5-fluorouracil, cisplatin and curcumin, were determined using CCK-8 assay. The results showed that cell viabilities of LoVo/CPT-11R cells treated with anticancer drugs (irinotecan, 5-fluorouracil, cisplatin and curcumin) were markedly increased compared with the control LoVo cells (Fig. 1A-D), herein the LoVo/CPT-11R cells acquired multi-drug resistance property.

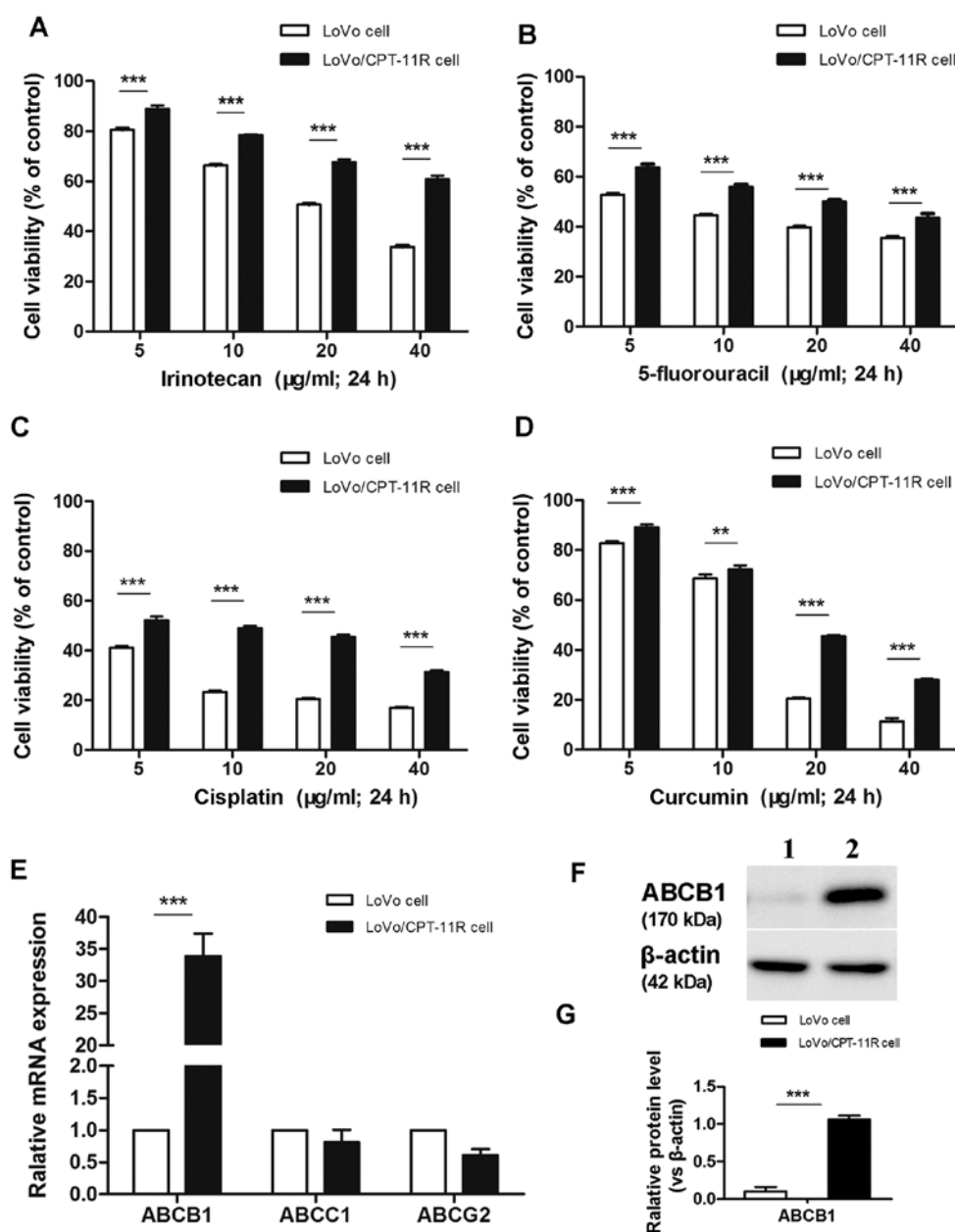


Figure 1. Cell viability to anticancer drugs and expression levels of ABC transports in LoVo/CPT-11R cells. (A-D) LoVo and LoVo/CTP-11R cells were treated with various concentrations of (A) irinotecan, (B) 5-fluorouracil, (C) cisplatin, or (D) curcumin for 24 h, and cell viabilities were determined using CCK-8 assay. (E) The mRNA expression of ABCB1, ABCC1 and ABCG2 in LoVo cells and LoVo/CPT-11R cells were analyzed by qRT-PCR assay. (F) ABCB1 protein (P-gp) expression was detected by western blotting assay. (G) Relative ABCB1 protein expression was measured by ImageJ. β -actin was used as a loading control. Each bar represents the mean \pm SD of the three independent experiments. ** $P < 0.01$, *** $P < 0.001$ vs. LoVo cells. Lane 1, LoVo cells; lane 2, LoVo/CPT-11R cells.

