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Increased expression of 5-lipoxygenase is common in clear cell renal cell carcinoma

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Summary. The clinical behaviour of Clear Cell Renal Cell Carcinoma (CC-RCC) is often unpredictable. To fully understand the signaling pathways involved in CC-RCC development, we examined whether the 5-Lipoxygenase (5-LO), which catalyzes the biosynthesis of proinflammatory leukotrienes, is involved in renal tumorigenesis. By analyzing 46 snap-frozen primary renal cell carcinomas and their corresponding normal renal cortex biopsies, 5-LO protein levels were found to be significantly increased in the majority of CC-RCCs ($P<0.001$). Quantitative 5-LO mRNA expression analysis revealed up to 3-fold increased expression in the tumor tissues. There was no association between 5-LO and gender, grade or vein invasion. In contrast, increased 5-LO protein and mRNA correlated with large tumor size (>4 cm) and age of patients ($P<0.001$). 5-LO was frequently overexpressed in von Hippel-Lindau protein (pVHL)–reduced tumors and in Vascular Endothelial Growth Factor (VEGF)-positive tumors, which represent two frequent alterations in CC-RCC. Cell culture experiments demonstrated that VEGF expression was strongly inducible by 5-LO metabolites in RCC cell lines. The loss of pVHL expression led to high basal 5-LO and VEGF expression, which were markedly reduced by transfection with 5-LO small interfering RNA (siRNA). These results suggest that 5-LO up-regulation is an important step in renal cancer progression.

Key words: 5-lipoxygenase, Renal cell carcinoma, Eiocasanoids, Tumor progression

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Introduction

5-Lipoxygenase (5-LO) is involved in the production of biologically active compounds such as 5(S)-hydroxyeicosatetraenoic acid (5(S)-HETE) and leukotriene B_4 (LTB_4) from arachidonic acid (Lewis et al., 1990; Soberman and Christmas, 2003). 5-LO is widely distributed in human tissues and is up-regulated by a variety of stimuli including cytokines, growth factors, and oncogenes (Catalano and Procopio, 2005; Catalano et al., 2005). Given the expression and function of 5-LO in human allergic and inflammatory diseases, recent evidence indicates that 5-LO is also implicated in carcinogenesis and tumor progression (Catalano et al., 2005). 5-LO is expressed in several cancer cells, and its inhibition blocks proliferation and induces apoptosis (Avis et al., 1996; Hong et al., 1999). In addition, 5-LO expression was reported in human prostate, breast, colon and pancreatic cancer tissues, where it is related to cell proliferation (Öhd et al., 2003; Matsuyama et al., 2004a; Hennig et al., 2005; Jiang et al., 2006). In human kidney, 5-LO is detected in both glomerular mesangial and epithelial cells under certain conditions, such as renal failure associated with diabetes and hypertension (Natarajan and Reddy, 2003). Recent studies pointed out the important role of 5-LO pathways in the regulation of human renal cell carcinoma (RCC) cell proliferation and survival (Matsuyama et al., 2004b, 2005). However, the expression of 5-LO has not been carefully investigated in human RCC tissues.

RCC is characterized by abundant neo-vascularization. Clear-cell (CC)-RCCs, which account for 85% of renal cancers, are more vascularized than non-clear-cell renal tumors, e.g. papillary or chromophobe RCC and oncocytomas (Jacobsen et al., 2000). The majority of CC-RCCs lack functional von Hippel-Lindau protein (pVHL) that leads to increased hypoxia-inducible factor 1α (HIF-1α) expression (Turner et al., 2002). The transcription factor HIF-1α is a key regulator of several pro-angiogenic factors such as

VEGF, platelet-derived growth factor and erythropoietin. Therefore, over-expression of VEGF may explain the hypervascular nature of CC-RCCs and stimulate the growth and progression of this malignancy (Na et al., 2003). In addition to HIF-1a, other genes relevant to cancer development and progression, such as vascularization factors, cell cycle regulators and growth factors may be involved in VEGF up-regulation of CC-RCCs. Recent studies have demonstrated that 5-LO can activate the transcription of VEGF (Romano et al., 2001). These findings suggest that 5-LO pathway may play an important role in the regulation of VEGF. In the present study, we investigated the expression of 5-LO in a series of human CC-RCCs and related the expression of 5-LO to clinical and pathologic variables. We show that up-regulation of 5-LO is a common and striking feature of CC-RCCs, and that 5-LO up-regulation in CC-RCC is correlated with reduced pVHL and enhanced VEGF expression.

