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Original article

Regulation and expression of aberrant methylation on irinotecan metabolic genes CES2, UGT1A1 and GUSB in the in-vitro cultured colorectal cancer cells

Fang-Wei Xie, Yong-Hai Peng, Xi Chen, Xiong Chen, Jie Li, Zong-Yang Yu, Wen-Wu Wang, Xue-Nong Ouyang *

Department of Medicine Oncology, Fuzhou General Hospital of Nanjing Military Command, 350025 Fuzhou, Fujian, China

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ABSTRACT

Objective: To evaluate the aberrant methylation gene expression related to the irinotecan (CPT-11) metabolic enzymes in different colorectal cancer cell strains; provide new thoughts and measures for reverse of tumor drug resistance.
Methods: Studied the aberrant methylation state of CES2, UGT1A1 and GUSB in eight colorectal cancer cell strains through MSP method; and analyze the expression of the target gene after being dealt with DAC.
Results: UGT1A1 showed methylation in five cell strains, while CES2 and GUSB respectively showed consistent unmethylation or hemimethylation. After being dealt with DAC, CES2 and GUSB mRNA showed different expressions but not significant. The expression quantity of UGT1A1mRNA in the low-expression cell strains increased significantly. The expression of UGT1A1 protein where POSITIVE presented low expression was up-regulated to different degrees. Negative tropism was found in CES2 and UGT1A1.
Conclusion: Methylation in UGT1A1 gene expression silencing as an important mechanism; methylation could provide an effective target for methylation regulation intervening in the treatment of CPT-11. Meanwhile, studies found that the changes in expressions of CES2 and GUSB might be resulted from some unknown target that still existed during the regulation, or from the influence of methylation in the non-core zone of promoters on the gene transcription.

1. Introduction

Colorectal cancer is considered as one of the malignant tumors with high incidence rate and mortality. As for the late, recurrent or metastatic colorectal cancer cases, pharmacotherapy serves as the main process for the current chemotherapy.

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 treatment for colorectal cancer. Drug resistance is considered as a big impediment to its efficacy. Relevant research on the drug resistance gene mechanism has always been the focus of relevant researches, but no desired effect has been achieved at present and there hasn't been an effective therapeutic method that can reverse drug resistance. So we try to change the thinking and enhance the effective time of it in vivo through regulation by starting from the metabolic pathways of CPT-11, irinotecan and its metabolites are mainly cleared by the liver and only a small amount (< 20%) by the kidney. In the liver tissue, it is further hydrolyzed by carboxylesterase (CES) to active metabolite, SN-38, which is then inactivated into inactive conjugate SN-38G by UDP-glucuronosyltransferase 1A (UGT1A) glucose aldehyde group before being excreted to the enteric cavity through the bile, and some SN-38G are changed into SN38 after losing glucose aldehyde group under the effect of GUSB. The GUSB in the tumor tissue also participate in this process.

Recent studies showed that the activation of irinotecan was of more significance at the tumor part than at the liver, thus regulation of the expression of CPT-11 metabolic enzymes in colorectal cancer cells was directly related to the SN38 concentration, and further affected the curative effect of chemotherapeutic drugs [1,2].

Studies have showed that metabolic enzymes of many chemotherapeutic drugs had genetic gene silencing, and methylation might be a kind of important mechanism responsible for inhibiting gene expression of these enzymes [3]. DNA methylation

* Corresponding author. Tel.: +86 138 0951 6120.
E-mail addresses: zjxfw@vip.tom.com (F.-W. Xie), oyxnong@163.com (X.-N. Ouyang).

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(DANT) was often closely related to the canceration of cells and was in direct proportion to the canceration of cells [4]. Studies have also shown that the mutation of DNMT caused demethylation of chromosome, which could lead to the instability of genome of mouse, thus resulting in lymphoma [5–7]. Gene silencing caused by excessive methylation could otherwise result in drug resistance. For this purpose, decitabine, a deoxycytidine analogue, has been developed currently to reverse drug resistance [8] through inhibiting the gene promoter relating to DNMT and hypermethylation state of topoisomerase. Therefore, methylation can be used as the marker for tumorigenesis, metastasis and diagnosis, and also as the marker for studying the regulation and expression of CPT-11 metabolic enzymes. However, no clear statement has yet been made on the relation and regulation mechanism of methylation and CPT-11 metabolic enzymes.

Therefore, this article intends to study the aberrant methylation of gene promoters relating to CPT-11 metabolic enzymes in colorectal cancer cell strains of different sources and the impact of aberrant methylation-mediated regulation on the gene expression relating to CPT-11 metabolic enzymes, verify the possible tolerant regulation targets of chemotherapeutic drugs of colorectal cancer, and explore the impact of aberrant methylation on the colorectal cancer metastasis and the regulation mechanism for the CPT-11 metabolic pathways in colorectal cancer cells, thus providing new thinking and countermeasures for reversing drug resistance of tumor cells and chemosensitization.

