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• GASTRIC CANCER •

# Expression and function of classical protein kinase C isoenzymes in gastric cancer cell line and its drug-resistant sublines

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## Abstract

AIM: **To investigate the expression and function of classical protein kinase C (PKC) isoenzymes in inducing MDR phenotype in gastric cancer cells.**

METHODS: **Two cell lines were used in the study: gastric cancer cell SGC7901 and its drug-resistant cell SGC7901/VCR stepwise-selected by vincristine 0.3, 0.7 and 1.0mg·L$^{-1}$, respectively. The expression of classical PKC (cPKC) isoenzymes in SGC7901 cells and SGC7901/VCR cells were detected using immunofluorescent cytochemistry, laser confocal scanning microscope and Western blot. The effects of anti-PKC isoenzymes antibody on adriamycin accumulation in SGC7901/VCR cells were determined using flow cytometric analysis.**

RESULTS: **(1) SGC7901 cells exhibited positive staining of PKC-α. SGC7901/VCR cells exhibited stronger staining of PKC-α than SGC7901 cells. The higher dosage vincristine selected, the much stronger staining of PKC-α was observed on SGC7901/VCR cells. (2) Both SGC7901 and SGC7901/VCR cells exhibited positive staining of PKC-βI and PKC-βII with no significant difference. (3) Compared with SGC7901, SGC7901/VCR cells had decreased adriamycin accumulation and retention. Accumulation of adriamycin in SGC7901 was 5.21±2.56mg·L$^{-1}$,in SGC7901/VCR 0.3 was 0.85±0.29 mg·L$^{-1}$, in SGC7901/VCR 0.7 was 0.81±0.32mg·L$^{-1}$, and in SGC7901/VCR 1.0 was 0.80±0.33mg·L$^{-1}$; Retention of adriamycin in SGC 7901 was 2.51±1.23mg·L$^{-1}$, in SGC7901/VCR 0.3 was 0.47±0.14mg·L$^{-1}$, in SGC7901/VCR 0.7 was 0.44±0.15mg·L$^{-1}$, and in SGC 7901/VCR 1.0 was 0.41±0.11mg·L$^{-1}$. (4) Fluorescence intensity presented adriamycin accumulation in SGC7901/VCR cells was increased from 1.14±0.36 to 2.71±0.94 when cells were co-incubated with anti-PKC-α but not with anti-PKC-βI, PKC-αII and PKCγ antibodies.**

CONCLUSION: **PKC-α, but not PKC-βI, PKC-βII or PKCγ, may play a role in multidrug resistance of gastric cancer cells SGC7901/VCR.**

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## INTRODUCTION

Multi-drug resistance (MDR), the principal mechanism by which many cancers develop resistance to a variety of chemotherapeutic drugs, is a major factor in the failure of many forms of chemotherapy[1-4]. It affects patients with numerous blood cancers and solid tumors. Cellular drug resistance is mediated by different mechanisms operating at different steps of the cytotoxic action of the drug from a decrease of drug accumulation in the cell to the abrogation of apoptosis induced by the chemical substance. Several different mechanisms will switch on in the MDR cells, but usually one major mechanism is operating. The most investigated mechanisms with known clinical significance are: (1) activation of transmembrane proteins effluxing different chemical substances from the cells, in which P-glycoprotein (P-gp) is the most known efflux pump; (2) activation of the enzymes of the glutathione detoxification system; (3) alterations of the genes and the proteins involved into the control of apoptosis (especially p53 and Bcl-2)[5-12]. PKC comprises a family of at least 13 distinct serine/threonine kinase isoenzymes involved in signal transduction pathways that govern a wide range of physiological processes including differentiation, proliferation, gene expression, brain function, membrane transport and the organization of cytoskeletal and extracellular matrix proteins[13-26]. Recently accumulated evidence indicates that PKC activity, especially cPKC, plays a significant role in the formation of tumor MDR. The isoenzymes possess distinct differences in localization in different cells. Within a single cell, PKC isoforms also exhibit differences in expression and function, so research on distinct function in tumor MDR of isoenzymes has important significance in screening drugs with high specificity that could reverse MDR and in disclosing the mechanism of MDR formation and its regularity of the reversion.

