

# Effects of cell membrane phospholipid level and protein kinase C isoenzyme expression on hepatic metastasis of colorectal carcinoma

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**BACKGROUND:** The molecular mechanism of hepatic metastasis of colorectal cancer is not well understood. The aim of this study was to assess the relations between phospholipid contents of cellular membrane and isoenzyme expression of protein kinase C (PKC) and their effects on hepatic metastasis of colorectal cancer.

**METHODS:** High performance liquid chromatography was used to detect contents of cell membrane phospholipids: phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) in primary foci, paratumor mucosa and hepatic metastatic foci in patients with colorectal carcinoma. The mRNA expression levels of PKC- $\alpha$ , - $\beta$ II, - $\delta$ , - $\epsilon$ , - $\lambda$ , - $\zeta$  isoenzymes were detected with the QRT-PCR technique.

**RESULTS:** The levels of PI, PC and PE in primary foci and hepatic metastatic foci were higher than those in paratumor mucosa. The level of PE in hepatic metastatic foci was much higher than that in primary foci ( $t=98.88$ ,  $P<0.01$ ); but the levels of PI and PC were not significantly different between primary foci and hepatic metastatic foci ( $t=1.73$ ,  $1.36$ ,  $P>0.05$ ). The expression levels of PKC- $\beta$ II, - $\delta$ , - $\epsilon$ , - $\lambda$ , - $\zeta$  were enhanced in primary foci and hepatic metastatic foci, but the level of PKC- $\alpha$  in primary foci was decreased as compared with that in paratumor mucosa. The levels of PKC- $\delta$ , - $\epsilon$ , - $\lambda$ , - $\zeta$  in hepatic metastatic foci were higher than those in primary foci. A positive correlation was observed between the expression levels of PI, PC and PKC- $\beta$ II and also between those of PE and PKC- $\delta$ , - $\epsilon$ , - $\lambda$ , - $\zeta$ . However, there was a close negative correlation between PE and PKC- $\alpha$ .

**CONCLUSION:** Increased levels of PI and PC and decreased ratio of PKC- $\alpha$  to PKC- $\beta$ II are related to colorectal

cancer genesis. Increased levels of PE, increased expression of PKC- $\delta$ , - $\epsilon$ , - $\lambda$ , - $\zeta$  isoenzymes and decreased level of PKC- $\alpha$  are related to hepatic metastasis in colorectal carcinoma.

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**KEY WORDS:** colorectal carcinoma; membrane phospholipid; protein kinase C; hepatic metastasis

## Introduction

Recurrence of colorectal carcinoma and hepatic metastasis causes death of patients who has undergone radical excision of primary carcinoma of the large intestine.<sup>[1-8]</sup> Approximately 50% to 60% of patients with colorectal cancer will develop hepatic metastasis during the course of disease. Hepatic metastasis of colorectal carcinoma is a multistage process involving epigenetic changes in signal transduction pathways, resulting in progressive deregulation of cell proliferation and survival mechanisms.<sup>[9-16]</sup> Protein kinase C (PKC) belongs to a family of 12 distinct serine/threonine kinases that participate in signal pathways involved in cellular proliferation, differentiation, and apoptosis in diverse cell systems.<sup>[17-21]</sup> Differences in tissue expression predict that individual PKC isoenzymes have distinct cellular functions. Studies of the expressions of PKC isoenzymes in human colorectal tumors<sup>[22-27]</sup> have shown the altered PKC isoenzyme levels in colorectal carcinomas.<sup>[28-31]</sup> Nevertheless, little is known about the correlations and the interactions of cellular membrane phospholipids and PKC isoenzymes and their effects on hepatic metastasis of colorectal carcinoma. To better understand signal pathways and biomolecular mechanisms of hepatic metastasis, we examined the levels of cell membrane phospholipids and PKC isoenzyme mRNA expressions in primary foci, paratumor mucosa, and hepatic metastatic foci of colorectal carcinoma.

## Methods

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### Patients and samples

Samples were taken from 58 colorectal cancer patients including 33 men and 25 women, aged from 26 to 85 years (average 56 years). Among them 25 out of 58 patients had hepatic metastasis. The samples were collected from fresh surgical specimens of primary foci, paratumor mucosa and hepatic metastatic foci. The samples were reserved in liquid nitrogen. Pathological examination was performed on all the colorectal cancer specimens.

