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人類肝細胞癌內蛋白激酵素 C 異構體的異常功能與基因調節之研究

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行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

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中　華　民　國　96 年 10　月　22　日

（一） 計畫中文摘要。(五百字以內)

關鍵字: 蛋白激酵素 C, 人類肝細胞癌

蛋白激酵素C(Protein Kinase C； PKC)是一種鈣和磷酸酯依賴的Ser/Thr 蛋白激酵素。至今已有10 種PKC 異構體 (α、βI、βI、γ、δ、ε、ζ、η、θ及ι) 存在，這些異構體在不同的組織或細胞中有不同的分佈及功能，且也被認為與腫瘤生長、移動和侵襲有關。一些證據顯示PKC異構體在癌瘤惡化和腫瘤形成過程中扮演重要角色。然而PKC在人類肝細胞癌形成過程的角色和機制，仍然未知。

最近我們的研究顯示低度分化的人類肝癌細胞株（HA22T/VGH、SKHep1）內PKCα mRNA的表現都比高度分化的人類肝癌細胞株（PLC/PRF/5、Hep3B 和HepG2）顯著增加，且PKCα也參與細胞增殖、移動和侵襲的作用。為了了解PKCα mRNA過度表現的機制，我們進行DNA放大和mRNA穩定度測試，結果發現PKCα mRNA過度表現與DNA放大和mRNA穩定度無相關性。因此我們尋找PKCα promoter上的轉錄因子，結果發現PKCα過度表現是藉由Elk-1和MZF-1轉錄因子互相結合調控。根據此觀點我們認為Elk-1和MZF-1轉錄因子調控PKCα表現可能與肝細胞癌形成有關。

為了深入了解人類肝癌細胞內 PKC 異構體與肝細胞癌形成關係，我們將擴大研究人類肝癌細胞內各種 PKC 異構體的異常功能和基因調控機制。本研究計畫檢測人類肝細胞癌和人類肝癌細胞株內 PKC 異構體 mRNA 的表現。結果顯示 PKCα 在人類肝癌組織表現量比正常組織高，並且與腫瘤大小、癌化期數和存活率有正相關。結果不僅幫助我們了解人類肝癌細胞內 PKC 異構體的的異常功能和基因調控機制，同時也將提供我們發展新的化學治療策略。

（二） 計畫英文摘要。（五百字以內）

Key Word: Protein kinase C, Hepatocellular carcinoma

Protein kinase C (PKC), a Ca^{2+}/phospholipiddependent Ser/Thr kinase, play an important role in transmembrane signal transduction. To date, ten isozymes of PKC (α, βI,βII, γ, δ, ε, ζ, η, θ, and ι) with distinct enzymological characteristics and intracellular localization have been identified, and it is also believed to be correlated with the tumor proliferation, migration and invasion. Some evidences that the PKC isoforms have been suggested to play an important role in tumorigenesis and tumor progression. However, the exact role of PKC isoforms in the progression of human HCC remains unclear.

Recent our data showed that the level of PKCα in the low differentiated human hepatocellular carcinoma (HCC) HA22T/VGH and SKHep1 cell lines were significantly higher than that in the high differentiated human HCC PLC/PRF/5, Hep3B and HepG2 cell lines and correlation with cell proliferation, migration and invasion. To understand of the mechanism of overexpression of PKCα, DNA amplification and mRNA stability assay were performed. The result indicated that PKCα expression enhancement in the poor-differentiated human HCC cells was found neither by DNA amplification nor by increasing mRNA stability. Furthermore, screening transcription factors in the putative cis-acting regulatory elements of human PKCα promoters, we demonstrated that PKCα expression is regulated by Elk-1 and MZF-1. Thus, we suggested that PKCα may be regulated by Elk-1 and MZF-1 cooperation involved in the progression of human HCC.

