

Original article:

## Gender specific expression of tumor suppressor PKC $\delta$ versus oncogenic PKC $\eta$ in renal cell carcinoma

Walburgis Brenner<sup>\*1</sup>, Christoph Wiesner<sup>1</sup>, Gloria Färber<sup>1</sup>, Thomas Herget<sup>2</sup>, Jan G. Hengstler<sup>3</sup>, Joachim W. Thüroff<sup>1</sup>

<sup>1</sup>Department of Urology, Telefax: ++49 6131 172986, e-mail: brenner@mail.uni-mainz.de (\*corresponding author), <sup>2</sup>Institute of Physiological Chemistry and Pathobiochemistry, University of Mainz, Langenbeckstr. 1, 55131 Mainz, <sup>3</sup>Institute of Pharmacology and Toxicology, Härtelstr. 16-18, 04017 Leipzig, Germany

### ABSTRACT

Tumor incidence for renal cell carcinoma is two-fold higher in males than in females. Members of the protein kinase C (PKC) gene family have been shown to be relevant for carcinogenesis. However, little is known about a possible gender specific role of PKC in renal cell carcinoma (RCC). In this study, we quantified expression of eleven PKC-isoforms in clear cell RCCs (ccRCC) and in the corresponding normal renal tissue. A possible association of PKC-isoforms with gender of the patients was examined. Tissue specimens of 27 patients, 14 males and 13 females, with ccRCC and of the corresponding normal renal tissue were examined. Expression of PKC-isoforms were detected by Western blot analysis and quantified by computer-aided integration. In ccRCCs, as well as in the corresponding normal renal tissue, all PKC-isoforms except PKC $\gamma$  and  $\theta$  were detectable. Clear associations with gender of the patients were observed: (i) PKC $\delta$  was reduced in tumor tissue of female patients ( $p = 0.023$ ), but not of male patients ( $p = 0.198$ ). (ii) A 3.6-fold enhanced expression of the oncogene PKC $\eta$  was found in tumor tissue of female compared to male patients ( $p = 0.049$ ). Gender specific differences in PKC $\delta$  as well as PKC $\eta$  expression suggest that different molecular mechanisms are relevant for carcinogenesis of ccRCC in male and female patients. This may become important for classification and treatment of ccRCC.

**Keywords:** Protein kinase C, gender specificity, renal carcinoma

### INTRODUCTION

The incidence of renal cancer is approximately two-fold higher in males than in females (deKernion et al., 1992). Furthermore, a gender specific difference in the prognosis of renal clear cell carcinoma has been shown: women had a more favorable prognosis with longer survival than men (Onishi et al., 2002; McNichols et al., 1981; Lieber et al., 1981). However, the

influence of gender on prognosis is discussed controversially (Nurmi, 1984).

Signal transduction pathways that regulate cell proliferation, apoptosis and migration are critical for development of renal cell cancer (Brenner et al., 1995). Usually, tumor development is caused by an upregulation of oncogenes and/or a downregulation of tumor suppressors.

The most important signal transduction pathways that regulate cell proliferation and apoptosis are the ras/MAP-kinase pathway

and the Akt/PKB pathway. Involved in these two pathways are the protein kinases C (PKC). PKC is a family of eleven serine/threonine kinases which play a critical role in the regulation of multiple cellular mechanisms such as cell differentiation, cell growth, migration, apoptosis and tumor development (Mellor et al., 1998; Brenner et al., 2000).

Because the PKC isoforms display substantial differences in enzymatic activities, tissue and intracellular distribution and cofactor requirements, they might have different physiological roles (Oehrlein et al., 1998; Goodnight et al., 1994). A point of interest is the identification of specific physiological meanings of each isoform for tumor cells. PKC $\epsilon$  overexpression results in general in tumorigenesis, leading to its classification as an oncogene (Cacace et al., 1993). Oncogenic properties are also known for the isoforms PKC $\eta$  and PKC $\zeta$  (Schonwasser et al., 1998; de Thonel et al., 2001; Nishizuka et al., 1995). In contrast, PKC $\alpha$  and PKC $\delta$  appear to play a role as tumor suppressors (Brooks et al., 1993; Lu et al., 1997; Fukumoto et al., 1997). Since ccRCC development is a multifactorial phenomenon and twice as often in men than in women, we investigated the role of the various PKC-isoforms and their expression pattern in male and female patients with ccRCC.