Additionally, we compared expression of the most known ABC transporter genes (ABCB1, ABCC1 and ABCG2) in mRNA level in LoVo and LoVo/CPT-11R cells. Results indicated that mRNA expression of ABCB1 was significantly increased in LoVo/CPT-11R cells, but the changes of expression of ABCC1 and ABCG2 were not significant (Fig. 1E). In addition, the expression of ABCB1 protein (P-gp) was detected by western blotting assay. Likewise, the ABCB1 was significantly overexpressed on protein level in LoVo/CPT-11R cells (Fig. 1F and G).

EMT-like morphology and altered localization of EMT markers in irinotecan-resistant cells. Light microscopy

revealed a marked alteration of cellular morphology in monolayer culture. LoVo/CPT-11R cells exhibited spindle shape, loose intercellular space, and irregular scattering, suggested a more 'mesenchymal' phenotype than parental LoVo cells (Fig. 2A). To further confirm our observation, the EMT marker proteins were stained by immunofluorescence assay. It was found that epithelial marker E-cadherin was located mainly in cell membrane of LoVo cells, but E-cadherin protein was located mainly in the cytoplasm of LoVo/CPT-11R cells (Fig. 2B). Moreover, compared to LoVo cells, the mesenchymal marker Vimentin protein was more distinctly stained in cytoskeletal localization in LoVo/CPT-11R cells (Fig. 2C).