Materials and methods

Patients and tissue specimens

RCC tumour and adjacent normal kidney specimens were analyzed from a total of 58 patients. There were 30 men and 20 women, with a mean age of 65.0 years (range, 39-88 years). Patients with RCC underwent potentially curative surgical resection at Department of Urology, Umberto I-Lancisi-Salesi University Hospital (Ancona, Italy) and Clinical Urology, University of Chieti, Medical School (Chieti, Italy) between 2003 and 2006. Informed consent was obtained from each patient. None of the patients included in this study had received chemo-, immuno- or radio-therapy before surgery. The medical history of each patient was obtained. We excluded patients with a history of inflammatory disease of the kidney or other malignancies. Tumours were classified according to the Heidelberg classification (Reuter and Presti, 2000). Of the 58 RCC tumours evaluated, 50 were diagnosed as conventional clear cell (CC-RCC), 6 as papillary RCC, and 2 as chromophobe RCC. Specimens of kidney cancer tissue obtained at surgery were immediately snap frozen in liquid nitrogen and stored until use.

Tumour stage and histological grade were determined in all patients. Pathological tumour stage was determined in accordance with the 1997 joint International Union Against Cancer (UICC) and American Joint Commission on Cancer staging system (Fleming et al., 1997). TNM criteria were: I-T1N0M0, II-T2N0M0, III-T3N0-1M0 and T1-2 N1M0, and IV-T4N0-2M0-1, any TN0-1M1 and any TN2M0. Tumour grade was determined according to the 1997 TNM grading system by light microscopy.

Cell cultures and treatments

Human RCC cell lines Caki-1, Caki-2, 786-O, the human adenocarcinoma A-498 cell line and the proximal tubular epithelial cell line HK-2 were obtained from American Type Culture Collection (ATCC). The cell line CRBM-1990 was previously isolated from a renal cell carcinoma bone metastasis (Avnet et al., 2004) and was a generous gift from Dr. Baldini (Istituti Ortopedici Rizzoli, Bologna, Italy). Caki-2 were cultured in RPMI-1640 medium (EuroClone, Milan, Italy), Caki-1 and CRBM in the HAM'S F12-K (Invitrogen, San Diego, CA), A-498 in EMEM (ATCC) and HK-2 in Keratinocyte SFM (Invitrogen, San Diego, CA). To *in vitro* assays, synthetic 5-HETE and other 5-LO metabolites were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Experimental conditions were as previously described (Romano et al., 2001), with all experiments repeated at least three times. Following treatment, conditioned medium was removed and analyzed for VEGF protein levels by ELISA (R&D Systems, Minneapolis, MN), and calibration curves were done for all experiments.

Protein extraction and quantification

Sections of the fresh frozen tissues were fractionated and homogenized in a buffer containing 150 mM NaCl, 10 mM Tris-Hcl (pH 7.4), 1 mM EDTA (pH 8), 1% Triton X-100, and a mixture of protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 1mM phenyl-methylsulfonil fluoride (PMSF) and 2 μg/ml proteinin). Protein expression levels were quantified using Bradford protein (Sigma, St. Louis, MO) detection using NanoDrop® ND-1000 Spectrophotometer (Celbio, Milan, Italy).

Western blot analysis

Electrophoresis with protein extract from each sample (40 μg) was done on 10.0% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Hybond-N, Amersham). The membranes were probed with anti-5-LO (1:500, BD Biosciences, Oxford, United Kingdom) and anti-VHL (1:250, BD Biosciences) primary antibodies. In parallel, membranes were also incubated with an anti-α-actin antibody (1:2,000, Sigma, Poole, United Kingdom). After washing, the blots were incubated in a horseradish peroxidase-conjugated secondary antibody (Amersham, Arlington Heights, IL). Specific protein bands were visualized with an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). To ensure that equal amounts of protein were loaded, the membranes were stained with Ponceau red and actin protein expression was detected.

Real-time quantitative reverse transcription-PCR

Total RNA from cell lines, primary RCCs, and normal renal cortex biopsies was extracted with Omnizol reagent (EuroClone, Milan, Italy) according to the instructions of the manufacturer. RNA concentrations

were determined with NanoDrop® ND-1000 Spectrophotometer (Celbio). The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide. The primers and probe used for quantitative RT-PCR were according to the publication of Catalano (Catalano et al., 2005) and purchased from Qiagen (Milan, Italy). The identities of PCR products were confirmed by DNA sequencing. Human 5-LO genomic DNA sequence GenBank was used as a reference sequence throughout. The reaction conditions for 5-LO detection were as described previously (Catalano et al., 2005). The fluorescence emitted by the reporter dye was detected using the Chromo 4™ Four-Color Real-Time System (Bio-Rad, Milan, Italy).