In the present study, we determined the expression of eleven PKC-isoforms in ccRCC tissue as well as in the corresponding normal renal tissue of 14 males and 13 females. For this purpose we extracted protein from 27 ccRCCs and quantified the PKC-isoforms by Western blot analysis. The resulting PKC values were correlated with the gender and age of the patients.

## MATERIALS AND METHODS

### *Patients and tissue specimens*

Tissue specimens were obtained under sterile conditions from 27 patients, 14 males and 13 females, with primary ccRCC. All patients had undergone nephrectomies at the

Department of Urology of the Johannes Gutenberg-University, Mainz, Germany. From these patients, tissue specimens from normal and malignant renal tissue (5x5x5 mm) were shock frozen in liquid nitrogen and stored at -80°C until needed (Reutzel et al., 2001). Normal and tumor tissue were taken from the same kidney, whereby the normal tissue was located at least 3 cm from the edge of the tumor. Because the precursor cells of ccRCCs are proximal tubulus epithelial cells, which are initially located in the cortex of the kidney, non malignant tissue specimens were obtained from the cortex for our experiments. The diagnosis of ccRCC and the histological classification of all tumor tissue specimens used in this study was confirmed by a pathologist. Grading and staging were done according to the International Union Against Cancer (Wittekind et al., 1997; Brauch et al., 2000). Because of limited amount of tissue, in five cases investigations could be determined only for part of the PKC isoforms.

### *Preparation of protein extract*

For preparation of protein extracts, the renal tumor tissue and normal renal tissue specimens were crushed with a mortar under liquid nitrogen (Schiffer et al., 2003) and suspended in lysis buffer at 4°C (20 mM HEPES, pH 7.7, 0.2 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.4 mM EDTA, 1% Triton X-100, 0.5 mM DTT, 100 µg/ml leupeptin, 100 µg/ml aprotinin, 10 mM benzamide, 2 mM phenylmethylsulphonyl fluoride, 20 mM  $\beta$ -glycerophosphate and 0.1 mM sodium-ortho-vanadate; Brenner et al., 2002). Protein concentrations of the supernatants were determined using Bicinchoninic acid reagents (Hengstler et al., 1999b; von Mach et al., 2003).

### *Western blot analysis*

The primary antibodies against the PKC-isoforms used for Western blotting as well as dilution factors and the apparent molecular mass of the antigen are listed in table 1. The horseradish peroxidase-conjugated goat-anti-rabbit or rabbit-anti-mouse antibodies were obtained from DAKO (Hamburg, Germany),

the horseradish peroxidase-conjugated donkey-anti-goat was obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA).

Protein was separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Brenner et al., 1995) in 10% acrylamide gels and transferred by semi-dry blotting onto polyvinylidene fluoride membranes (PVDF, Immobilon P, Millipore, Bedford, MA) for Western blotting. For Western blot analysis of PKC isoforms, a protein extract of the embryonic carcinoma stem cell line PCC7-Mz1, which expresses all PKC isoforms (Oehrlein et al., 1998), was always run in parallel as positive control. The membrane was blocked in 0.1% Triton X-100, 5% low fat milk powder in PBS for one hour (Fritz et al., 1997). The primary antibodies were incubated overnight at 4°C, diluted according to table 1 in 0.1% Triton, 1% low fat milk powder and 0.1% BSA (bovine serum albumine) in PBS. After washing three times with 0.1% Triton X-100

in PBS, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for one hour at room temperature. The bound antibodies were visualized by enhanced chemiluminescence (ECL) detection system using Fuji medical X-ray films. To check the specificity of the signals detected by Western blot, the primary antibodies were preadsorbed with the corresponding blocking peptide for 10 min. Afterwards, Western blot analyses were performed as described above. Signals were considered as specific when the bands reflecting PKC did not show up after this pretreatment. After exposure to X-ray film, the Western blot membrane was stained with Coomassie brilliant blue (Sigma, Deisenhofen, Germany) and the amount of total protein was calculated by integration of the bands of each lane using Sigma gel 1.0 software (Brenner et al., 1999; Hengstler et al., 1998).