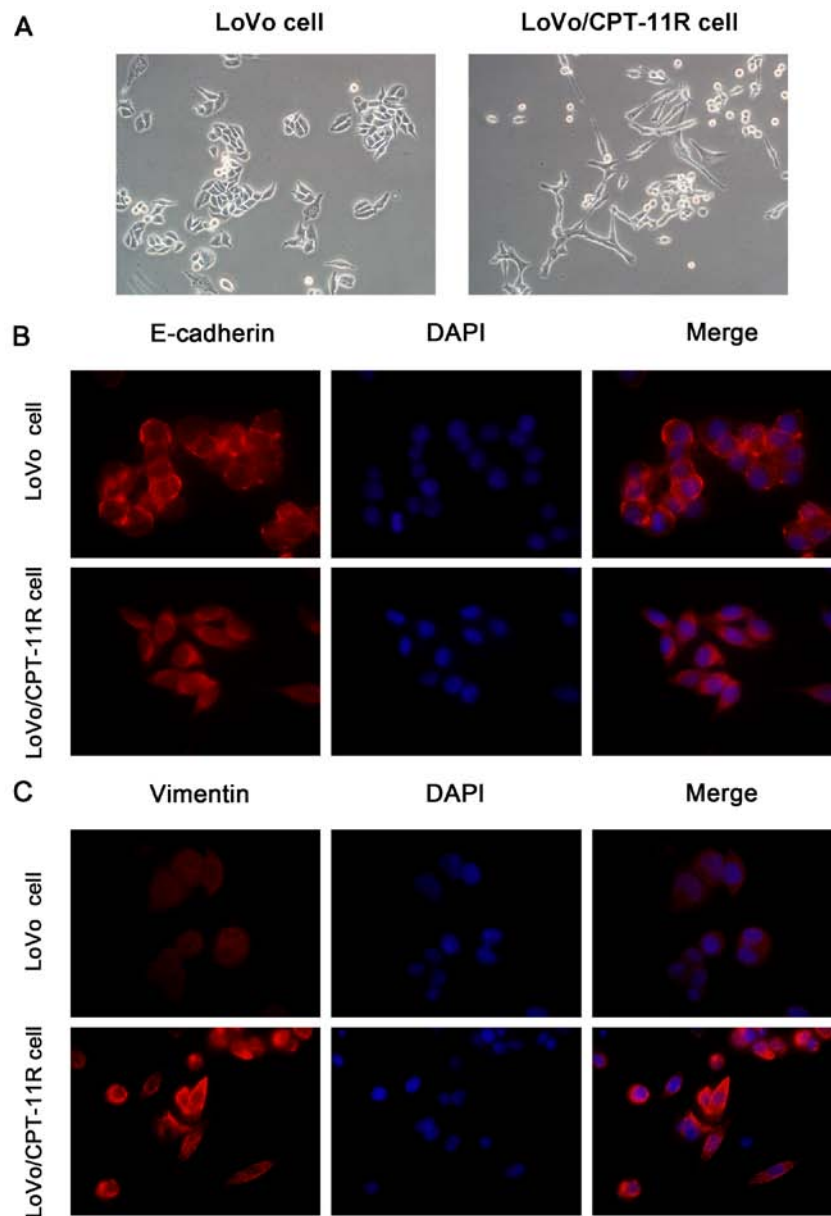


Figure 2. Cellular morphology and altered localization of EMT markers. (A) Cellular morphology was detected by Light microscopy in monolayer culture. (Original magnification, x200). Cellular localization of (B) E-cadherin and (C) vimentin were determined by immunocytochemistry assay in LoVo cells and LoVo/CPT-11R cells. Cells were stained with DAPI (blue) and second antibody (red). Fluorescent images were captured under an epifluorescence microscope. (Original magnification, x1000).

Irinotecan-resistant cells overexpress EMT markers, Twist1 and CSC markers. The observed morphological changes implied that the irinotecan-resistant cells had transitioned to a mesenchymal phenotype. To determine whether these morphological changes were associated with EMT, we examined respectively the expression of EMT markers in mRNA and protein levels. Consistent with morphological changes, LoVo/CPT-11R cells showed downregulation of the epithelial markers E-cadherin and ZO1, and upregulation of the mesenchymal markers vimentin and N-cadherin (Fig. 3A-C). These results suggested that EMT could be associated with acquired drug resistance after long-term exposure to irinotecan. In addition, EMT-inducing transcription factors were also detected by the above methods. The expression of Twist1 was higher in LoVo/CPT-11R cells than LoVo cells, the changes of expres-

sion of Snail, Slug and Zeb1 were not statistically significant (Fig. 3D-F).

The relation of aggressive cell characteristics and CSC continue to be under intense investigation. Therefore, we detected respectively the expression of CSC identification markers. It was founded that the expression of CD44 and CD133, most widely studied CSC biomarkers, were increased in mRNA and protein levels in LoVo/CPT-11R cells compared to LoVo cells (Fig. 3G-I).

Overexpression of Twist1 contributes to irinotecan resistance, EMT and CSC-like phenotype of LoVo cells. Considering the expression of Twist1 was significantly increased in LoVo/CPT-11R cells, we built overexpressed Twist1 LoVo cells by lentivirus transfection assay, to confirm whether