## MATESIALS AND METHODS

### *Materials*

Human gastric cancer cell line SGC7901 was reserved by our institute and its drug-resistant sublines SGC7901/VCR were stepwise-selected by vincristine 0.3, 0.7 and 1.0mg·L$^{-1}$, respectively. RPMI 1640 medium was the product of Gibco (U.S.A.). Newborn bovine serum was purchased from Hyclone (U.S.A.). Chemical drugs vincristine and adriamycin were purchased from Farmitalia Carlo Erba

(U.S.A.) and Minsheng (Hangzhou, China). Rabbit-anti-human polyclonal antibody PKC-α, PKC-βI, PKC-βII and PKCγ were the products of Santa Cruz Biotechnology. SABC immunohischemistry kit and the HRP labeled goat-anti-rabbit IgG was purchased from Boste (Wuhan, China). FITC labeled goat-anti-rabbit IgG was purchased from Zhongshan (China).

### Methods

**Immunofluorescent cytochemistry** The expression of PKC isoenzymes were detected by routine immunocytochemical fluorescence method[27,28].The procedures were as follows. Cells were maintained at 37℃ in a 50mL·L$^{-1}$ $CO_2$-humidified incubator in RPMI 1640 medium supplemented with 25mmol·L$^{-1}$ HEPES buffer and 100mL·L$^{-1}$ new born bovine serum. SGC7901/VCR cells were cultured in the medium with extra adding vincristine at the concentration of 0.3, 0.7 and 1.0mg·L$^{-1}$, respectively. Cells at exponential phage were harvested, digested by 2.5g·L$^{-1}$ trypsin and then cultured on the slides in the medium described above at 37℃ for further 24h; RPMI 1640 medium was then washed by PBS and cells were fixed in cold acetone for 5min; 50mL·L$^{-1}$ $H_2O_2$ was added and incubated at room temperature for 10-15min and added 3g·L$^{-1}$ TritonX-100 for another 15min; normal goat serum (1:10) was added and incubated at room temperature for 30min; rabbit-anti-human polyclonal antibody PKC-α, PKC-βI, PKC-βII and PKCγ (1:100) were added respectively and incubated at 4℃ over night; FITC labeled goat-anti-rabbit IgG was added and incubated at 37℃ for 1h; the slides were sealed by 500mL·L$^{-1}$ glycerin buffer and observed with a fluorescence microscope. Unrelated monoclonal antibody and PBS were used as negative controls.

**Laser confocal scanning microscope** The laser confocal scanning microscope protocols used were as described[29,30].Methods of cell culture and stain with Ab were carried out as described in immunocytochemical fluorescence method. FITC labeled goat-anti-rabbit IgG was added in the darkness and incubated at room temperature for 3h; the slides were sealed by 500mL·L$^{-1}$ glycerin buffer and observed with a laser confocal scanning fluorescence microscope. Unrelated monoclonal antibody and PBS were used as negative controls.

**SDS-PAGE** According to Chen et al and Xiao *et al*[31-33], cells at exponential phase were harvested and washed by cold PBS and suspended in extraction buffer (50mmol·L$^{-1}$ Tris-Cl (pH7.5), 150mmol·L$^{-1}$ NaCl, 0.2mmol·L$^{-1}$ EDTA, 1mmol·L$^{-1}$ PMSF and 10g·L$^{-1}$ NP-40). The homogenate was heated for 5min in a boiling water bath and then centrifuged. The supernatants were harvested and the protein concentrations were assayed by Bradford method. 150μg total protein were electrophoresed on SDS-polyacrylamide gels with the stacking and the separating gels containing 50 and 100g·L$^{-1}$ acrylamide, respectively, and the gels were stained with Coomassie brilliant blue dye.

**Western blot analysis** According to She *et al* [34],after SDS-PAGE, proteins were transferred onto nitrocellulose membrane under a constant current of mA for 1h. Non-specific binding sites were blocked by PBS with 50mL·L$^{-1}$ milk plus 1g·L$^{-1}$ Tween-20 at room temperature. Primary and secondary antibodies were rabbit-anti-human polyclonal antibody PKC and HRP labeled goat-anti-rabbit IgG, respectively. Films were exposed in DAB detection reagent to develop color of bands.

**Flow cytometric analysis** According to Jiang *et al*[35] and Feng *et al*[36], cells were cultured in 6-well culture plates at 37℃ for 48h, adriamycin was added to the final concentration of 5mg·L$^{-1}$. After further culture for 1h, rabbit-anti-human polyclonal antibody against different cPKC isoenzymes was added and incubated for 40min, PBS and normal rabbit serum were used as negative controls. And then, cells were harvested or cultured in drug-free medium for another 30 min and harvested. The harvested cells of the phases were suspended in cold PBS, intracellular adriamycin fluorescence intensity was determined by flow cytometric analysis with the stimulative and acceptant wave length at 488nm and 575nm, respectively.