Therefore, for further understanding whether PKC isoforms play important roles in the malignant of human HCC, this proposal is to investigate the abnormal function and gene-regulation mechanism of PKC isoforms in human HCC. In this study, we deterimine the mRNA expressions of PKC isoforms in human HCC. The results found that the level of PKCα in the HCC tissues were significantly higher than that in the nontumor tissues, and it positive correlation with tumor size, tumor stage and survival rate. The results not only help us to understand the abnormal function and gene-regulation mechanism of PKC isoforms in human HCC, but also provide us to develop novel chemotherapy strategies.

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Overexpression of protein kinase Cα mRNA in human hepatocellular carcinoma: A potential marker of disease prognosis

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Abstract

Abstract: Members of the protein kinase C (PKC) isoenzyme family play a central role in the tumorigenesis of several tissues. However, little is known about subtype specific intracellular expression of PKC in human hepatocellular carcinomas.
Methods: We investigated PKC isoforms mRNA expression in 42 HCC specimens using reverse transcription polymerase chain reaction analysis, and the correlation between PKC isoforms expression and clinicopathologic parameters.
Results: We found that PKCα, PKCδ and PKCι mRNA were significantly increased in HCCs as compared to the corresponding non-cancerous liver tissues. PKCα expression also significantly correlated with tumor size ($P<0.05$) and TNM stage ($P<0.05$), but PKCδ and PKCι did not. The log-rank analysis revealed that patients with higher PKCα mRNA expression in the HCC tissues had significantly shorter survival rate than patients with lower PKCα mRNA expression ($P<0.01$).
Conclusions: Our results suggested that the PKCα may be a prognostic factor for the survival of patients with HCC.

Keywords: Protein kinase Cα; Hepatocellular carcinoma

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world, and is especially prevalent in parts of Asia and Africa. The most common cause of death in patients with HCC is metastasis due to disease recurrence. It is important for tumor control to identify specific predictive markers that predispose patients to death. Although many factors, including tumor-associated antigens, molecular markers, and soluble markers in a variety of body fluids have been used to diagnose and monitor disease in patients with HCC [1], at present, none of these factors has demonstrated sufficient diagnostic specificity for use as prognostic markers.

Protein kinase C (PKC) is a serine/threonine kinase that plays a key role in several steps of the signal transduction pathway, including cellular proliferation, differentiation, and apoptosis [2–4]. PKC consists of a family of 10 related isotypes with different cofactor requirements, tissue and subcellular distribution, and substrate specificity [5]. According to their primary sequence, PKC isoforms are subdivided into 3 main classes: conventional, novel, and atypical. Conventional PKC isoforms includePKCα, PKCβI, PKCβII, and PKCγ are activated by diacylglycerol (DAG), phosphatidylserine (PS), and calcium. Novel PKC isoforms include PKCδ, PKCε, PKCη, and PKCθ which are calcium independent but are dependent upon DAG and PS for optimal enzyme activity. Atypical PKC isoforms include PKCζ and PKCλ/ι which are stimulated by PS but not by DAG and calcium. PKC isoforms are ubiquitously expressed in tissues. PKC isoforms have been shown to display variable expression profiles during cancer progression depending on the particular cancer type [6]. Several immunohistochemical studies have shown that PKCα is overexpressed in high grade urinary bladder, prostate, and endometrial cancers [7–10]. In contrast,

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breast, colon, and basal cell cancers display downregulation of PKCα expression [11–13]. PKCβ expression has been shown to be upregulated in colon and prostate cancers [9,13] and downregulated in bladder cancer [10]. However, the expression of PKCι, PKCε and PKCλ is decreased in pancreatic cancers [14] and PKCη is increased in renal cancer [15]. The expression of PKCι has been suggested to be a negative prognostic factor in ovarian carcinoma [16] and PKC as a positive prognostic factor in B-cell lymphoma [17]. These studies provide supporting evidence for the role of a specific PKC isotype in different kinds of malignant tumors. However, to date, limited data are available on the significance of PKC isoforms in HCC.

