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Original Article

Influence of *UGT1A1* gene methylation level in colorectal cancer cells on the sensitivity of the chemotherapy drug CPT-11

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ABSTRACT

Objective: To study the influence of the methylation level of *UGT1A1* gene related to CPT-11 metabolic enzymes in colorectal cancer cells on the sensitivity of chemotherapy drugs.
Methods: Test the changes in sensitivity of seven colorectal cancer cell strains that have been/not been subject to DAC treatment to CPT-11, analyze its correlation with *CES2*, *UGT1A1* and *GUSB* mRNA expression according to IC_{50}; screen the effective interference sequence of *UGT1A1* siRNA, test the changes in cytotoxicity of CPT-11 after *UGT1A1* siRNA is transfected, select RK0 cells and make them transfected with the chemosynthetic *UGT1A1* siRNA after their *UGT1A1* expression is restored with or without demethylation treatment.
Results: The sensitivity of different colorectal cancer cell strains to CPT-11 showed difference ($P < 0.05$), *UGT1A1* expression in colorectal cell lines had a negative correlation with the IC_{50} ($r = 0.790648$, $P < 0.05$), the interference efficiency of the screened *UGT1A1* siRNA was up to 78%. The IC_{50} value of siRNA decreased by nearly one time after transfected with HT-29 ($P < 0.01$); which of methylated RK0 cells of *UGT1A1* gene increased instead after the demethylation treatment. However, the IC_{50} value of the demethylation treatment group increased compared with the non-demethylation treatment group after *UGT1A1* siRNA was transfected.
Conclusions: The cytotoxicity of CPT-11 to colorectal cancer cells has a negative correlation with *UGT1A1* expression, and positive correlation with *CES2* and *GUSB*. The specific silencing *UGT1A1* gene of siRNA could significantly increase the sensitivity of CPT-11 to the chemotherapy of colorectal cancer cells. *UGT1A1* methylation was an important factor affecting the chemosensitivity of CPT-11.

Irinotecan (CPT-11) is a major drug to treat colorectal cancer in recent years, but it is common that CPT-11 is still insensitive to chemotherapy of colorectal cancer since it is used in this area. Drug resistance is one of the main causes resulting in the chemotherapy failure, the death of 90% cancer patients has a relation with the drug resistance of tumor, and there is no effective way to solve this problem. For many years, people have carried out a large number of studies on drug resistance mechanisms of tumors, and found that the generation of chemotherapeutic drug resistance involves a series of mechanisms. Some of such studies have shown that chemotherapeutics metabolic enzymes have their own epigenetic gene silencing, while methylation is an important mechanism to control the gene expression of these enzymes [1].

During the activation of CPT-11, CES2 has the highest affinity with CPT-11 in carboxylesterase (CES) family, and it is the key enzyme to hydrolyze CPT-11 into SN-38 (Fig. 1) [2] that is then inactivated into the inactive conjugate SN-38G by UDP-glucuronosyltransferase (UGT) glucose aldehyde group, of which, UGT1A1 is a major member in the UGT family, playing a key role in the generation of SN-38G. The main way to remove the active SN-38 is to change it into inactive SN-38G [3] through the glycosylation [4] of UGT1A1. Based on our previous study involving the expression of CPT-11 metabolic enzyme-related genes *CES2*, *GUSB* and *UGT1A1* in colorectal cancer tissue and cells as well as the methylation level [1,2], it has been found that the expression of *CES2* and *GUSB* has a positive correlation with the sensitivity of CPT-11. Methylation exists in the *UGT1A1* gene promoter of methylated colorectal cancer cells of

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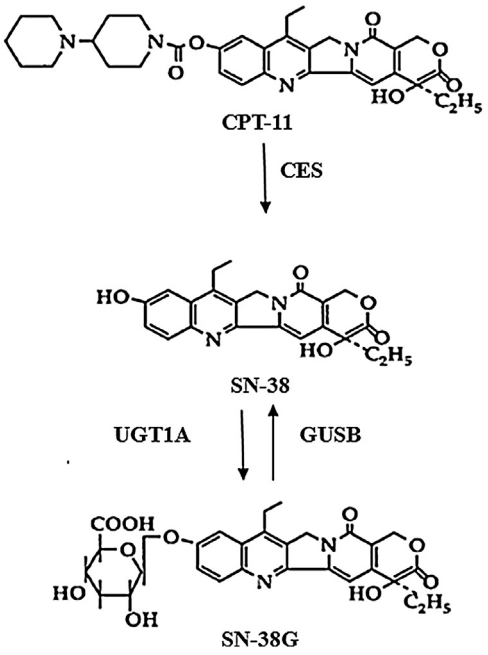

Fig. 1. The metabolism process of CPT-11 in liver.

UGT1A1, which is the important mechanism of the *UGT1A1* gene expression silencing. While *CES2* is of hypomethylation and *GUSB* is hemi-methylated, both of which are not involved in the regulation of the expression of the corresponding gene. Therefore, the purpose of this study is to investigate the influence of the *UGT1A1* gene methylation on the sensitivity of the chemotherapy drug CPT-11, intending to reveal possible epigenetic mechanisms of the colorectal cancer chemotherapy drug tolerance, and providing new ideas and measures for the reversal of tumor resistance and chemotherapy sensitizing.

1. Material and methods

1.1. Main material

1.1.1. Cell lines

Colorectal cancer cell strain: LoVo, HT-29, ls174T, DLD-1, HCT-116, RK0 and HCT-15.