### Cell membrane phospholipid extraction and detection

Tissue sample (0.3 g) was taken respectively from each specimen. Five ml of Tris-HCl buffer was added to each sample for homogenation. The solution was centrifuged at 2000 rpm for 15 minutes. The supernatant was taken for centrifugation again at 17 500 rpm for 20 minutes. After removal of the supernatant 5 ml Tris-HCl buffer was added to the pellet and resuspended. Two ml of suspension was added into 5 ml of CHCl<sub>3</sub>-CH<sub>3</sub>OH (2:1, V/V) for mixation. Then 1 ml of distilled water was added for centrifugation at 3000 rpm for 10 minutes. The supernatant was removed for drying with nitrogen gas, and preserved at -20 °C. After dissolution with chloroform, the solution was ready for testing.

A PE LC-235 array detector and a PE LC-250 duality gradient pump (Perkin Elmer, Inc., USA) were used. Acetonitrile-methyl-phosphoric acid (100:3:1, V/V/V) was used as the flowing phase of chromatography.  $\mu$ Porasil column (3.9 mm i. d.  $\times$  300 mm) was applied, with a flow rate of 1.5 ml/min, a wavelength of 205 nm, a detection sensibility of 0.05 Auf $\ddot{s}$ , and a column pressure of 0.05 Auf $\ddot{s}$ . Each sample was tested repeatedly three times. The coefficient of variation (CV %) of cusp value of cell membrane phospholipids detected was less than 3% on average.

### Assessment of PKC isoenzyme mRNA expression

Total cellular RNA was extracted with Trizol reagent (Life Technologies, Inc., USA) from specimens of primary foci, paratumor mucosa, and hepatic metastatic foci. QRT-PCR was performed according to the instructions for detecting PKC isoenzyme mRNA expressions.<sup>[32]</sup>  $\beta$ -actin mRNA expression level was used as a reference. Primers used for  $\beta$ -actin and PKC isoenzyme-specific PCR and the expected fragment length of the amplification products are listed in Table 1. PCR products were separated in 1.8% agarose gel. Ethidium bromide staining was carried out after electrophoresis. And the intensity of ethidium bromide fluorescence (OD value) was quantitated with the 1D image analysis system (Kodak Digital Science<sup>TM</sup>, USA).

### Statistical analysis

Statistical significance was analyzed by using one-

**Table 1.** PKC isoenzyme primer sequences for QRT-PCR and amplification fragment length

PKC isoenzyme	Primer sequence	Fragment length (bp)
PKC- $\alpha$	5'-TGAATCCTCAGTGGGAATGAGT-3'	325
	5'-GGTTGCTTCTGTCTTCTGAA-3'	
PKC- $\beta$ II	5'-CATCTGGGATGGGGTGACAACC-3'	420
	5'-CGGTGGAAGTTTTCAGCGTTTC-3'	
PKC- $\delta$	5'-CACTACATCAAGAACCATGAGT-3'	432
	5'-ACTTGGTTCAGTGGGTCTCT-3'	
PKC- $\epsilon$	5'-ACGCAAGATCGAGCTGGCTGT-3'	440
	5'-ATTAGTGTTCACACGACCCA-3'	
PKC- $\lambda$	5'-GCTTATGTTGAGATGATGGCGG-3'	201
	5'-TGACAACCCAATCGTTCCCTTTG-3'	
PKC- $\zeta$	5'-CGATGGGGTGGATGGGATCAAAA-3'	561
	5'-GTATTCATGTCAGGGTGTCTGGA-3'	

**Table 2.** Cell membrane phospholipid levels of primary foci, paratumor mucosa and hepatic metastatic foci of colorectal cancer tested with high performance liquid chromatography

Phospholipids (mg/g)	Primary foci (n=58)	Paratumor mucosa (n=58)	Hepatic metastatic foci (n=25)
PI	1.56 $\pm$ 0.15 *	0.93 $\pm$ 0.14	1.55 $\pm$ 0.14 *
PS	0.48 $\pm$ 0.08	0.45 $\pm$ 0.06	0.47 $\pm$ 0.07
PE	42.32 $\pm$ 4.35 *	19.45 $\pm$ 3.72	78.36 $\pm$ 5.63 *#
PC	108.64 $\pm$ 7.58 *	55.76 $\pm$ 5.63	112.26 $\pm$ 7.55 *

\*: compared with paratumor mucosa,  $P < 0.01$ ; #: compared with primary foci,  $P < 0.01$ .

way ANOVA, Student's  $t$  test, and Pearson's product-moment correlation coefficient for cell membrane phospholipid levels and PKC isoenzyme mRNA expression among the groups of primary foci, paratumor mucosa, and hepatic metastatic foci.

