Expression of protein kinase C isoforms in cancerous breast tissue and adjacent normal breast tissue

Yueh-Min Lin[1], Cheng-Chuan Su[2,3], Wei-Wen Su[4], Jin-Ming Hwang [5], Hsi-Hsien Hsu[6], Chang-Hai Tsai[7], Fuu-Jen Tsai[8], Chih-Yang Huang[8, 9, 10,*], Jer-Yuh Liu[11,12,*], and Li-Mien Chen[13,14,*]

[1] Department of pathology, Changhua Christian Hospital, Changhua

[2] Departments of Clinical Pathology and Anatomic Pathology, Buddhist Dalin Tzu Chi General Hospital, Chiayi

[3] Department of Pathology, School of Medicine, Tzu Chi University, Hualien

[4]Comprehensive Breast Cancer Center, Changhua Christian Hospital, Changhua

[5]School of Applied Chemistry, Chung-Shan Medical University, Taichung

[6]Division of Colorectal Surgery, Mackay Memorial Hospital, Taipei

[7]Department of Healthcare Administration, Asia University, Taichung

[8]Graduate Institute of Chinese Medical Science, [9]Graduate Institute of Basic Medical Science, China Medical University, Taichung

[10]Department of Health and Nutrition Biotechnology, Asia University, Taichung

[11]Center for Molecular Medicine, China Medical University Hospital, Taichung

[12]Graduate Institute of Cancer Biology, China Medical University, Taichung

[13]Departments of Internal Medicine, Armed Force Taichung General Hospital, Taichung and

[14]Center of General Education, Central Taiwan University of Science & Technology, Taichung, Taiwan, R.O.C.

---

* These authors contributed equally to this paper.

Corresponding author: Li-Ming Chen MD, Departments of Internal Medicine, Armed Force Taichung General Hospital, Taichung, Taiwan. Tel: +886-4-; Fax: +886-4-. E-mail address:.

Abstract

The role of PKC in the carcinogenesis of human breast cancer has been studied at the molecular level for more than two decades. In this study, we employed Western blotting to determine the existence of PKC isoforms in cancerous breast tissue and normal breast tissue. The results contained significant expressions of a conventional PKC (PKCα) and two atypical PKCs (PKC ζ and λ/ι) in both breast tumors and adjacent normal breast tissue. For the isoforms α, ζ, and λ/ι, the expression of individual isoforms was higher in the breast tumors than in the adjacent normal breast tissue. Although the correlation coefficient was low, significant linear correlation was found among the activities of the isoforms. This data suggests a new direction in cancer chemotherapy, namely the blockage of the signal transduction pathway of the specific isoforms.

Key Word: protein kinase C (PKC), human breast cancer

Introduction

Protein kinase C (PKC), a lipid-regulated and calcium-dependent protein kinase, has 10 isoforms. According to their cofactors, the isoforms can be divided into three main classes: Ca^{++}-dependent or conventional PKCs (α, βI, βII, and γ), Ca^{++}-independent or novel PKCs (δ, ε, η and θ), and Ca^{++}-independent and DAG and phosphatidylserine activated PKCs (ζ and λ/ι) (3, 12). These isoforms may exist in some organs or universally in all organs. In different organs, they may exhibit differences in structure and also differ in cell signaling function.

It has been reported that overexpression of PKC may lead to disorders in cell proliferation and differentiation (8, 9). The expression of PKC in human breast tumor biopsies has been found to be significantly higher than that in adjacent normal breast tissue. Overexpression of c-myc, Ha-ras, erb13, and HER-2/neu in breast cancer have been attributed to the overexpression of this enzyme (14). In addition, PKC mediates phosphorylation of membrane-associated oncogene products such as $pp60^{src}$ and Ki-ras protein, and activates nuclear pro-oncogenes such as TPA (4, 7). These findings suggest that PKC may play an important role in the regulation of proto-oncogenes and oncogenes involved in the carcinogensis of human breast cancer.

Although the role of PKC in the carcinogensis of human breast cancer has been studied at the molecular level, there is no available information concerning the changes in the activities of the isoforms in the breast tissue. In this study, we employed Western blotting to determine the existence of the isoforms of PKC in cancerous breast tissue

and normal breast tissue. The relationships between the isoforms of PKC and various demographic and clinical factors were also investigated.