In this study, we investigated the mRNA expression of PKC isoforms in tissue specimens from 42 patients with HCC using the reverse transcription polymerase chain reaction (RT-PCR) technique. Our data provide the first definitive evidence of a correlation between PKCα expression and prognosis in patients with HCC.

2. Materials and methods

2.1. Patients and sample

Tissue specimens were obtained from 42 patients (6 women; 36 men) with primary HCC who underwent tumor resection at Department of General Surgery, Taichung Veterans General Hospital. The mean age of all patients was 57.6 y (range: 18–76 y). Among these patients, 26/42 (61.9%) were positive for hepatitis B surface antigen (HBsAg); 14/42 (33.3%) were positive for hepatitis C virus infection and negative for HBsAg negative; 2/42 (4.8%) were negative for both hepatitis C and B virus infection. The extent of hepatocellular disease in our patients was staged according to the tumor-node-metastasis (TNM) system [18]. Among our patients, 4/42 (9.5%) had stage I disease; 19/42 (45.2%) had stage II disease; 10/42 (23.8%) had stage III disease; and, 9/42 (21.4%) had stage IV disease. Tumors were also grouped according to size as< 3.5 cm [13/42 (30.9%)] and ≥ 3.5 cm [29/42 (69.1%)]. In all cases, the diagnosis of HCC was confirmed histologically, based mainly on examination of tissue sections stained with hematoxylin and eosin and examined microscopically by an antatomic pathologist. The tissue samples were snap-frozen in liquid nitrogen immediately after resection and stored at −70 °C.

2.2. RNA extraction

Total RNA was extracted from HCC tissue specimens using the guanidinium thiocyanate-phenol-chloroform method [19]. The specimens consisting of HCC and non-tumorous liver tissues, were homogenized using 4 mol/l guanidine thiocyanate, 25 mmol/l sodium citrate, 0.5% (w/v) sodium lauryl sarcosinate, and 0.1 mol/l-mercaptoethanol) in a polypropylene tube, followed by isolation of total RNA using a standard method [19]. The extract integrity was assessed by 1.5% agarose gel electrophoresis and RNA was visualized by ethidium bromide staining. The total amount of RNA was determined spectrophotometrically.

2.3. Oligonucleotide synthesis

The oligodeoxyuncleotide (ODN) primers used in RT-PCR were: PKCα, PKCδ, and PKCζ as described previously [20]; PKCβI, PKCβII, PKCγ, PKCε, PKCη, PKCθ, and PKCι as described previously [21].

2.4. Reverse transcription-PCR

The RT-PCR assay was performed according to De Petro et al. [22] with slight modifications. An aliquot of total RNA (1 μg) was reverse transcribed. The RT product (4 μL) was diluted to a final volume of 50 μl with PCR buffer (50 mmol/l KCl10 mmol/l Tris–HCl2 mmol/l $MgCl_2$) containing 0.5 μmol/l dNTPs (final concentration, 0.8 mmol/l) and 0.5 unit of Super-Therm Taq DNA polymerase (Southern Cross Biotechnology, Cape Town, South Africa). Thirty-three cycles of PCR were performed on a GeneAmp PCR system 2400 (Applied Biosystems, Foster City, CA). The PCR products were analyzed by 1.2% agarose gel electrophoresis and direct visualization after SYBR Green I (Cambrex Bio Science Rockland, Inc., Rockland, ME) staining. The agarose gels were scanned and analyzed using the Kodak Scientific 1D Imaging System (Eastman Kodak Company, New Haven, CT). The relative levels of PKC isoform mRNA was expressed as the ratio of PKC isoform mRNA to beta-2-microglobulin (β_2-MG) mRNA (PKC isoform/β_2-MG). The accuracy of the amplification reaction for each set of primers was determined by amplifying several dilutions of the same cDNA with the same cycling profiles and amplifying the same cDNA dilution with different cycling profiles. The specificity of the cDNA was validated using DNA sequence analysis (data not shown).

