World Journal of Gastroenterology

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DOI: 10.3748/wjg.v2.i4.232

World J Gastroenterol 1996 December 15; 2(4): 232-235
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Preliminary study on mechanism of drug resistance in human colon cancer cell line HCT$_{V2000}$

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Author contributions: All authors contributed equally to the work.

Supported by the Key Project of the 8th Five-Year Plan of China, No. 85-914-03-10

Original title: *China National Journal of New Gastroenterology* (1995-1997) renamed *World Journal of Gastroenterology* (1998-).

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Received: July 31, 1996
Revised: August 26, 1996
Accepted: September 18, 1996
Published online: December 15, 1996

Abstract

AIM: To investigate the mechanisms of multidrug resistance in human colon cancer cell line HCT$_{V2000}$.

METHODS: P-glycoprotein (P-GP) was detected by immunocytochemical staining. Expression and amplification of *mdr1* gene were detected by nucleic acid hybridization. Cytotoxicity was measured by MTT method.

RESULTS: HCT$_{V2000}$ cells showed a positive response to monoclonal antibodies against P-GP encoded by the *mdr1* gene. Overexpression of *mdr1* mRNA was found, but no evidence of *mdr1* gene amplification or rearrangement was suggested. Verapamil increased the accumulation of ^{3}H-VCR inside the cells and partially reversed drug resistance to VCR.

CONCLUSION: Overexpression of *mdr1* mRNA is one of the important mechanisms of drug resistance in HCT$_{V2000}$ cell line; however, there may be some other mechanisms which are also involved in this.

Key words: Colon neoplasms; Drug resistance; Cell line

Zhang XH, Li XT, Ji XJ, Zhu YJ. Preliminary study on mechanism of drug resistance in human colon cancer cell line HCT$_{V2000}$. *World J Gastroenterol* 1996; 2(4): 232-235 Available from: URL: http://www.wjgnet.com/1007-9327/full/v2/i4/232.htm DOI: http://dx.doi.org/10.3748/wjg.v2.i4.232

INTRODUCTION

Investigating the mechanisms of multidrug resistance (MDR) is one of the methods of raising chemotherapy effects in cancer. Drug-resistant cell lines are an important model system for the analysis of *mdr1* gene in human cancer cells. The protein product of *mdr1* gene, P-glycoprotein (P-GP), is an energy-dependent drug efflux pump, which decreases intracellular drug accumulation. MDR cell lines have wide cross-resistance to hydrophobic compounds[1]. Verapamil (VRM) may reverse MDR in these cells by competing with antitumor drugs for binding to P-GP[2]. HCT$_{V2000}$, an MDR cell line of human colon cancer HCT-8 cells, was developed by stepwise selection on exposure to increasing doses of vincristine (VCR). In this report we aimed to study expression and amplification of *mdr1* gene, and the effect of reversal of VCR resistance with verapamil, as well as to analyse the mechanisms of drug resistance in HCT$_{V2000}$ and its parent line.

MATERIALS AND METHODS

Cell lines and cell culture

The HCT-8 line of human colon cancer cells was obtained from the American Type Culture Collection. HCT$_{V2000}$ cells were 147-fold more resistant to VCR than HCT-8 cells. Cells were grew in monolayer in RPMI-1640 with 10% newborn calf serum, penicillin (100 U/mL) and streptomycin (100 μg/mL). The cultures were incubated at 37℃ in a humidified atmosphere of 5% CO_2.

Survival assay by MTT assay[3]

Cells were seeded onto 96-well plates at a density of 2000 cells per well. Drugs were added 24 h later. After incubation at 37℃ for 96 h, 100 μL of 0.5 mg/mL MTT reagent (FLUKA) was added to each well. The plates were then incubated for 4 h, and DMSO was added before absorption at 540 nm was measured with a Dynatech MR700 ELISA plate reader.

Immunocytochemical staining

JSB-1 (1:120) and MRK-16 (5 μg/mL), monoclonal antibodies against P-GP were provided by Dr. Scheper and Dr. Tsuruo, respectively. Expression of P-GP was detected with avidin-biotin peroxidase complex (ABC) method as described by Grogan *et al*[4]. ABC kits were produced by Vector Laboratories, Inc.