Materials and Methods

Specimen Collection

Written consent was obtained from each patient. Surgical specimens of breast tumor (infiltrating ductal carcinoma) and normal human breast tissue were obtained by mastectomy from the operating rooms of Chang Hua Christian Hospital in Chang Hua and Chung Shan Medical University Hospital in Taichung, both of which are in Taiwan. After resection, these specimens were stored at -70°C for the analysis.

PKC Extraction

PKC extraction was performed according to Nishizuka (13) with slight modifications. All operations were carried out at 4°C. The specimen (80 mg) was sectioned into small pieces and washed with homogenized buffer A (pH 7.5; 20 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), 2 mM ethylenedianmine-tetraacetic acid (EDTA), 50 mM phenylmethylsulfonyl fluoride (PMSF), 10% glycerol, 50 mM β-mercaptoethanol). The tissue was then mixed with 200 µl buffer B (pH 7.5; 20 mM Tris-HCl, 2 mM EDTA, 50 mM PMSF, 10% glycerol, 0.1% Triton X-100). After homogenization, 200 µl homogenized buffer B was added to the homogenates before carrying out homogenization again. The homogenates were then transferred to another vial and then a further 330 µl of homogenized buffer B were added. This mixture was incubated for 1 h with stirring at intervals of 5-10 min. The homogenates were then centrifuged at 15,000 *g* for 3 hs. The supernatant (1.5 ml) was then transferred into vials and stored at -70°C for further experiments.

Determination of Protein Contents

Protein contents of the sample preparation were determined by the Bradford protein assay (6). The protein assay reagents were purchased from Bio-Rad Lab (Richmond, CA, USA). Coomassie brilliant blue G-250 was used for staining and bovine serum albumin (BSA) was employed as the standard. Changes in optical density were monitored at 595 nm.

Western Blotting

PKC isoforms in the samples were analyzed by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (11). The extracts were standardized to the same volume with PBS. The extracts were standardized to the same volume with PBS. After adding a treatment buffer in a ratio of 1 to 5 to the extract, the mixture was boiled for 10 min and then rapidly placed in an ice bath. The mixture was then spin-down in a centrifuge and loaded onto slab polyacrylamide gels, using a 4% stacking gel (pH 6.8) and 10% separating gel (pH 8.8). Electrophoresis was run on a mini vertical slab gel unit (Biorad Scientific Instruments, Richmond, California, USA) at 140 V and 35 mA for 3.5 h.

After electrophoresis, the gels were equilibrated in a cold transfer buffer and proteins were transferred onto nitrocellulose papers (Amersham, Hybond-C Extra Supported, 0.45 m) using a Hoefer Scientific Instruments Transphor Unit at 100 mA overnight. The nitrocellulose papers were washed with a washing buffer and incubated in 50 ml 3% FBS blocking buffer (3% FBS, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) at room temperature for 1 h. Primary antibodies against individual PKC isoforms (1:100 dilute) in 20 ml 3% FBS blocking buffer was then added and incubated at room

temperature for 3 h. The nitrocellulose papers were washed in the washing buffer for 10 min in triplicate and then immersed in the secondary antibody of the corresponding isoform (1:1,000 dilute) containing 20 ml 3% FBS blocking buffer. The papers was washed in triplicate with the washing buffer for 10 min. Color was developed using 20 ml of a color developing substrate in color reagents (700 µl nitrobluetrazolium and 780 µl 5-bromo-4-chloro-3-indolyl-phosphate (10 mg/ml)). The reaction was terminated by addition of deionized water. Color changes on the nitrocellulose papers were determined using a destimetor (Alphalmager 2000 version 3.2, Alpha Innotech Corp., San Leandro, CA, USA).

Results

To determine whether PKC isoforms were associated with the breast cancer development, we scanned for ten types of PKC isoforms (α, βI, βII, γ, δ, ε, η, θ, ζ and λ/ι) using the Transduction Laboratory antibodies in both the adjacent normal breast tissue and breast tumors taken from the mixed tissue samples of 10 patients (Fig. 1). Analysis revealed significant immunostaining in both adjacent normal breast tissue and cancerous breast tissue and PKCα and γ antibodies at -80 KDa, and PKC ζ and λ/ι antibodies at -72 KDa, whereas the PKC β, δ, ε, η, and θ antibodies did not show any immunoreactivity. A band at 48 KDa was detected with all types of PKC isoforms as compared with the marker of brain cell lysates (data not shown). From this data, the following Western blotting tests focused on the detection of the 3 most abundant isoforms in the breast cancer tissues.