2.5. Statistical analysis

Relative mRNA expression levels (PKC isoform/β_2-MG) were expressed as the mean ± SEM. We used an unpaired Student's *t*-test to analyze the difference in PKC isoform expression levels between HCC and non-cancerous liver tissue. To analyze the correlation between PKC isoform levels and clinicopathological parameters, differences in PKC isoform mRNA expression between the two groups were evaluated using the Fisher exact test. Survival rates were calculated using the Kaplan–Meier method, and the difference in survival curves was analyzed using the log-rank test. $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Expression of PKC isoforms genes in human HCC

Among 10 PKC isoforms we studied, 8 (PKCα, PKCβI, PKCβII, PKCδ, PKCε, PKCη, PKCι, and PKCζ) of these

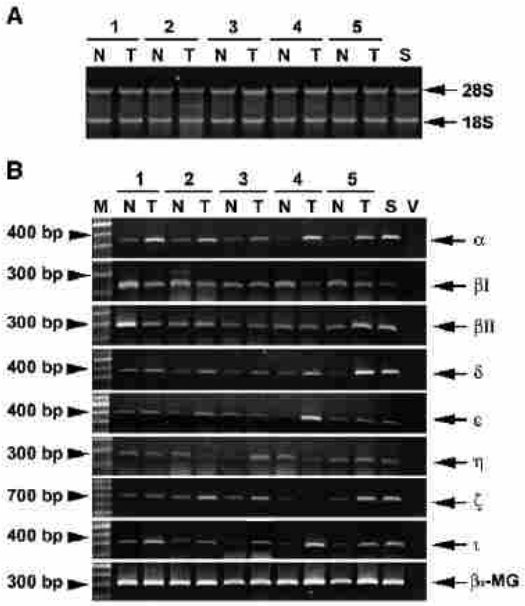

Fig. 1. Expression of PKC isoform (α, βI, βII, δ, ε, η, ζ and ι) and β_2-MG mRNA was detected by semiquantitative RT-PCR. N, non-tumorous liver tissues; T, HCC tissue; S, positive control (from mixtures of ten random HCC tissues); V, negative control for PCR without RT product; M, 100 bp ladder as the DNA size marker. Ethidium bromide-stained blot shows 28 S and 18 S ribosomal RNAs as an internal control. The data represent 1 of 3 independent experiments with similar results.

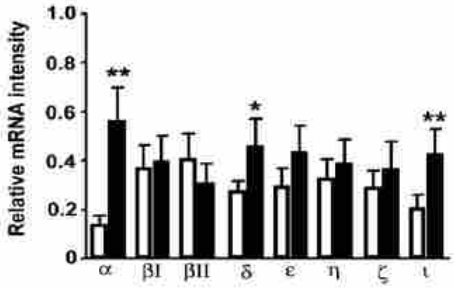

Fig. 2. The relative expression of PKC isoform (PKCα, PKCβI, PKCβII, PKCδ, PKCε, PKCη, PKCζ, and PKCι) mRNA in human non-tumorous liver tissue (□; $n=42$) and HCC tissue (■; $n=42$). Data are the mean±SEM. *, $P<0.05$; **, $P<0.01$.

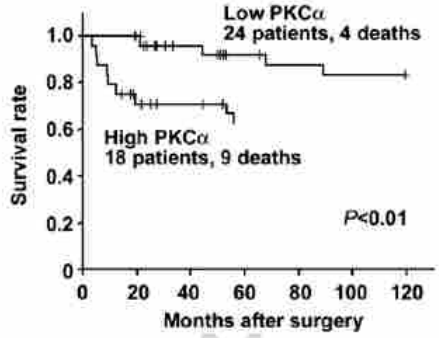

Fig. 3. Survival rate of patients with HCC. High expression of PKCα mRNA was associated significantly with worse prognosis ($P<0.01$).