1.1.2. Main reagents

RPMI1640 and DMEM purchased from Gibco; Fetal bovine serum purchased from Hyelone Company; MTT purchased from Sigma; Lipofectamine2000 purchased from Invitrogen Company; ExTaq PCR kit purchased from TaKaRa Company; Rever Tre Ace-a-reverse transcription kit and SYBR Green Realtime PCR kit purchased from TaKaRa Company.

1.1.3. Main instruments

Flow cytometry from BD Company of the USA; fluorescence microscopy from Olympus Company of Japan; CO_2 incubator from Heraeus Company of the USA; Microplate reader, PCR, UV gel imaging system from Bio-Rad Company of the USA; DU 800 nucleic acid/protein detector from Siemens of the USA; and fluorescence quantitative PCR instrument from ABI Company of the USA.

1.2. Main methods

1.2.1. Testing the cytotoxicity of CPT-11 with different concentrations to colorectal cancer cell strain via MTT

The colorectal cancer cell strains LoVo, HT-29, ls174T, DLD-1, HCT-116, RK0 and HCT-15 were respectively inoculated into 96-well plates on a 4000 pieces/hole basis. After 4 hours and when adherent cells were obtained, CPT-11 was added to each well till the final concentrations respectively reached 1.25, 2.5, 5, 10, 20, 40, 80 and 160 μg/mL. Four parallel wells were set for each concentration, with the final volume of 200 μL for each well. After 72 hours, the drug reaction was stopped, and 20 μlMTT solution (5 mg/mL) was added to each well. Next, they were incubated for another 4 hours at 37 °C, with 5% CO_2, then incubating was suspended, and the supernatant in the wells was extracted carefully. An amount of 150 μL DMSO was added to each well and we measured the optical density of each well using the microplate reader in the wavelength of 490 nm. This experiment was repeated for three times and the average values were taken. The inhibition rate of CPT-11 to colorectal cancer cells was calculated according to the following formula: inhibition rate (fa) = [1 – average *D* (490) value of experiment groups/average *D* (490) value of cell control groups] × 100%, then the IC_{50} value was obtained through the calculation of the inhibition rate.

1.2.2. Testing the expression of the metabolic enzyme mRNA related to CPT-11 via real time PCR

The seven types of colorectal cell lines (DLD-1, HCT-15, LoVo, HT-29, LS174T, HCT-116 and RK0) were inoculated into 6-well culture plates on a 1 × 105 basis, and then cultivated in the culture medium of DMEM or RPMI1640 containing 10% calf serum in the incubator at 37 °C, with 5% CO_2. CPT-11 was added to make the final concentration reach IC_{50} when the cells grew to 40–50% confluent culture. DAC was added to each type of cell strain. Since the aqueous solution of DAC was instable and easily biodegradable, after the treatment by DAC for 24 hours, replaced the culture medium and continued the culture by adding the drug with the same concentration. The drug concentration gradient was respectively 5 μm. For the blank group, an equal volume of PBS was used as the control. After 72 hours, the cells were collected and RNA was extracted according to the operation instructions of the Trizol reagent manual. After being digested by DNAase, 5 × RT Buffer 2 μL, RNA Ace 0.5 μL, RNase inhibitor 0.5 μL, dNTPs 2 μL and Oligo (dT) 1 μL were added according to the instructions of the Rever Tre Ace-a-reverse transcription kit with reaction conditions: at 42 °C, for 10 min; at 30 °C, for 20 min; at 99 °C, for 5 min; at 4 °C, for 5 min; and stored at –20 °C. The reaction system SYBR Premix Ex Taq™ 10 μL stated in TaKaRa SYBR Green I manual were adopted, with 0.8 μL, cDNA 2 μL, ROX 1 μL and ddH_2O 5.4 μL forward/reverse primers respectively and a total volume of 20 μL. The reaction conditions were: denaturated at 95 °C for 30 s; at 95 °C for 5 s, at 55 °C for 20 s and 72 °C for 20 s, 40 cycles; finally, cooled at 37 °C for 20 min, till to the end of the reaction. Metabolic enzymes *CES2*, *GUSB* and *UGT1A1* related to CPT-11 were used as genes, and GAPDH as the internal reference gene. CES2 gene primers: sense primer 5′-GTAGCA-CATTTTCAGTGTTCC-3′, antisense on primer 5′-GTAGTTGCCCC-CAAAGAA-3′, with products of 151 bp; *GUSB* gene primers sense primer 5′-GGACTTCAACCTGCTTCGC-3′, antisense primer 5′-ACG-CACCACTTCTTCCATCA-3′, with products of 202 bp; *UGT1A1* gene primers: sense primer 5′-TCTTGCGAACAACACGATA-3′, antisense primer 5′-ATTCAGGGTCACTCCAGCT-3′, with products of 211 bp; *GAPDH* gene primers: sense primer 5′-ACGGATTTGGTCGTATTGGG-3′, antisense Primer 5′-GTAGTTGCCCCCAAAGAA-3′, with products of 218 bp. Use the relative quantitative PCR for comparison.

1.2.3. siRNA design for UGT1A1 gene of colorectal cancer cells

Appropriate target base pairs were selected according to *UGT1A1* mRNA sequences in GenBank (Accession Number: NM-000 463) based on following principles:

- the most appropriate length should be 21 to 23 bp;
- GC content should be about 30% to 50%;