**Statistical analysis** Data were presented as $\bar{x}\pm s$. Significant differences were determined by using ANOVA in statistical software SPSS10.0.

## RESULTS

### Immunofluorescent cytochemistry

To investigate the expression of PKC isoenzymes of SGC7901 cells and its drug-resistant cell subline SGC7901/VCR. immunofluorescent cytochemistry was performed. The positive signals were of fluorescent signals. Both SGC7901 cells and SGC7901/VCR cells expressed PKC-α, PKC-βI, PKC-βII and PKCγ. The expression of PKC-α was stronger in SGC7901/VCR cells than that in SGC7901 cells. There was no significant difference in the expression of PKC-βI and PKC-βII between SGC7901/VCR cells and SGC7901 cells. And the expression of PKCγ in SGC7901/VCR cells was positive as strongly as that in SGC7901 cells, and also no significant difference was found.

### Laser confocal microscope analysis

PKC-α expressed in both SGC7901 cells and SGC7901/VCR cells. The positive signals were localized in cytoplasm and membrane. Compared with SGC7901 cells, the intensity of fluorescence in SGC7901/VCR cells was increased significantly when analysed by the intensity of pixel by using computer, which was 100 in SGC7901/VCR cells and 80 in SGC7901 cells.

### Western blot

The expression of PKC-α was significantly higher in SGC7901/VCR cells than that in SGC7901 cells, in which expression increased with the increase of drug-dose-resistance of SGC7901/VCR cells. No significant difference was found in the expression of PKC-βI, PKC-βII and PKCγ between SGC7901/VCR cells and SGC7901 cells (Figure 1).

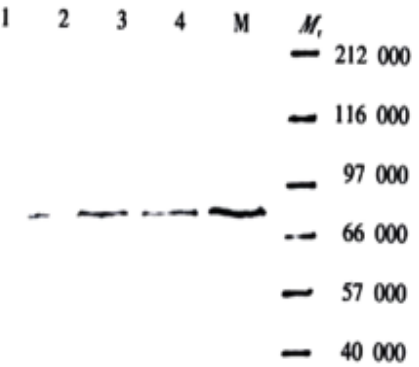


**Figure 1** Western blot (detected with anti-PKC-α antibody)

### Flow cytometric analysis

The effects of anti-PKC-α or βI antibody on adriamycin accumulation and retention in SGC7901/VCR cells were determined by flow cytometric analysis. When cells were cultured in drug-RPM1640, intracellular drug concentration would increase and finally stabilised at the highest plateau value, which was called adriamycin accumulation. When cells were cultured in drug-free medium, drug was effluxed from cells and subsequently, drug concentration stabilised at a lower plateau value, which was still higher than the

initial value and so called adriamycin retention. The results presented by the values of fluorescence intensity showed that adriamycin accumulation and retention decreased in SGC7901/VCR cells than that in SGC7901 cells. When co-incubated with anti-PKC-α antibody, the accumulation of adriamycin in MDR cells increased and showed partly dose-dependent effect, while PKC-βI, PKC-βII and PKCγ could not influence the ADR accumulation in SGC7901/VCR cells (Table 1,2).

**Table 1** Adriamycin accumulation and retention in cells by flow cytometric analysis ($\bar{x}\pm s$ fluorescence intensity)

| Adriamycin | SGC7901 | SGC7901/VCR in different resistant drug dose (VCR.mg·L$^{-1}$) | | |
|---|---|---|---|---|
| | | 0.3 | 0.7 | 1.0 |
| Accumulation | 5.21±2.56 | 0.85±0.29[b] | 0.81±0.32[b] | 0.80±0.33[b] |
| Retention | 2.51±1.23 | 0.47±0.14[b] | 0.44±0.15[b] | 0.41±0.11[b] |

[b]$P$<0.01, *vs* SGC7901.