Table 1: Antibodies for Western blot analysis of the eleven PKC-isoforms (p = polyclonal, m = monoclonal), Antibodies marked with P.P. were kindly provided by Peter Parker, London, Great Britain.

| PKC Antibodies               | Supplier                  | Type | Species | Dilution Factors | Apparent molecular mass of Antigen (kDA) |
|------------------------------|---------------------------|------|---------|------------------|--|
| anti-PKC $\alpha$            | UBI                       | m    | Mouse   | 1:2000           | 82                                       |
| anti-PKC $\beta$ I           | P.P                       | p    | Rabbit  | 1:2000           | 96                                       |
| anti-PKC $\beta$ II          | Santa Cruz                | p    | Rabbit  | 1:2000           | 80                                       |
| anti-PKC $\gamma$            | Sigma                     | m    | Mouse   | 1:2000           | 80                                       |
| anti-PKC $\delta$            | Santa Cruz                | p    | Rabbit  | 1:2000           | 80                                       |
| anti-PKC $\epsilon$          | Santa Cruz                | p    | Rabbit  | 1:2000           | 93                                       |
| anti-PKC $\eta$              | Santa Cruz                | p    | Rabbit  | 1:2000           | 80                                       |
| anti-PKC $\theta$            | Santa Cruz                | p    | Rabbit  | 1:2000           | 79                                       |
| anti-PKC $\zeta$             | P.P.                      | p    | Rabbit  | 1:2000           | 75                                       |
| anti-PKC $\iota$ / $\lambda$ | Transduction Laboratories | m    | Mouse   | 1:2000           | 74                                       |
| anti-PKC $\mu$               | Santa Cruz                | p    | Rabbit  | 1:2000           | 116                                      |

#### *Evaluation of PKC content*

The amount of separated whole protein was calculated after staining of the Western blot membrane with Coomassie brilliant blue and integration of all spots by Sigma gel 1.0 software. The intensities of the signals of PKC-isoforms on the X-ray film were referred to the whole amount of analyzed

protein on the respective sample. The in this way calculated values were defined as “densitometric units”. To assess a potentially development of expression during carcinogenesis of ccRCC, the relative expression of PKC was calculated. Therefore, the densitometric units of tumor tissue were divided through the densitometric units of the

corresponding normal renal tissue of the same patient.

#### Statistical analysis

The ratio between PKC expression in tumor tissue and in the corresponding normal renal tissue was calculated. Possible association of these ratios with gender and age were evaluated using the two-sided Mann-Whitney test (Hengstler et al., 1999a). Statistical analysis was performed using SPSS 10.0 software (SPSS Inc., Chicago, USA).

## RESULTS

#### Association of PKC expression with sex and age

Expression of all eleven PKC-isoforms was determined in ccRCC tissue specimens and in the corresponding normal renal tissues. We observed that two of the PKC-isoforms, namely PKC $\delta$  and  $\eta$ , were influenced by patient's gender. In females, a decrease in PKC $\delta$  expression was observed in ccRCC versus normal renal tissue (median 82.9%; Q<sub>1</sub> 62.6%; Q<sub>3</sub> 100.8; p = 0.023). No major difference in PKC $\delta$  was measured for male patients between ccRCC and normal tissue (median 109.2%; Q<sub>1</sub> 90.7%; Q<sub>3</sub> 141.7%; p = 0.198). The difference in ratios of PKC $\delta$  expression (ccRCC divided by normal tissue) between male and female patients was significant with p = 0.019 (Fig. 1).