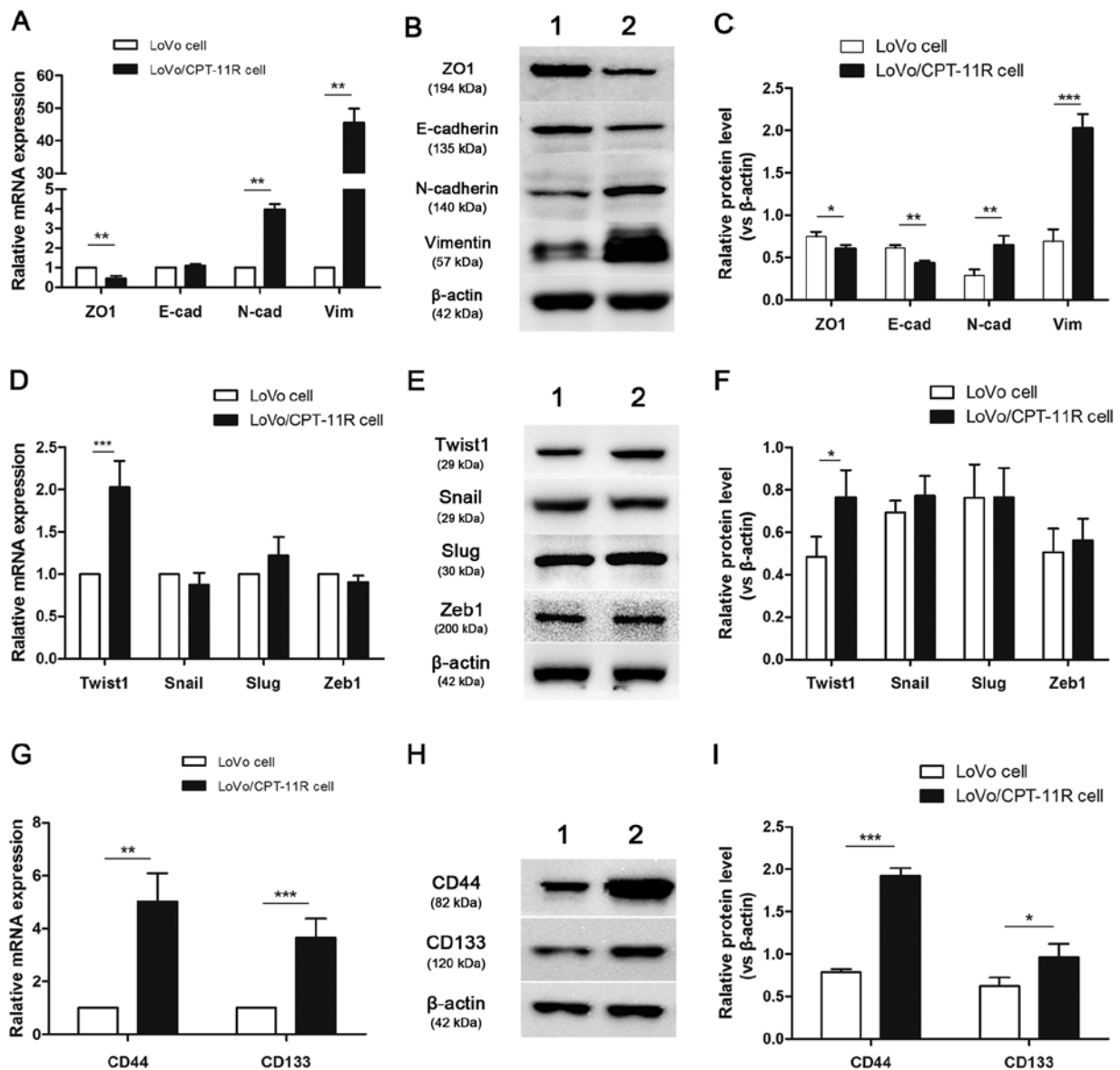


Figure 3. Expression of EMT markers, EMT-inducing transcription factors and CSC markers. The mRNA expression levels of EMT markers (A), EMT-inducing transcription factors (D) and CSC markers (G) were analyzed by qRT-PCR assay in LoVo cells and LoVo/CPT-11R cells. Protein expression and relative protein expression levels of EMT markers (B and C), EMT-inducing transcription factors (E and F) and CSC markers (H and I) were detected by western blotting assay and measured by Image J. β -actin was used as a loading control. Values present the mean \pm SD of three independent experiments. * P <0.05, ** P <0.01, *** P <0.001 vs. LoVo cells. Lane 1, LoVo cells; lane 2, LoVo/CPT-11R cells. E-cad, E-cadherin; N-cad, N-cadherin; Vim, vimentin.

overexpression of Twist1 contributed to resistance to irinotecan, EMT and CSC-like phenotype of LoVo cells. The control LoVo cells (transfected with empty vector lentiviruses) and LoVo/Twist1 cells (transfected with overexpressed Twist1 lentiviruses) were treated with different concentrations of irinotecan and CCK-8 assays were performed. The results showed that overexpression of Twist1 led to a distinct increase in cell viability compared with control cells (Fig. 4A). Moreover, we detected respectively the expression of EMT and CSC markers in protein level by western blotting assay. The results showed that the expression of E-cadherin was decreased and that the expression of CD44 was increased (Fig. 4B and C).

Overexpression of Twist1 enhances migration and invasion potential by regulating MMP2 expression. To assess the

migratory and invasive potentials of overexpressed Twist1 LoVo cells, *in vitro* cell migration and invasion assay were performed. Compared with the controls, LoVo/Twist1 cells demonstrated a 1.74-fold increase at 24 h in migration (Fig. 5A and B), and a 2.63-fold increase at 48 h in invasion (Fig. 5C and D). Metalloproteinases (MMPs) are crucial to invasion and migration, a significant association has been reported between tumor aggressiveness and increased levels of MMP2 and MMP9 in many experimental and clinical studies. Our results detected that the expression of MMP2 was significantly increased in the protein level, but the MMP9 was not significantly changed in LoVo/Twist1 cells compared to control cells.