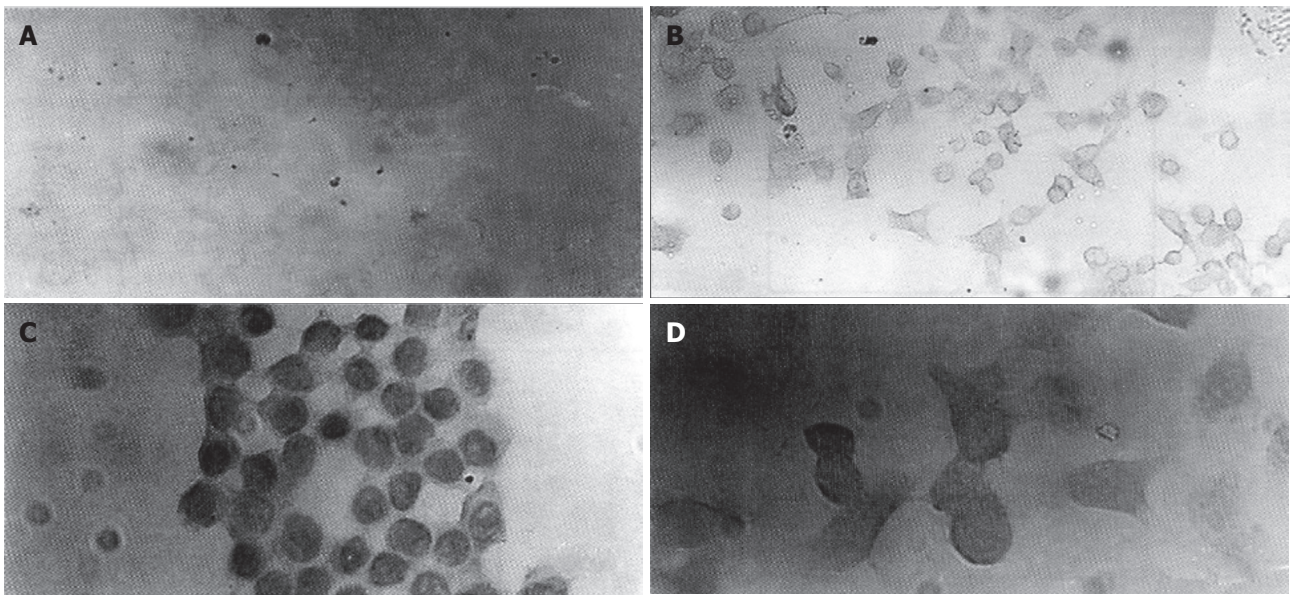

Figure 1 Demonstration of P-glycoprotein in HCT-8 (A and C) and HCT$_{V2000}$ cells (B and D) by immunocytochemical staining with JSB-1 (A and B) and MRK-16 (C and D).

DNA and RNA isolation

Total cellular RNA was prepared by acid-phenol extraction method[5]. Genomic DNA was isolated from cells by the method of Maniatis *et al*[6].

DNA and RNA analysis[6]

DNA (10 μg) was digested with the restriction endonuclease *Eco*R Ⅰ, electrophoresed on a 0.7% agarose gel, and transferred to nylon filters for Southern blot analysis. Slot blotting filters were prepared with DNA using a BRL blot apparatus. RNA (10 μg) was separated on 1.2% agarose gels containing formaldehyde, and then transferred to a nylon filter. DNA and RNA were cross-linked to the filter by baking for 1 h at 80℃ in an oven. A ^{32}P-labeled DNA probe, a 1.383-kb *Eco*R Ⅰ fragment containing the *mdr1* coding regions from PHDR5A, was labeled by the random priming method. PHDR5A was provided by Dr. Gottesman (NCI, Bethesda, MD, United States).

Drug accumulation

Cells were plated at a density of 5 × 10^5 cells/mL and incubated overnight at 37 ℃. Then the medium was replaced with serum-free RPMI1640, and the cells were incubated for 1 h with ^{3}H-VCR (4.6 × 10^{10} Bq) in the absence or presence of VRM. The cells were washed three times with cold PBS. NaOH solution (1 mol/L) was added to dissolve the cells, and the solution was neutralized with acetic acid. The radioactivities were determined by liquid scintillation counting.

RESULTS

Expression of P-GP

Immunocytochemical staining showed that P-GP could be detected in plasma membrane and cytoplasm of HCT$_{V2000}$ cells with both JSB-1 and MRK-16. However, in HCT-8 cell line, a low level of P-GP expression was detected with MRK-16, but not with JSB-1 (Figure 1).

Expression of mdr1 mRNAt

Northern blot analysis was conducted with RNA isolated from the two cell lines. The *mdr1* specific probe was hybridized to a 4.5 kb RNA species, which showed high expression in HCT$_{V2000}$, but no *mdr1* mRNA was detected in HCT-8 (Figure 2).