A further difference between male and female patients was observed for PKC $\eta$ . The ratios (ccRCC divided by normal tissue) were higher in female (median 361.9%; Q<sub>1</sub> 99.4%; Q<sub>3</sub> 1000.0%; p = 0.035) as compared to male patients (median 93.2%; Q<sub>1</sub> 29.8%; Q<sub>3</sub> 185.0%; Fig. 2). A difference between ccRCC and normal tissue could be detected only for females (p = 0.049). A correlation between the PKC expression and the age of the patients was not detectable.

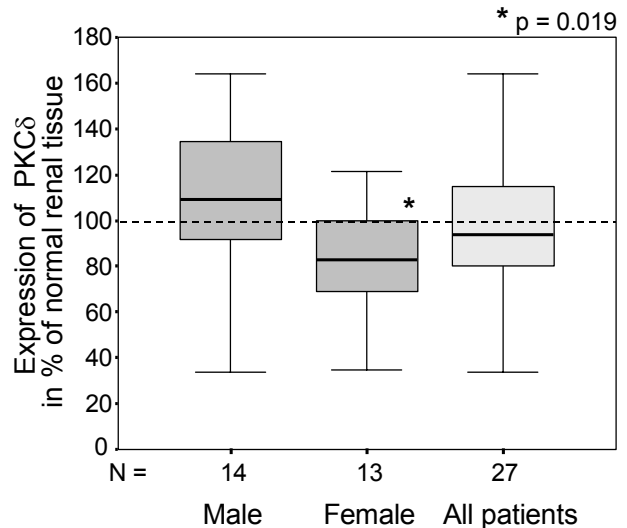


Figure 1: Expression of PKC $\delta$  in relation to gender. PKC $\delta$  expression in tumor tissue in percent of the corresponding normal renal tissue. In females, PKC $\delta$  was reduced by about 24% (p = 0.019) compared to the nearly unaltered expression in males. Box plots show medians (central lane), 25% and 75% percentiles (lower and upper side of the box) and minimum and maximum (lower and upper bars).

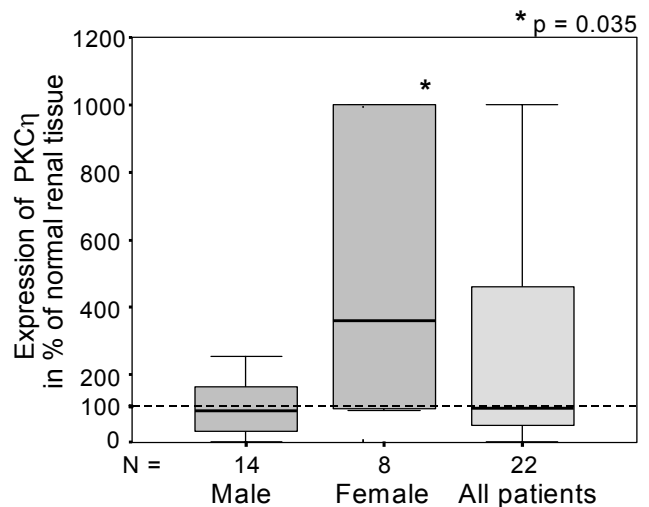


Figure 2: Expression of PKC $\eta$  in relation to gender. PKC $\eta$  expression in tumor tissue in percent of the corresponding normal renal tissue. Expression of PKC $\eta$  was 3.9-fold higher in females compared to males (p = 0.035). In addition, PKC $\eta$  was 3.6-fold enhanced compared to normal renal tissue in females. Box plots show medians (central lane), 25% and 75% percentiles (lower and upper side of the box) and minimum and maximum (lower and upper bars).

## DISCUSSION

We studied the expression of eleven PKC-isoforms in ccRCC tissue specimens and the corresponding normal renal tissues of 27 patients. PKC is involved in regulation of cell growth, differentiation and apoptosis. However, the role of the different PKC isoforms in gender specific carcinogenesis is not investigated until now. We examined whether PKC expression differed between normal and tumor tissue and if it was associated with gender.