Significant expressions of a conventional PKC α and two atypical PKCs (PKC ζ, λ/ι) were detected in both breast tumors and adjacent normal breast tissue (Fig. 1A and 1B, Table 1). Since PKCγ exists only in the brain and may cross-react with the antibody which acts against PKCα, the positive finding for this isoform in these tissues is questionable.

For the isoforms α, ζ, and λ/ι, the expression of individual isoforms was higher in the breast tumors than in the adjacent normal breast tissue (data not shown). Although the correlation coefficient was low, significant linear correlation was found among the activities of the isoforms (Table 2). However, there were no significant correlation in the activities of individual isoforms attributable to lymphatic metastasis, stage of carcinoma, age of the patient, or the presence of estrogen receptors (Table 3).

Discussion

It has been reported that the activity of PKC elevates significantly in human breast tumor than in the adjacent normal breast tissue (14). Using Western blotting, we not only confirmed these reports but also found that four endogenous isoforms (α, ζ, λ/ι) exist in the breast tumor. These findings suggest that PKC isoforms are important in the regulation of tumor cells and carcinogensis of human breast cancer.

In this study, the expression of PKCα in breast tumor is overexpressed, similar to that of PKCα-transfected breast cancer cell line MCF-7 (16). The finding is due to the fact that overexpression of this isoform may increase the expression of PKCβ and decrease the amount of PKCη and PKCδ. This change in PKCα activity may also lead to a more aggressive neoplastic phenotype. Therefore, overexpression of PKCα may be an important factor in the carcinogensis of human breast tumor.

Overexpression of PKC isoforms α, βI, δ, ε, and ζ have also been reported in uterine cancer cells where they increase the rate of proliferation of uterine tumor cells (5). In this study, we demonstrated that the expression of PKCζ was significantly higher in the breast tumor than in the adjacent normal breast tissue. This finding suggests the role of PKCζ as an important enzyme in the proliferation of breast cancer cells.

PKCλ and PKCζ belong to the atypical PKC isoforms in human breast tumors and have a 72% similarity in their structures. These two isoforms exist universally in all kinds of tissue. The existence of PKCλ has been demonstrated in an undifferentiated mouse embryonal carcinoma cell line P19. In the P19 cells, the expressions of PKCα and PKCε are elevated and that of PKCλ is significantly decreased after differentiation

(1). Overexpression of this isoform not only leads to a higher degree of undifferentiation in breast cancer cells but can also be used as a marker for the prognosis of patients.

Although PKCι does not affect the proliferation of cells, this isoform has been considered to have a significant association with drug resistance. This phenomenon has been demonstrated in the apoptosis of leukemia cells where inhibiting the PKCι lessened the drug resistance (10). The existence of PKCι in our specimens indicates that breast tumors may have chemopreventative effects against anti-cancer drugs. However, this suggestion requires further investigation.

Although PKCα, δ, ζ, and λ are age-dependent in a developing kidney (15), we did not find significant differences in the expression of PKC isoforms in breast tumors by age group. Moreover, there were no significant associations between the levels of expression of PKCα, ζ, and λ/ι in the breast tumor at the stage of carcinoma, nor at the stage of lymphatic metastasis. There were also no significant associations regardless of the existence or absence of estrogen or progesterone receptors. PKCα, βI and βII have already been reported to be associated with human breast tumors (2). The identification of PKC α, ζ, and λ/ι in human breast tumors in this study suggests that chemotherapeutically blocking the signal transduction pathway of these specific isoforms may be a new direction in the treatment of breast cancer.

Acknowledgements

This work was supported by the grants from the National Science Council, Republic of China (NSC 98-2320-B-039-042-MY3), as well as by the Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH99-TD-B-111-004) and in part by the Taiwan Department of Health Cancer Research Center of Excellence (DOH99-TD-C-111-005).

References

1. Akimoto K, Mizuno K, Osada S, Hirai S, Tanuma S, Suzuki K, Ohno S. A new member of the third class in the protein kinase C family, PKC lambda, expressed dominantly in an undifferentiated mouse embryonal carcinoma cell line and also in many tissues and cells. *J. Biol. Chem.* 269: 12677-12683, 1994.

2. Ali, S., Al-Sukhun, S., El-Rayes, B.F., Sarkar, F.H., Heilbrun, L.K. and Philip, P.A. Protein kinases C isozymes are differentially expressed in human breast carcinomas. Life Sci. 84: 766-771, 2009.

3. Assoka, Y., Nakamura ,S.I. Yoshida ,K.and Nishizuka, Y. Protein kinase C, calcium and phospholipid degradation. *Trends Biochem. Sci.* 17: 414-417, 1992..