isoforms were detectable in HCC and adjacent non-tumorous tissue from our patients (Fig. 1). The housekeeping gene, β2-MG, was expressed in both HCC and non-tumorous liver tissue. Relative mRNA levels associated with PKCα, PKCι and PKCδ isoforms were significantly higher in HCC tissue compared with non-tumorous liver tissue (PKCα, 0.57±0.17 vs. 0.14±0.03, $P<0.01$; PKCδ, 0.48±0.16 vs. 0.27±0.07, $P<0.05$; PKCι, 0.43±0.13 vs. 0.21±0.11, $P<0.01$) (Fig. 2). Although relative levels of mRNA associated with other PKC isoforms (βI, ε, η, and ζ) were also increased in HCC tissues, they were not significantly different between HCC and non-tumorous liver tissue. Moreover, PKCγ and PKCθ mRNA was not detected in any of the HCC and non-tumorous liver tissues.

Table 1
Correlation between the clinicopathologic features and the mRNA expression of PKCα, PKCδ, and PKCι in 42 HCC patients

Clinicopathological parameters	PKCα[a] (n)			PKCδ[a] (n)			PKCι[a] (n)		
	−	+	P^b	−	+	P^b	−	+	P^b
Age (years)									
<60	11	10	0.3779	17	4	0.1529	8	13	0.1084
≧60	13	8		13	8		13	8	
Sex									
Female	4	2	0.4814	4	2	0.5608	2	4	0.3314
Male	20	16		26	10		19	17	
HBs-Ag									
Negative	11	5	0.1923	11	5	0.5149	7	9	0.3757
Positive	13	13		19	7		14	12	
Anti-HCV									
Negative	14	13	0.2743	20	7	0.4337	13	14	0.5000
Positive	10	5		10	5		8	7	
Tumor size (cm)									
<3.5	11	2	0.0170	10	3	0.4457	7	6	0.5000
≥3.5	13	16		20	9		14	15	
TNM stage									
I–II	19	4	0.0003	18	5	0.2309	11	12	0.5000
III–IV	5	14		12	7		10	9	
Liver cirrhosis									
Absent	16	13	0.4834	21	8	0.5543	14	15	0.5000
Present	8	5		9	4		7	6	

[a] −, designed as low expression; +, designed as high expression.
[b] P value determined using the Fisher exact test.

3.2. Association of PKCα, PKCδ and PKCι mRNA expression with the clinicopathologic characteristics of patients with HCC

To elucidate the role of PKCα, PKCδ, and PKCι isoforms in HCC, the patients were divided into 2 groups based on the level (high or low) of expression of mRNA associated with each of these isoforms and using a cutoff level of the median value for mRNA expression associated with each of these isoforms in all patients ($n=42$) with HCC. We then correlated the relative level of PKCα, PKCδ and PKCι mRNA expression with the clinicopathologic characteristics of our patients with HCC. As shown in Table 1, although the expression of PKCα mRNA did not correlate with age, sex, HCV or HBV infection, or cirrhosis, high expression of PKCα mRNA was associated with tumor size ($P<0.01$) and TNM stage ($P<0.01$). In contrast, PKCδ and PKCι mRNA expression did not correlate with any of the clinicopathologic parameters we evaluated (Table 1).

3.3. Analysis of PKC isoforms with potential prognostic significance in patients with HCC

We analyzed the survival rates in our cohort of patients with HCC to assess the prognostic significance of PKCα, PKCδ and PKCι mRNA expression. Patients who had higher PKCα mRNA expression levels had shorter survival rates than patients who had lower PKCα mRNA expression levels (median survival rate, 53.1% vs. 89.2%, $P=0.0006$, Fig. 3), when assessed by Kaplan–Meier curves. Whereas, the survival rates were not significantly different between patients who had higher PKCδ or PKCι mRNA expression and those who had lower PKCδ or PKCι mRNA expression, respectively (median survival rate, 53.1% vs. 68.0%, $P=0.1803$ for PKCδ; median survival rate, 53.1% vs. 89.2%, $P=0.1386$ for PKCι).