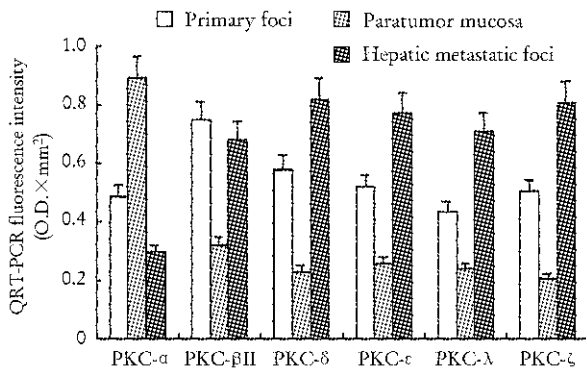
## Results

### Cell membrane phospholipid level variation

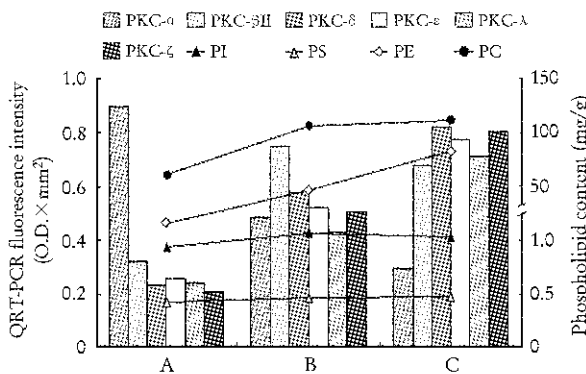
The phospholipid levels of cellular membrane in primary foci, paratumor mucosa and hepatic metastatic foci from 58 patients with colorectal cancer are listed in Table 2. The levels of PI, PE and PC in primary and hepatic metastatic foci were higher than those in paratumor mucosa. Comparison of primary foci with hepatic metastatic foci showed no significant differences in PI and PC levels ( $t = 1.73, 1.36, P > 0.05$ ); but the PE level in hepatic metastatic foci was higher than that in primary foci ( $t = 98.88, P < 0.01$ ). The levels of PS were not significantly different among the three different tissues.

### Alteration of PKC isoenzyme mRNA expression

QRT-PCR was used to detect the mRNA expression levels of PKC- $\alpha$ , - $\beta$ II, - $\delta$ , - $\epsilon$ , - $\lambda$ , - $\zeta$  isoenzyme



**Fig. 1.** PKC isoenzyme mRNA expressions in 58 patients with colorectal cancer.



**Fig. 2.** Correlations between cell membrane phospholipid levels and PKC isoenzyme mRNA expressions. A: Paratumor mucosa; B: primary foci; C: hepatic metastatic foci.

in primary foci, paratumor mucosa and hepatic metastatic foci (Fig. 1). PKC- $\alpha$  mRNA expression levels in primary and hepatic metastatic foci were lower than those in paratumor mucosa ( $F=60.30, P<0.01$ ). High levels of PKC- $\beta$ II,  $-\delta, -\epsilon, -\lambda, -\zeta$  isoenzyme mRNA expression were detected in both primary and hepatic metastatic foci, and the levels of these isoenzymes in paratumor mucosa were lower ( $F=55.71, 114.61, 107.24, 91.75, 65.43, P<0.01$ ). PKC- $\beta$ II levels in primary and hepatic metastatic foci were not significantly different ( $t=4.28, P>0.05$ ); but PKC- $\delta, -\epsilon, -\lambda, -\zeta$  mRNA expression levels in hepatic metastatic foci were higher than those in primary foci ( $t=4.31, P<0.01$ ).

**Correlation of cell membrane phospholipid level with PKC isoenzyme expression in hepatic metastasis**

Correlations of phospholipid levels of cell membrane with PKC isoenzyme mRNA expressions and their effects on hepatic metastasis of colorectal carcinoma (Fig. 2) showed that the levels of PI and PC and PKC- $\beta$ II expression in primary foci were higher than those in paratumor mucosa, but the same in hepatic metastatic foci. The levels of PI and PC were closely correlated

with PKC- $\beta$ II expression ( $r=0.715, P<0.05; r=0.901, P<0.01$ ). The high levels of PI, PC and expression of PKC- $\beta$ II were related to colorectal cancer genesis; the level of PE and expressions of PKC- $\delta, -\epsilon, -\lambda, -\zeta$  were stepwisely increased in paratumor mucosa, primary and hepatic metastatic foci in turn, whereas PKC- $\alpha$  expressions decreased gradually. The PE level was correlated with expressions of PKC- $\delta, -\epsilon, -\lambda, -\zeta$  ( $r=0.954, 0.987, 0.997, P<0.01; r=0.832, P<0.05$ ). Both of them were closely related to hepatic metastasis of colorectal carcinoma.