Downregulation of Twist1 increases the irinotecan sensitivity, and reverses EMT and CSC-like phenotype of LoVo/CPT-11R

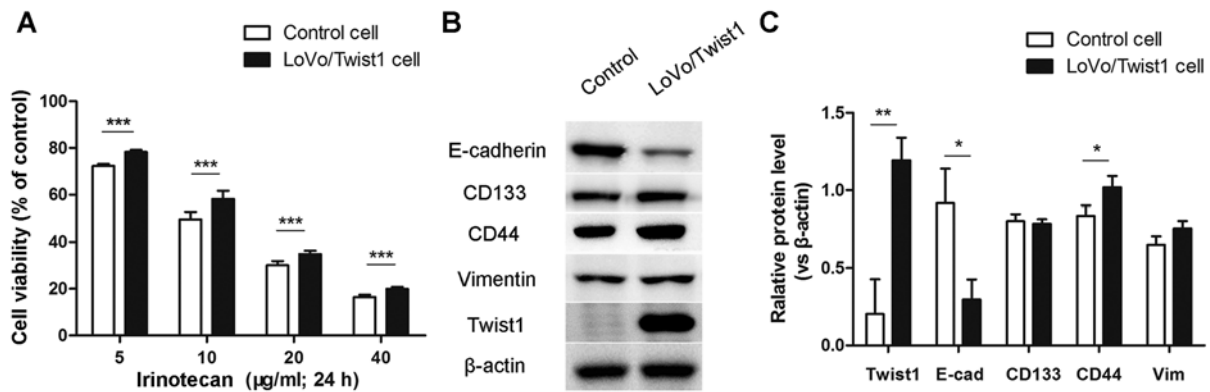


Figure 4. Overexpression of Twist decreases the sensitivity to irinotecan and induces EMT and CSC-like phenotype of LoVo cells. (A) The control cells and LoVo/Twist1 cells were respectively treated with various concentrations of irinotecan (5, 10, 20 and 40 µg/ml) for 24 h, and cell viabilities were determined by CCK-8 assay. (B and C) Protein expression of EMT and CSC markers were detected by western blotting assay and relative protein expression levels measured by ImageJ. β-actin was used as a loading control. Each bar represents the mean ± SD of the three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. LoVo cells. Control, LoVo cells transfected with empty vector lentiviruses; LoVo/Twist1, overexpressed Twist1 LoVo cells.