Amplification of mdr1 gene

To determine the possibility that overexpression of *mdr1* mRNA in HCT$_{V2000}$ cell line might result from *mdr1* gene amplification or rearrangement, genomic DNA from HCT$_{V2000}$ and HCT-8 was analyzed. DNA slot blot analysis showed that the signal intensity from HCT$_{V2000}$ was the same as that from HCT-8 cells, and there was no apparent amplification of *mdr1* gene in HCT$_{V2000}$ cells (Figure 3). Hybridization of the Southern blot revealed that *mdr1* probe, pHDR5A, was hybridized to five *Eco*R Ⅰ fragments in both cell lines and the size and the signal intensity of the five bands from the two cell lines were similar (Figure 4). It is therefore suggested that neither amplification nor rearrangement exists in the HCT$_{V2000}$ cell line and that overexpression of the *mdr1* mRNA in HCT$_{V2000}$ cells may be correlated with activated transcription of *mdr1* gene.

Effect of VRM

Reversal of multidrug resistance. Nontoxic or slightly toxic (growth inhibition did not exceed 10%) concentrations of VRM were used in these experiments. The IC$_{50}$ values of HCT$_{V2000}$ and HCT-8 cells to VCR were 3651.8 and 24.9 nmol/L respectively, and the index of resistance was approximately 167 when compared with each other. VRM enhanced the cytotoxicity of VCR in both cell lines. A 84- and 4-fold increase in cytotoxicity was produced by 10 μg/mL VRM in HCT$_{V2000}$ and HCT-8 cells, respectively. Reversal of MDR by VRM in the HCT$_{V2000}$ cell line was more effective than that in the HCT-8 cell line (Figures 5 and 6).

Increase of ^{3}H-VCR intracellular accumulation. The intracellular accumulation of ^{3}H-VCR in HCT$_{V2000}$ cells was lower than that in HCT-8 cells. VRM increased the accumulation of ^{3}H-VCR in both cell lines. According to the drug sensitivity test (Table 1), the drug resistance of HCT$_{V2000}$ cells was related to the decrease in intracellular drug accumulation. The ability of VRM to enhance VCR cytotoxicity was directly related to the ability to increase intracellular drug accumulation. VRM at 10 μg/mL increased ^{3}H-VCR accumulation in HCT$_{V2000}$ to a level comparable to HCT-8. Although the intracellular concentration of ^{3}H-VCR was similar in both cell lines, HCT$_{V2000}$ cells maintained 8-fold higher resistance to VCR than HCT-8 cells did.

DISCUSSION

Human colon cancer is intrinsically resistant to chemotherapeutic agents and the exact mechanism of drug resistance is not clear yet. There existed different results in studying the mechanisms of multidrug resistance in the human colon cancer cell line HCT-8. Klohs's study on HCT-8 cells showed that although verapamil increased adriamycin accumulation and cytotoxicity, surface labelling failed to detect a glycoprotein with appropriate molecular size due to the insensitivity of the technique used[7]. Hunter *et al*[8], however, detected the expression of P-GP in HCT-8 cells by immunoprecipitation with MAb JSB-1. In this report overexpression of P-GP in both cell lines was observed, and the intensity of the protein staining in HCT$_{V2000}$ cells was much stronger than that in HCT-8 cells. Drug resistance in both cell lines can be overcome by VRM, which inhibits the function of P-GP. These results can be used to explain the P-GP mechanism of intrinsic drug resistance and acquired drug resistance in this kind of colon cancer cells. JSB-1 and MRK-16 recognize different epitopes in plasma membrane or within cytoplasm and the results would be different under different experimental conditions[4].

Table 1 Effect of Verapamil on intracellular accumulation and cytotoxicity of vincristine (mean ± SD)

VRM (μg/m)	HCT-8 cell line		HCT$_{V2000}$	
	Drug accumulation[1]	Relative ID50	Drug accumulation[1]	Relative ID50
0	0.60 ± 0.07	1	0.42 ± 0.03	146.66
5	1.49 ± 0.05	0.32	1.08 ± 0.05	2.05
10	2.06 ± 0.21	0.2	1.89 ± 0.13	1.73

[1]Pmol/5 × 10^5 cells, n = 3.

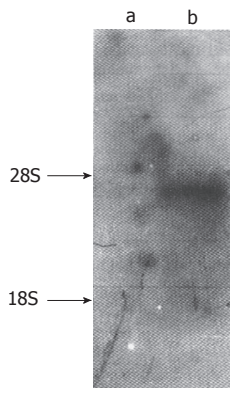

Figure 2 Northern hybridization. Ten micrograms of total cellular RNA were loaded for each line. a. HCT-8 cell line; b. HCT$_{V2000}$ cell line.

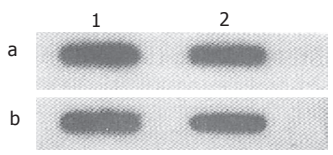

Figure 3 Slot blot hybridization. Twenty micrograms (1) or 10 μg (2) DNA from HCT-8 cell line (a) and HCT$_{V2000}$ cell line (b) were analyzed.