**Table 2** Effects of adriamycin accumulation in SGC7901/VCR cells by anti-PKC isoenzymes Ab ($\bar{x}\pm s$ fluorescence intensity)

| Group | ρ(anti-PKC isoenzymes Ab)/(μg·L$^{-1}$) | | | |
|---|---|---|---|---|
| | 0 | 25 | 250 | 500 |
| Anti-PKC-α Ab | 1.14±0.36 | 1.09±0.32 | 2.49±0.84[b] | 2.71±0.94[b] |
| Anti-PKC-βIAb | 1.14±0.36 | 1.13±0.38 | 1.14±0.39 | 1.14±0.39 |
| Anti-PKC-αIIAb | 1.14±0.36 | 1.14±0.38 | 1.14±0.40 | 1.14±0.39 |
| Anti-PKC-γAb | 1.14±0.36 | 1.14±0.39 | 1.14±0.40 | 1.14±0.39 |

[b]$P$<0.01, *vs* 0μg·L$^{-1}$ anti-PKC isoenzymes Ab

## DISCUSSION

The development of resistance to chemotherapeutic agents remains one of the major obstacles for successful cure of cancer patients. Tumor cells may acquire MDR in the course of exposure to various compounds that are used in modern anticancer therapy, including cytotoxic drugs and differentiating agents. Therefore, the recurrence of the disease after the initial treatment may be associated with establishment of secondary MDR in the residual tumor. Research on resistance to cancer treatment was mainly focused for 20 years on MDR. No useful method of reversing MDR, suitable for clinical use, has yet emerged from this large quantity of work. The reason could be an complicated mechanism involved in it. There are several ways for cancer cells to develop resistance or defense mechanisms against cytotoxic drugs[37-43].

Resistance to therapy has been correlated to the presence of at least two molecular "pumps" that actively expel chemotherapeutic drugs from the tumor cells. This action thus spares tumor cells from the effects of the drug, which has to act inside the cells at the nucleus or the cytoplasm. The two pumps commonly found to confer MDR in cancer are P-gp and multidrug resistance-associated protein (MRP). But they can not explicate the phenomenon of MDR fully. It also reported that some cancer cells are resistant to signal of apoptosis and so making cell life longer might confer to the MDR phenotype.

Recent studies have indicated that the signal of phosphorylation might be an important part of MDR mechanisms. PKC isoforms are often overexpressed in disease states such as cancer and play a critical role in regulation of long term cellular events such as proliferation, differentiation and tumorigenesis. An increase in PKC activity might result in an oncogenic role and in MDR. Several studies indicate a role for PKC in the regulation of the MDR phenotype, since several PKC inhibitors are able to partially reverse MDR and inhibit P-gp phosphorylation.

The PKC family consists of several isoforms comprising three groups: classical, novel and atypical. PKC isoforms are widely distributed in mammalian tissues and have many important physiological functions[44-47]. cPKC subfamily shows significant specificity in tissue distribution. The isoenzymes possess distinct differences in localization in different cells. Within a single cell, PKC isoforms also exhibit differences in their distribution before and after their translocation following activation. For example, thymus cells express PKC-α and PKC-βI but not PKC-βII and PKCγ; Cortical and medullary cells of suprarenal gland express PKC-α, while the cortical cells also express PKC-βI and PKCγ.

To date in recent years, the MDR phenotype is also associated with variation in content of PKC isoenzymes. Different isoforms possess distinct differences in expression and function in different MDR cells. It has been confirmed that sensitive cells show the phenotype of MDR when transfected with cDNA encoding PKC-α, which indicats the effect of PKC on MDR. Resistance to ADR of mouse leukemia MDR cell-line could be reversed by anti- PKC-βmAb when it was incubated with anti-PKC-α or anti-PKC-βmAb[48-60].

This study confirmed that PKC-α, PKC-βI,PKC-βII and PKCγ were expressed in both SGC7901 cells and SGC7901/VCR cells. Our results showed that the expression of PKC-α was significantly higher in SGC7901/VCR cells than that in SGC7901 cells and the expression increased with the increase of drug-dose-resistance of SGC7901/VCR cells. There was no significant difference in the expression of PKC-βI, PKC-βII and PKCγ between SGC7901/VCR cells and SGC7901 cells. The result of flow cytometric analysis showed that ADR accumulation decreased in SGC7901/VCR cells much than that in SGC7901 cells, together with increase of the expression of PKC-α. Further study confirmed that anti-PKC-α antibody could reverse ADR accumulation in MDR cells to some degree and showed partly does-dependent effect, while PKC-βI, PKC-βII and PKCγ could not influence the ADR accumulation in SGC7901/VCR cells. The results suggested that the formation of MDR in SGC7901/VCR cells was associated with over expression of PKC-α but not with PKC-βI, PKC-αII and PKCγ. Since isoenzymes of PKC possess only 1-10 amino acid in there pseudo substrate action site in C1 domain, research on distinct function in tumor MDR of isoenzymes has important significance in screening effective drugs with high specificity that could reverse MDR and in disclosing the mechanism of MDR formation and its regularity of the reversion. Because gastric cancer is common in China and some areas in the world[61-80], this results may be important for further study.

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