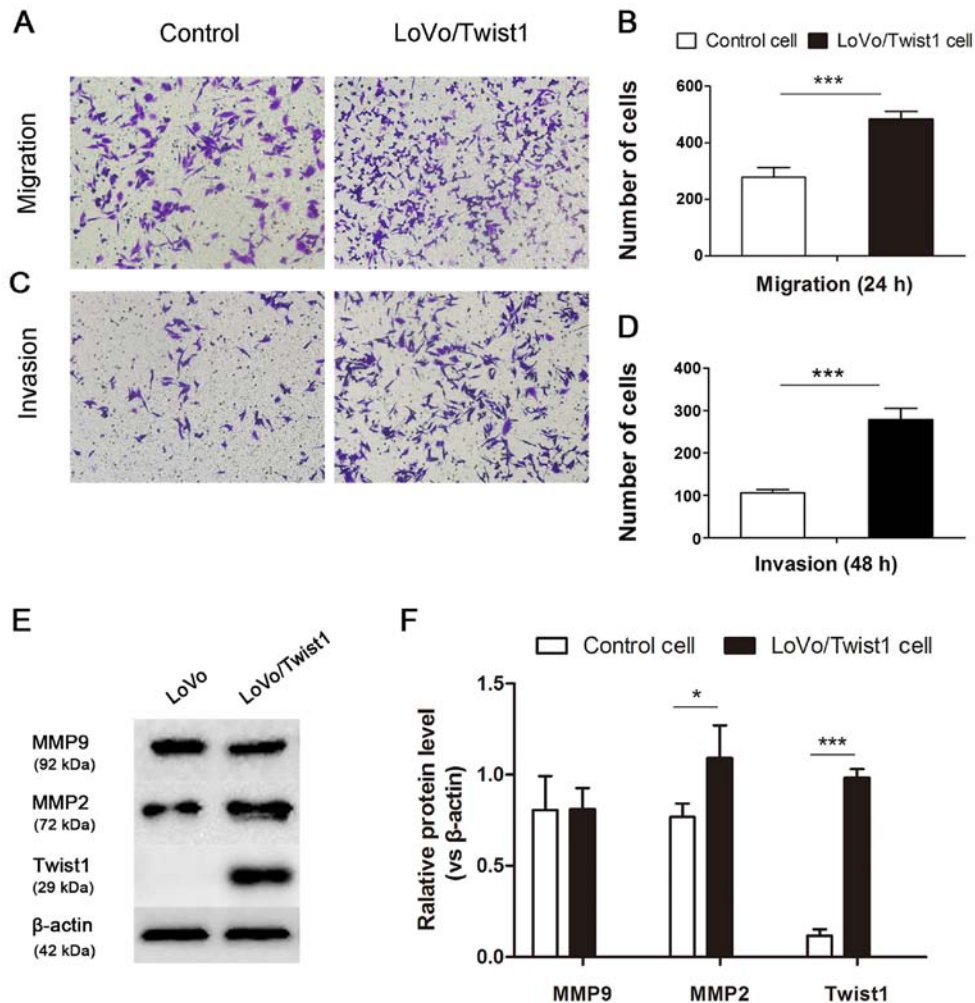


Figure 5. Overexpression of Twist1 enhances migration and invasion potential and increases MMPs expression. (A and B) The number of overexpressed Twist1 LoVo cells are 1.74-fold increased at 24 h in migration. (C and D) The number of overexpressed Twist1 LoVo cells are 2.63-fold increased at 48 h in invasion. (Original magnification, x200). (E and F) The expression of Twist1, MMP2 and MMP9 protein in overexpressed Twist1 LoVo cells compared to control cells. Values are presented as the mean ± SD of three independent experiments. *P<0.05, ***P<0.001 vs. control cells. Control, LoVo cells transfected with empty vector lentiviruses; LoVo/Twist1, overexpressed Twist1 LoVo cells.

cells. To further discover the molecular link between irinotecan resistance and Twist1, we inhibited the expression of

Twist1 in LoVo cells and LoVo/CPT-11R cells by RNAi. After transfected respectively with NC and si-Twist1 in LoVo cells