4. Balleste, R., Furth, M.E. and Rosen, O.M. Phobol ester and protein kinase C mediated phosphorylation of cellular kirsten ras gene product. *J. Biol. Chem.* 262: 2688-2695, 1987.

5. Bamberger, A.M. Bamberger, C.M. Wald,M., Kratzmeier, M. and Schulte, H.M. Protein kinase C (PKC) isoenzyme expression pattern as indicator of proliferative activity in uterine tumor cells. *Mol. Cell. Endocrinol.* 123: 81-88, 1996.

6. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254, 1976.

7. Gould, K.L. Woodgett, J.R. Cooper ,J.A. Buss, J.E. Shalloway ,D. and Hunter T. Protein kinase C phosphoylation pp60^{src} at novel site. *Cell* 42: 849-857, 1985.

8. Housey, G.M. Johnson, M.D. Hsiao, W.L. O'Brian, C.A. Murphy, J.P, Kirschmeier, P. and Weinstein ,I.B. Overproduction of protein kinase C causes disordered growth control in rat fibroblast. *Cell* 52: 343-354, 1988.

9. Krauss, R., Housey ,G., Johnson ,M. and Weinstein, I.B. Disturbances in growth control and Gene expression in a C3H10T1/2 cell line that stably overproduces protein kinase C. *Oncogene* 4: 991-998, 1989.

10. Murray, N.R. and Frield, A.P. Atypical protein kinase C iota protects human leukemia cells against drug apoptosis. *J. Biol. Chem.* 272: 27521-27524, 1997.

11. Newton, A.C. Protein kinase C: Structure, function, and regulation. *J. Biol. Chem.* 270: 28495-28498, 1995.

12. Nishizuka, Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 258: 607-614, 1992.

13. Nishizuka, Y. Protein kinase C and lipid signaling for sustained cellular responses. *Faseb J.* 9: 484-496, 1995.

14. O'Brian, C.A. Vogel ,V.G. Singletary ,S.E. and Ward ,N.E. Elevation protein kinase C expression in human breast tumor biopsies relative to normal breast tissue. *Cancer Res.* 49: 3215-3217, 1989.

15. Serlachius ,E., Svennilson, J., Schalling, M. and Aperia ,A. Protein kinase C in the developing kidney : Isoform expression and effects of ceramide and PKC inhibitors. *Kidney Int.* 52: 901-910, 1997.

16. Ways, D.K. Kukoly, C.A. deVente, J., Hooker, J.L. Bryant ,W.O., Posekany, K.J. and Fletcher, D.J. Cook PP Parker PJ MCF-7 breast cancer cells transfected with protein kinase C-α exhibit altered expression of other protein kinase C isoforms and display a more aggressive neoplastic phenotype. *J. Clin. Invest.* 95: 1906-1915, 1995.

Table 1 Clinical characteristics of the patients with breast tumor (infiltrating ductal carcinoma) analyzed in this study

ID	PKCα	PKCζ	PKCλ	PKCι	LN	Grade	ER	PR	Age (years)
1	256	163	159	166		3	-	-	
2	235	163	196	181		3			
3	246	124	153	154		3			
4	214	163	116	133		2	+	+	63
5	107	101	104	133		1	+	+	56
6	256	147	294	217		2	-	-	58
7	150	202	165	136		2			46
8	150	240	116	102		3	+	+	74
9	65	63	18	30	1	3	-	-	46
10	175	188	200	144	1	2	+	+	63
11	104	169	36	52	1	3	-	-	46
12	91	200	100	106	1	3	+	+	75
13	78	94	5	29	1	3	-	-	48
14	58	19	5	16	0	2	-	-	67
15	150	194	232	163	0	3	+		
16	100	100	100	100	1	2	+	+	82
17	71	88	45	57	0	2	+		57
18	136	119	141	88	0	2	+	-	49
19	80	125	255	132	1	2	+		43
20	129	131	86	57	1	2	-	-	58
21	85	169	67	88	1	2	+		40
22	85	219	153	101					51
23	85	183	92	105	0	2	-		45
24	111	219	55	92	0	2	-	-	66
25	170	261	123	123	1	2	+		64