**Discussion**

The liver is one of the organs that are subject to blood route metastasis of colorectal carcinoma.<sup>[33-36]</sup> Cancer cells from colorectal primary foci get to the liver through the portal vein pathway. Most of the cancer cells are captured or dormant in the liver; but it is by no means indicated that all cancer cells in the liver would destine to develop clinical metastatic lesions. Our findings suggested that phospholipid levels of cell membrane and PKC isoenzyme mRNA expressions are closely related to genesis and progression of colorectal cancer. PI and PC levels in primary and hepatic metastatic foci may increase more prominently than in paratumor mucosa; but they are not significantly different in primary foci and hepatic metastatic foci. We conclude that the increase of PI and PC levels is related to occurrence of colorectal carcinoma. PE levels have a stepwise increase from paratumor mucosa to primary cancer, and hepatic metastatic foci. This indicates a correlation of PE level increase with hepatic metastasis of colorectal cancer.

The results of PKC isoenzyme mRNA test showed that the level of PKC- $\alpha$  expression is decreased in primary and hepatic metastatic foci; but that of PKC- $\beta$ II expression is increased in primary and hepatic metastatic foci. Enhanced expression of PKC- $\beta$ II and decreased expression of PKC- $\alpha$  are correlated with occurrence of colorectal cancer, but they may be weakly associated with hepatic metastasis. Nevertheless, the high expression levels of PKC- $\delta, -\epsilon, -\lambda, -\zeta$  in hepatic metastatic foci are closely related to metastasis of colorectal cancer to the liver.

Phospholipids of cellular membrane play important roles in signal transduction and cycle regulation of cells.<sup>[37-41]</sup> Our study has proved that the levels of membrane phospholipid are correlated with PKC isoenzyme expressions and they exert significant effects on the occurrence of colorectal cancer and hepatic metastasis. PI metabolism produces intracellular messenger of inositol triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> activates the signal pathway of IP<sub>3</sub>-Ca<sup>2+</sup> and enhances the concentration of Ca<sup>2+</sup>, so it influences cell proliferation.<sup>[42,43]</sup> DAG as a special activator of PKC makes tar-

get proteins phosphorylated and it is related to a comprehensive pathophysiological process.<sup>[44,45]</sup> Because PKC isoenzymes depend on  $Ca^{2+}$  and DAG, PKC- $\alpha$  promotes cell apoptosis and PKC- $\beta$ II inhibits apoptosis process.<sup>[32,46,47]</sup> A suitable ratio of PKC- $\alpha$  to PKC- $\beta$ II is crucial to regulating cell growth. Compared with paratumor mucosa, primary and hepatic metastatic foci have a lower level of PKC- $\alpha$  expression and a higher level of PKC- $\beta$ II expression. Maladjustment of the PKC- $\alpha$  to PKC- $\beta$ II ratio makes cell apoptosis weakened and leads to abnormal proliferation of cells, resulting in biological behavior changes or carcinomatous changes.

PC hydrolysis produces abundant DAG, but PI metabolism engenders DAG. The activities of PKC- $\delta$  and PKC- $\epsilon$  are dependent on DAG, which activates PKC- $\delta$  and PKC- $\epsilon$  continuously.<sup>[48,49]</sup> Increased PC level and high activity of PKC- $\delta$  and PKC- $\epsilon$  promote abnormal cleavage and de-differentiation of cells, thus facilitating occurrence and progression of colorectal cancer. The higher PE level in hepatic metastatic foci than in paratumor mucosa and primary foci suggests that PE increase is correlated with hepatic metastasis of colorectal cancer. As an important component of lipid bilayer, PE keeps cell membrane stable in addition to its important roles in ion transmembrane transport and membrane protein functioning.<sup>[50]</sup> PE activates not only PKC- $\delta$  and PKC- $\epsilon$  isoenzymes through the DAG pathway, but also PKC- $\lambda$  and PKC- $\zeta$ , which are of independence of  $Ca^{2+}$  and DAG. PKC- $\lambda$  and PKC- $\zeta$  expressions increase markedly in hepatic metastatic foci, so PE activates PKC more powerfully. With the preceding mechanisms, colorectal cancer cells get changed in biological behavior and develop hepatic metastasis. This helps us to understand the signal pathway and biomolecular mechanisms of hepatic metastasis of colorectal carcinoma and to explore effective interventional methods.

## Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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Addresses are given to us to conceal our whereabouts.

— “Sake” (H. H. Munro)