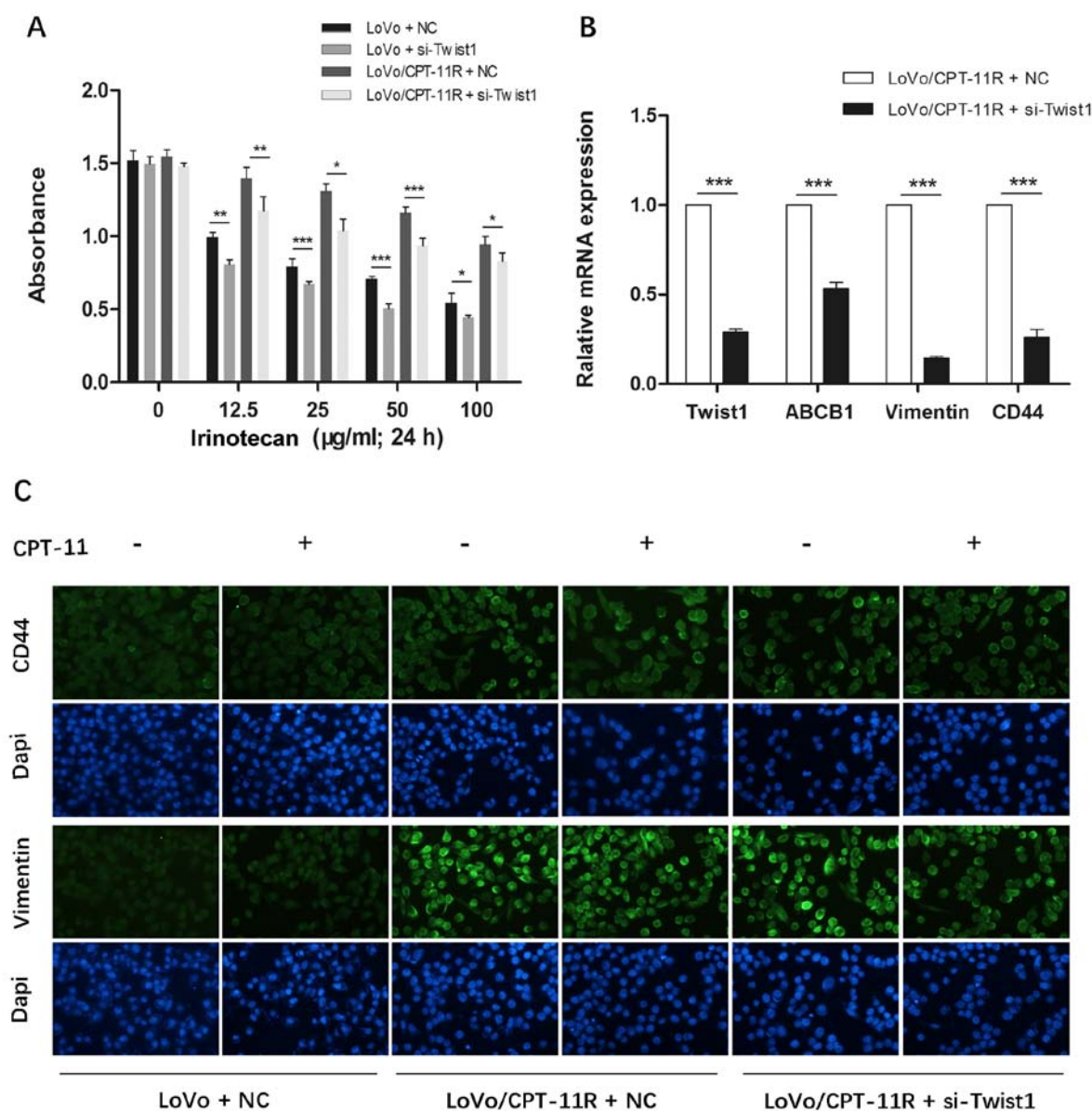


Figure 6. Downregulation of Twist1 increases the sensitivity to irinotecan and reverses EMT and CSC-like phenotype of the LoVo/CPT-11R cells. (A) After transfected respectively with NC and Twist1-targeted siRNA in LoVo cells and LoVo/CPT-11R cells, cells were treated with different concentrations of irinotecan (0, 12.5, 25, 50 and 100 $\mu\text{g/ml}$) for 24 h, then every group absorbance at 450 nm was detected by CCK-8 assays. (B) The mRNA expression of Twist1, ABCB1, vimentin and CD44 were analyzed by qRT-PCR assay. (C) The protein expression of vimentin and CD44 were detected by immunocytochemistry assay. Cells were stained with Dapi (blue) and second antibody (green). (Original magnification, x200). Each bar represents the mean \pm SD of the three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. NC, cells transfected with N Control; si-Twist1, cells transfected with Twist1-targeted siRNA.

and LoVo/CPT-11R cells, cells were treated with different concentrations of irinotecan (0, 12.5, 25, 50 and 100 $\mu\text{g/ml}$) for 24 h, then absorbance of each group at 450 nm was detected by CCK-8 assays. The results showed that the inhibition of Twist1 expression led to a distinct decrease in absorbance (Fig. 6A). It indicated that the downregulation of Twist1 could increase the irinotecan sensitivity in LoVo cells and LoVo/CPT-11R cells.

Moreover, we detected respectively the expression of EMT and CSC-like markers by RT-PCR and immunofluorescence assays. The results showed that the inhibition of Twist1 could downregulate the mRNA expression of vimentin, CD44 and P-gp (Fig. 6B), and distinctly decreased the protein expression of vimentin and CD44 of LoVo/CPT-11R cells when adding irinotecan (Fig. 6C). It indicated that the downregulation

of Twist1 could reverse EMT and CSC-like phenotype of LoVo/CPT-11R cells.

Discussion

We established irinotecan-resistant subline of human colon cancer LoVo cells by stepwise adaptation to increasing drug concentrations. In addition, LoVo/CPT-11R cells also displayed cross-resistance to non-camptothecin anticancer drugs (5-fluorouracil, cisplatin and curcumin), indicated the presence of the multidrug resistant (MDR) phenotype. Numerous published studies have showed that ATP-binding cassette (ABC) transporters play a crucial role in the development of multidrug resistance by the efflux of anticancer agents outside the cancer cells (24). The most extensively characterized