26	196	233	233	167	0	2	-		55
27	179	268	86	107					63
28	60	106	61	78	0	3	+		49
29	119	219	37	53	0	3	+		55
30	94	176	4	25	0	3	+		55
31	196	226	110	94	1	3	-		76
32	94	247	104	96	1	2	+	+	48
33	238	261	199	136	1	3	-	-	31
34	162	220	164	99	1	2	-	-	56
35	264	318	216	123	0	3	+	+	26
36	153	153	150	102	0	3	-	-	38
37	102	148	66	45	0	2			58
38	221	294	258	163	0	1	+		72
39	187	85	94	60	0	2	-		23
40	340	245	251	154	0	2	+		62
41	179	148	129	93	0	3	+		40
42	102	107	52	45	1	2			43

Table2. Correlations among activities of PKCα、PKCζ、PKCι、PKCλ in the tissue of human breast tumor

	PKCα	PKCζ	PKCι
PKCζ	0.001		
PKCι	0.001	0.0039	
PKCλ	0.001	0.0025	0.0001

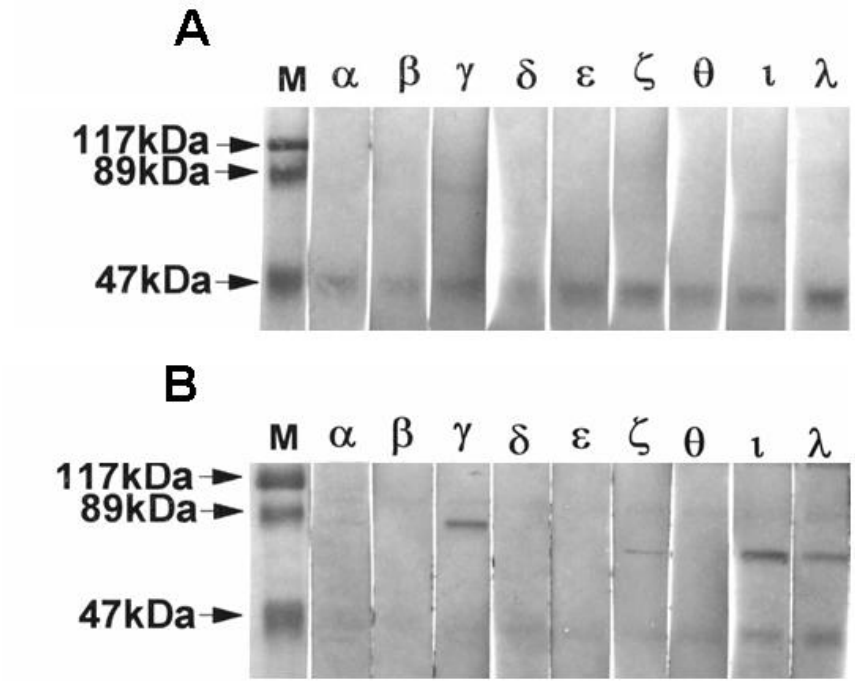
Table 3. Comparison of activities of PKCα、PKCζ、PKCι、PKCλ by lymphatic metastasis, grade of carcinoma, age of the patient, and the presence of estrogen receptor

	PKCα	PKCζ	PKCι	PKCλ
Lym.N + (n=15)	124.4±13.7	170.6±17.4	108.0±19.0	88.7±10.2
- (n=17)	148.4±19.1	173.3±19.5	121.7±22.0	93.3±12.0
Grade 3 (n=15)	146.2±18.2	181.9±17.8	104.8±20.5	99.2±12.5
2 (n=17)	144.3±18.9	163.9±16.9	126.8±19.5	102.6±10.6
Age >45 (n=29)	138.2±12.4	175.9±13.1	111.6±14.9	96.4±9.1
<45 (n=9)	152.4±24.5	172.2±26.3	139.4±25.0	98.4±10.9
ER + (n=20)	144.8±16.5	185.0±15.6	133.0±17.7	107.7±8.5
- (n=15)	151.5±18.3	157.7±18.6	113.3±23.0	94.8±15.4

Legend

Fig. 1. Immunoblot analysis of protein kinase C iosoforms in both adjacent normal breast tissue (A) and breast tumors (B). Specimens was prepared as described in “Materials and Methods”. Aliquots of the homogenates were separated on denaturing polyacrylamide gels and transferred to nitrocellulose paper. The blots were stained with PKC isoenzyme-specific antibodies. M, the molecular weights are indicated on the left

Fig. 1.


A
M α β γ δ ε ζ θ ι λ
117kDa
89kDa
47kDa
B
M α β γ δ ε ζ θ ι λ
117kDa
89kDa
47kDa