4. Discussion

It is well known that PKC plays an important role in tumor carcinogenesis. However, little is known about the specific role of each PKC isoform in the pathogenesisis of human HCC. To clarify the role of PKC isoforms in human HCC, we measured PKC isoform mRNA expression in 42 patients with HCC and

compared it to the clinicopathological characteristics of these patients. Our results indicate that the expression of PKCα, δ and ι mRNA was dramatically increased in HCC tissues (Fig. 2). The elevated expression of PKCα mRNA in HCC was consistent with previous results by immunohistochemical analysis [23]. The elevated expression of PKCα mRNA occurred predominantly in tissue from patients with higher stage (stage III or IV) HCC and larger tumor size (≥3.5 cm). Moreover, in the recent study, we also found that PKCα was higher in the poorly differentiated SK-Hep-1 and HA22T/VGH HCC cells as compared with that in the well-differentiated HCC cell lines (PLC/PRF/5, Hep3B and HepG2), and antisense ODN PKCα and siPKCα and antagonist Go6976 significantly decreased the proliferation, migration and invasion in the SK-Hep-1 and HA22T/VGH cell lines (summit for publication). These observations are consistent with previous findings in other malignant tumors, such as gliomas [24] and prostate [25] and breast [26] cancers, suggesting that PKCα may also be involved in the malignant progression of disease in patients with HCC.

The PKCα gene is located on the long arm of chromosome 17 (17q24) [27] and allelic gain in this region is seldom found in human HCCs [28]. Previously, we reported that DNA amplification of the PKCα gene may occur rarely in human HCC cell lines [29]. Using the antisense knockout assay and ChIP (chromatin immunoprecipitation) assays, we found that PKCα expression was regulated by Elk-1 and MZF-1 proteins at the transcriptional level. Although additional studies are therefore required to delineate in greater detail the physiological functions of Elk-1 and MZF-1 proteins in the etiology of human HCC, it is likely that the increase in PKCα mRNA levels in human HCC cells may be due to an increase in transcription.

In contrast to previous reports [30,31] which found that the level of membrane-bound PKCα was significantly lower in HCC than in adjacent normal tissue, we obtained an inconsistent pattern in the level of PKCα mRNA expression in HCC cells. The reduction in the membrane-bound level of PKCα was mainly due to stimulation and down-regulation of PKC under general anesthesia [23], because the levels of PKCα were significantly higher in cells from liver biospies than the corresponding levels obtained in cells from hepatectomy specimens in patients who underwent general anesthesia with pentothal or propofol. Halothane and propofol have been reported to stimulate purified brain PKC activity *in vitro* when assayed with physiologically relevant lipid bilayers in the absence or presence of Ca^{2+} [32] This effect appears to be mediated through the lipid-binding regulatory domain of PKC [32]. These 2 anesthetics have been demonstrated to stabilize the active conformation of PKC [33,34]. Moreover, halothane has been shown to promote translocation of PKCα, $PKC\beta_{I/II}$, and PKCγ from the cytosol to the membrane fraction of synaptosomes and down-regulation of their immunoreactivity [35]. This finding may explain the inconsistency between results for PKCα mRNA levels by RT-PCR analysis and PKCα protein levels by kinase activity assay and western blot analysis [30,31].

The log-rank analysis showed that PKCα mRNA expression was significantly associated with patient survival rates. This result indicates that PKCα over-expression might help identify HCC patients with a poor prognosis, although the mechanism by which PKCα overexpression contributes to the poor prognosis of patients with HCC must be elucidated by further study.

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計畫成果自評

1. 研究內容與原計畫相符程度：90%程度相符。

2. 達成預期目標情況：已達預期目標。

3. 研究成果之學術或應用價值：結果幫助我們了解人類肝癌細胞內 PKC 異構體的的異常功能和基因調控機制。

4. 是否適合在學術期刊發表或申請專利：已在學術期刊發表。

5. 主要發現或其他有關價值：將提供我們發展新的化學治療策略。