MDR transporters included ABCB1 (also known as MDR1 or P-glycoprotein), ABCC1 (also known as MRP1) and ABCG2 (also known as BCRP or MXR) (25). When compared with the genes expression in LoVo cells, we found only the ABCB1 was significantly higher in LoVo/CPT-11R cells, and the expression of P-gp was also increased. Indeed, ABCB1 encoded the xenobiotic transporter P-gp that has been extensively investigated *in vitro* and *in vivo* as a predictor of MDR in various tumors (26,27). The expression of ABCB1 is regulated at different levels by multiple signaling pathways, including hypoxia-inducible factor-1 α (HIF-1 α), p53, chromosomal rearrangement, methylation, acetylation and microRNA (28). Recently, a potential transcriptional regulatory role of Twist1 has been identified in chemotherapy drug resistance (29). In the present study, the positively correlated downregulation of ABCB1 and Twist1 in LoVo/CPT-11R cells was detected, which also indicate a novel role of Twist1 in maintaining the irinotecan-resistant phenotype of colon cancer through regulating ABCB1 expression.

EMT program is now known to facilitate the metastatic spread and progression of cancer cells from the site of the primary tumor to the surrounding tissues and distant organ(s). It is also generally considered that EMT is associated with cancer aggressiveness, invasive and metastatic behavior, and chemotherapeutic resistance (30). However, comprehensive studies of EMT and irinotecan resistance in colon cancer are lacking. In the present study, we found that LoVo/CPT-11R cells exhibited a more mesenchymal phenotype and location alteration of epithelial marker E-cadherin from cell membrane to cytoplasm than parental LoVo cells. To confirm the role of EMT and resistance to irinotecan, we examined the expression of EMT markers in mRNA and protein levels, respectively. As expected, LoVo/CPT-11R cells showed downregulation of E-cadherin and ZO1 and upregulation of vimentin and N-cadherin. In addition, the expression levels of EMT-inducing transcription factors (Twist1, Snail, Slug and Zeb1) were detected. Notably, results showed that only Twist1 was significantly higher in LoVo/CPT-11R cells than LoVo cells. Increasing evidence suggested that EMT not only enables cancer cells to disseminate but also to acquire the ability to self-renew by inducing a CSC trait (31-33). In experimental models, CSCs are more resistant than differentiated tumor cells to chemo- and radiotherapy and they can escape from the effects of conventional cytotoxic treatments (34). It has been reported that CD133 and CD44 are two proposed stem cell markers in various metastasized cancers including colorectal cancer (35-37). Therefore, we detected respectively the expression of CSC identification markers. The expression of CD44 and CD133 were increased both in mRNA and protein levels in LoVo/CPT-11R cells. Therefore, it was identified that the irinotecan resistance cells presented EMT-like and CSC-like phenotype. In order to confirm whether Twist1 contributes to formation of resistance to irinotecan and alteration of EMT, CSC-like phenotype in colon cancer cells, we established overexpressed Twist1 LoVo cells, named LoVo/Twist1 cells, by lentivirus transfection assay. After treated with different concentrations of irinotecan, we found that the overexpression of Twist1 led to a distinct resistance to irinotecan compared with control cells transfected with empty vector. Moreover, the decreased expression of E-cadherin and the increased

expression of CD44 demonstrated the formation of EMT, CSC-like phenotype in LoVo/Twist1 cells. In contrast, we found that downregulation of Twist1 increased the irinotecan sensitivity, reversed EMT and CSC-like phenotype of LoVo/CPT-11R cells transfected with NC and si-Twist1. Therefore, it was identified that EMT and CSC-like phenotype induced by Twist1 contribute to acquire resistance to irinotecan.

In addition, compared with the controls, LoVo/Twist1 cells performed a significant enhancement both in migration and invasion. It is known that MMPs are a group of matrix-degrading proteins implicated in several pathological processes, including invasion and metastasis of CRC (38). Increased MMP2 and MMP9 expression have also been documented to correlate with cancer invasion (39). Consistent with previous reports, MMP2 protein was increased in LoVo/Twist1 cells, but MMP9 was not significantly changed compared to control cells, which indicated that overexpressed Twist1 contribute to the migratory and invasive potentials of colon cancer by upregulated MMP2 expression. In summary, the development of a highly aggressive colon cancer phenotype requires the coordination of many different molecular changes, which are a consequence of genomic alterations. We have demonstrated that EMT and CSC-like phenotype induced by Twist1 contribute to acquiring resistance to irinotecan, and high expression of Twist1 enhanced migration and invasion potential. It suggests that Twist1 may be a potential molecular target for overcoming the irinotecan resistance and metastasis in colon cancer. However, it would make the resistance mechanisms associated with Twist1 more fruitful if gene expression profiling of LoVo/CPT-11R cells was added to the analysis.

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