2. Materials and methods

2.1. Reagents and cell lines

Chemicals used in this study were products of Sigma (St. Louis, MO, USA), unless otherwise indicated. RPMI1640, DMEM and Fetal Bovine Serum (FBS) were obtained from Gibco Co. (USA). RNAprep pure Cell/Bacteria Kit, TIANamp Genomic DNA Kit and 2 × Taq RCR Master Mix were purchased from TIANGEN Biotech Co. (Beijing, China). Primary antibodies against CES2, UGT1A1, GUSB, β-actinand HRP-Conjugated secondary antibodies were obtained from Santa Cruz (USA). CpGenome DNA Modification Kit was purchased from Chemicom Co. (USA). Green Realtime PCR Master Mix-Plus were purchased from Toyobo Co. (Japan).BCA Protein Assay Kit and Enhanced chemiluminescence (ECL) Western Blot Kit was purchased from Pierce Co. (USA). Human colorectal cancer cell HCT-15, human colorectal epithelial cell DLD-1, human colon cancer cell LoVo, HT-29, LS174T, human colorectal cancer cell HCT-116, human colonic adenocarcinoma cell RKO, SW480 cell lines were stocks of the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

2.2. Cell culture

DLD-1 and HCT-15 were cultured in RPMI1640 culture solution containing 10% fetal calf serum, while LoVo, HT-29, LS174T, HCT-116, RKO, and SW480 were cultured in DMEM culture solution containing 10% fetal bovine serum; all were cultured at 37 °C in the incubator (Heraeus, USA) containing 5% CO_2. Conducted passage at a cell proportion of 1:4 when cells covered 80%∼90% culture flask surface. Changed the solution once after the cells were adherent to the wall, and then changed the solution once every 2 days. Cells in the period of logarithmic growth would be taken for experiment.

Inoculated a 6-hole culture plate with eight colorectal cancer cell strains (DLD-1, HCT-15, LoVo, HT-29, LS174T, HCT-116, RKO, SW480) by 1×10^5, cultured in incubator at an atmosphere of 5% CO_2 with DMEM or RPMI1640 culture solution containing 10% fetal calf serum at 37 °C; medicated when cells grew to cover 40∼50% culture flask surface. Experimental group: added 5-aza-2′-deoxycytidine (DAC) (final concentration: 5 μM), changed the culture solution every 24 h, and increase the final concentration of DAC to 5 μM; control group: added PBS of equal quantity and changed the solution every 24 h. Collect cells when cells grew to cover 80%∼90% culture flask surface after 72 h.

2.3. Detection of methylation of CES2, UGT1A1 and GUSB genes in colorectal cancer cell strains

The design for methylation specific PCR (MSP) primers is in accordance with the following principles:

- at least one CpG island is in the primers sequences. Furthermore, this CpG island is located near **3**′-region;
- many **C**-terminals without CpG island are in the primers sequences;
- both the number and position of CpG island are identical in DNA methylated and unmethylated primers;
- other priciples are consistent to regular PCR.

Collect the genome DNA of eight cell strains after treatment by using TIANamp Genome DNA Kit respectively, took 2 μg genome DNA and used CpGenome DNA Modification Kit to modify the DNA sample with hydrosulfite. Its principle is that: hydrosulfite enables deamination of unmethylated cytosine in the DNA sequence, thus becoming uracil, however, the methylated cytosine cannot be deaminized, so the basic group will not change. A master mix containing the reaction buffer, dNTPs, Taq polymerase, and 1.6 μl cDNA in 20 μl reaction mixture was transferred to different PCR tubes. Forward and reverse primers corresponding to different individual genes were added to the PCR tubes and subjected to PCR amplification using primer sets directed against CES2M/UGT1A1M/GUSBM, and CES2U/UGT1A1U/GUSBU. The annealing temperature was 53 °C for these primers. The primers are showed in Table 1. Methylated or unmethylated were simultaneously amplified in same reaction tube using the above mentioned. When methylated and unmethylated products were amplified in a specimen, simultaneously was considered hemimethylation. Meanwhile, hemimethylation is also called positive methylation. Placenta DNA has been proved to be unmethylated DNA. Therefore, placenta tissue DNA dealt with M.SssI methyltransferase would be used for modifying DNA CpG island methylation kit and PCR amplification would be used as methylated positive control, while placenta tissue DNA not dealt with M.SssI methyltransferase

Table 1
MSPCR primers for CES2, UGT1A1 and GUSB gene.

Name of primer	Sence primer	Antisence	Primer size (bp)
CES2/M	GTCGTTATAGGTCGTTTTTTAGAGC	CAACGATAATAATTCCGCGAT	108
CES2/U	TGTTATAGGTTGTTTTTTAGAGTGT	AAATCAACAATAATAATTCCACAAT	110
UGT1A1/M	AATATAAGGTAGGTAGGTTTTACGG	TTTTATAATTAAAATTTTCAACGCT	211
UGT1A1/U	AATATAAGGTAGGTAGGTTTTATGG	TTTTATAATTAAAATTTTCAACACT	211
GUSB/M	TGGGGAGTAGATTTCGTTTTTATC	GTAATACGCCTAAAACCATCCG	173
GUSB/U	GGGAGTAGATTTTGTTTTTATTGG	TCATAATACACCTAAAACCATCCAC	173

M: methylation; U: unmethylation.