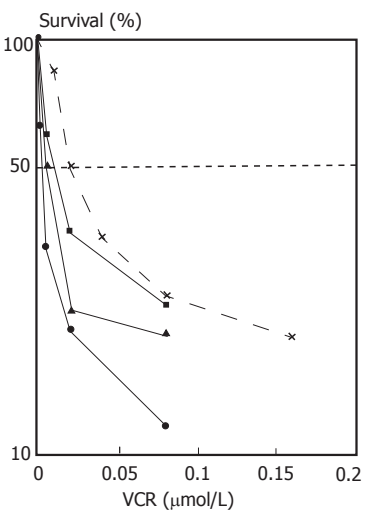

Figure 5 Effect of VRM on sensitivity of HCT-8 cells to VCR. The doses of VRM were 0 (x), 2.5 (■), 5 (▲) and 10 (●) μg/mL.

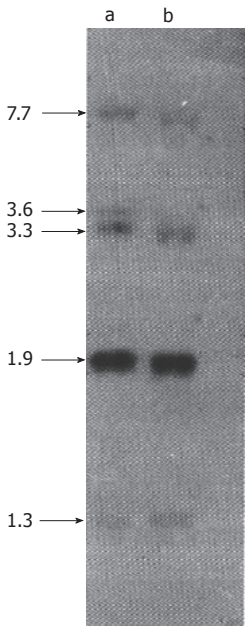

Figure 4 Southern hybridization of mdr1 gene. Ten micrograms of DNA extracted from HCT-8 cell line (a) and HCT$_{V2000}$ cell line (b) were digested with *Eco*R Ⅰ and separated on a 0.7% agarose gel. The arrows indicate the position of hybridization.

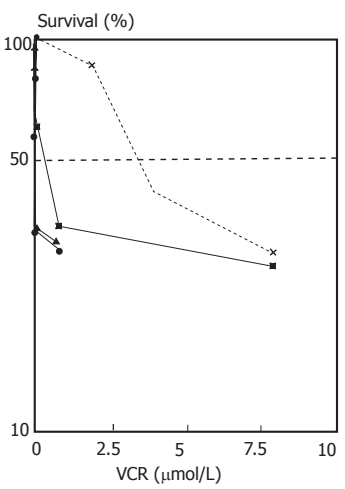

Figure 6 Effect of VRM o-n sensitivity of HCT$_{V2000}$ cells to VCR. The doses of VRM were o (x), 2.5 (■), 5 (▲) and 10 (●) μg/mL.

Our results suggest that combination of different monoclonal antibodies could reduce false negative rate.

The drug resistance mediated by P-GP is considered as typical MDR. P-GP is encoded by *mdr1* gene in human MDR cells. Overexpression of *mdr1* gene is frequently found in some types of human tumors, including colon cancer, but the amplification of *mdr1* gene has not been reported yet. In this study, we failed to detect the expression of *mdr1* mRNA in HCT-8 cells by Northern blot hybridization probably due to the low levels of mRNA in this cell line and insensitivity of the method. Expression of *mdr1* mRNA was at a high level but no gene amplification or rearrangement was observed in HCT$_{V2000}$ cells, being consistent with observations of clinical specimens. The overexpression of P-GP in this cell line may be due to activated transcription of *mdr1* gene. Shen *et al* reported that KB cell lines with low level of drug resistance did overexpress *mdr1* mRNA without *mdr1* gene amplification. However, expression of *mdr1* mRNA was increased simultaneously with amplification of *mdr1* gene in high-level drug resistant KB cell lines (40-fold)[9]. No *mdr1* gene amplification was found in HCT$_{V2000}$ cell line, which is about 146-fold more resistant to VCR than HCT-8 cell line. There may be two possibilities: expression of *mdr1* mRNA may not always be accompanied by amplification of *mdr1* gene in cells with high levels of drug resistance, and the activation of *mdr1* gene may not be the only mechanism for the drug resistance in this cell line.

Drug accumulation and cytotoxicity assays showed that VRM increased VCR intracellular accumulation and reversed drug resistance in HCT$_{V2000}$ cell line. This provides evidence for the P-GP mediated mechanisms of drug resistance of colon cancer cells. But there is not much correspondence between the amount of ^{3}H-VCR intracellular accumulation and the level of drug resistance in HCT$_{V2000}$ cell line. VRM can only reverse drug resistance partially while VCR intracellular accumulation is increased perfectly in this cell line. All of these assays demonstrate that other mechanisms of drug resistance besides P-GP may exist in this cell line. The cell line HCT$_{V2000}$ may be a useful model

for studying the mechanisms of drug resistance in human colon cancer.

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S- Editor: Yang ZD **L- Editor**: Wang TQ **E- Editor**: Li RF

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ISSN 1007-9327
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