Statistical analysis

The statistical analysis used for making the comparison among the groups was performed through the Whitney U tests for independent samples or the Wilcoxon signed-rank test for related samples, as appropriate. Comparisons between proportions were assessed using the chi-squared test or the Fisher's exact test, when appropriate. The correlation analysis was performed using the Spearman's rank test. Two-tailed tests were used for all comparisons and $p<0.05$ was considered statistically significant. SPSS 11.0 software was used for all calculations.

Results

Clinical findings

We examined tumours from 30 men and 20 women, ranging from 39 to 88 years of age (median age, 65 years). With regard to treatment, 31 patients underwent radical nephrectomy, whereas 27 underwent partial resection including one that underwent bilateral partial resection. The histological cell type, tumour grade, pathological tumour stage of 58 RCC samples are summarized in Table 1.

Expression of 5-LO protein in RCC tissues and cell lines

In the RCC tissue samples, 5-LO was firstly detected by Western blot. The typical pattern of 5-LO protein expression in RCC and corresponding non-malignant kidney cortex is shown in Fig. 1A. The amount of 5-LO markedly varied between different tissue samples. However, 5-LO protein expression in tumour tissue was significantly increased in the majority of CC-RCCs (31 of 50; 62%) compared with their corresponding non-malignant kidney cortex tissues (Fig. 1B). Papillary and