Expression of PKC $\delta$  in females was significantly lower in ccRCC tissue compared to the respective normal renal tissue ( $p = 0.023$ ). In male patients, no difference of PKC $\delta$  expression in ccRCC tissue compared to normal tissue was observed. PKC $\delta$  is a well known tumor suppressor with several established anti-carcinogenic mechanisms: (i) inhibition of cell cycle progression by antagonisation of the Jak-STAT signaling pathway (Saharinen et al., 1997); (ii) inhibition of phospholipase D (antagonizing Raf activation; Goekjian and Jirousek 1999) and (iii) phosphorylation of lamin B leading to apoptosis (Cross et al., 2000). Interestingly, expression of PKC $\delta$  has been reported to be estrogen dependent in some cell types (Shanmugam et al., 1999), a possible explanation for gender specific expression. Furthermore, induction of PKC $\delta$  in transgenic mice resulted in a more pronounced reduction of papilloma in males than in females (Reddig et al., 2000), which lead to the speculation that tumors of males are more sensitive against alteration of PKC $\delta$  expression than of females. Perhaps, female tumors require a stronger reduction of the tumor suppressor PKC $\delta$  to trigger carcinogenesis, whereas in male patients tumor development may occur even with unaltered PKC $\delta$  levels. This theory would explain the reduced expression of PKC $\delta$  in female renal cell carcinomas (Fig. 2).

Expression of PKC $\eta$  did not differ between ccRCC and normal renal tissue in males. However, a more than three-fold increase in mean PKC $\eta$  expression was

observed in females in ccRCC compared to normal renal tissue ( $p = 0.049$ ). PKC $\eta$  has been described to be associated with histological grading: tumors with grading 3 and 4 expressed about three-fold more PKC $\eta$  than grade 1 and 2 tumors (Brenner et al., 2003). PKC $\eta$  has been discussed to function as an oncogene because it is involved in activation of Raf (Schonwasser et al., 1998). This might explain the role of PKC $\eta$  in progression of ccRCC, although an association with increased Raf signaling and proliferation has not yet been examined in ccRCC. In contrast to PKC $\eta$ , expression of other oncogenes like c-met or bcl-2 did not correlate with gender (Nakopoulou et al., 1997; Takayama et al., 1996).

The described gender specific expression of PKC isoenzymes seems to be dependent on cell type and species. In rat liver, Zangar et al. found a gender specific expression of the oncogenic PKC $\alpha$  isoform with an enhanced expression in females (Zangar et al., 1995).

The more distinct downregulation of the tumor suppressor PKC $\delta$  and high level of oncogene PKC $\eta$  in females does not imply a worse outcome. In contrast, female patients with ccRCC have a better survival rate than male patients (Onishi et al., 2002; McNichols, 1981), although other studies describe no differences in prognostic outcome of males versus females (Kirkali et al., 2001, Congregado et al., 2001). However, males seem to be more sensitive against treatment with Interleukin 2 compared to females (Elias and Hunt, 2001). Obviously, different molecular mechanisms contribute to renal carcinogenesis in male and females. For renal carcinogenesis in females, a stronger reduction of tumor suppressors and increase of oncogenes is perhaps required. This may be one reason for the two-fold higher incidence of RCC in males than in females. Furthermore, PKC $\delta$  seems to represent a tumor suppressor that is relevant for pathogenesis of ccRCC in females.

The main findings of this study, a more pronounced reduction of the tumor

suppressor PKC $\delta$  in tumors of female compared to male patients and an increased expression of the oncogene PKC $\eta$  in tumors of female but not male patients suggest gender specific molecular mechanisms of carcinogenesis in ccRCC. In females, carcinogenesis of ccRCC seems to be associated with a stronger reduction of the tumor suppressor PKC $\delta$  and a stronger enhancement of the oncogene PKC $\eta$ . This may play a role for classification of ccRCC and for females treating with PKC $\eta$  specific inhibitors.

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