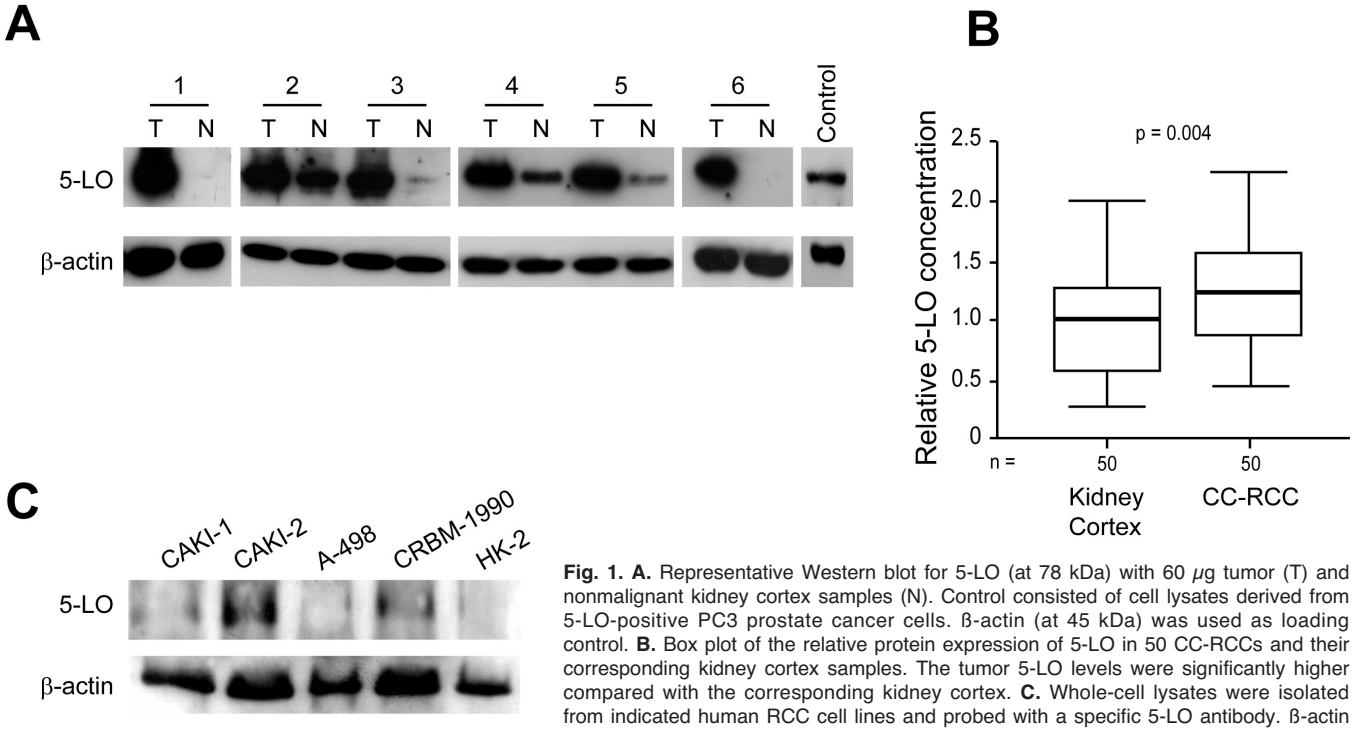


Fig. 1. A. Representative Western blot for 5-LO (at 78 kDa) with 60 μg tumor (T) and nonmalignant kidney cortex samples (N). Control consisted of cell lysates derived from 5-LO-positive PC3 prostate cancer cells. ß-actin (at 45 kDa) was used as loading control. **B.** Box plot of the relative protein expression of 5-LO in 50 CC-RCCs and their corresponding kidney cortex samples. The tumor 5-LO levels were significantly higher compared with the corresponding kidney cortex. **C.** Whole-cell lysates were isolated from indicated human RCC cell lines and probed with a specific 5-LO antibody. ß-actin was used as loading control. These cell lines include clear-cell RCC (CAKI-1, CAKI-2), renal adenocarcinoma (A-498), carcinoma bone metastasis (CRBM-1990) and proximal tubule epithelial (HK-2) cells.

chromophobe RCCs also expressed significantly higher values of 5-LO compared with kidney cortex (data not shown).

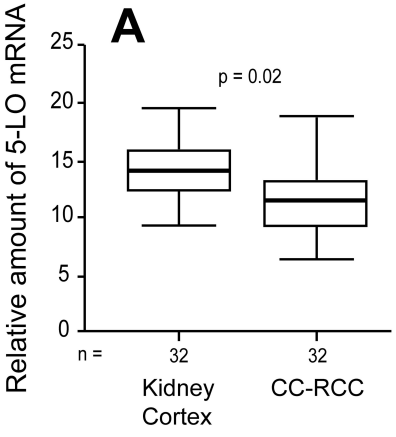
To support our data on increased 5-LO expression in RCC tissues, we analyzed the 5-LO protein levels in human RCC cell lines. 5-LO protein was readily detected in RCC cells CAKI-1 and CAKI-2, and in a cell line isolated from a renal carcinoma bone metastasis (CRBM-1990) (Fig. 1C). In contrast, 5-LO was undetectable in the adenocarcinoma-derived A-498 cells and proximal tubule-derived HK-2 cells (Fig. 1C).

Expression of 5-LO mRNA in RCC tissues and cell lines

Expression of 5-LO mRNA was then assessed by Real-time quantitative reverse transcription-PCR in a subset of 32 cases. The threshold cycle (CT) was defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe exceeds a fixed threshold above baseline. For a chosen threshold, a

Table 1. Clinical and pathological information of renal cell carcinoma.

	Stage				Grade			
	I	II	III	IV	1	2	3	Total
Conventional (clear cell subtype)	31	4	10	5	24	23	3	50
Papillary renal cancer	4	0	1	1	2	3	1	6
Chromophobe renal cancer	2	0	0	0	0	2	0	2
Total	37	4	11	6	26	28	4	58



C

RT-PCR product sequence of 5-LO mRNA derived from 15 CC-RCC

From 201
5'–

201 TGACTGTGGA	CGAGGAACTG	GGCGAGATCC	AGCTGGTCAG
241 AATCGAGAAG	CGCAAGTACT	GGCTGAATGA	CGACTGGTAC
281 CTGAAGTACA	TCACGCTGAA	GACGCCCCAC	GGGGACTACA
321 TCGAGTTCCC	CTGCTACCGC	TGGATCACCG	GCGATGTCGA
361 GGTTGTCCTG	AGGGATGGAC	GCG	

–3'

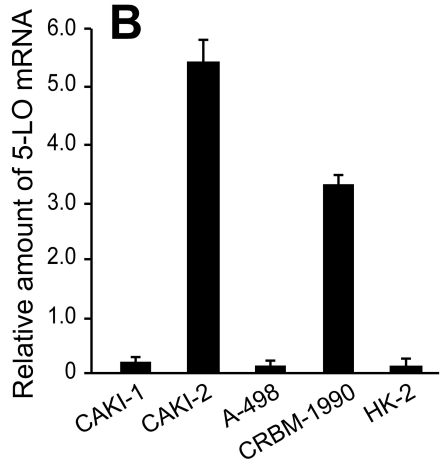


Fig. 2. A. Box plot of the relative mRNA expression of 5-LO in 32 CC-RCCs and their corresponding kidney cortex samples as determined by real-time quantitative RT-PCR. The tumor 5-LO mRNA levels were significantly higher compared with the corresponding kidney cortex. **B.** Real-time quantitative RT-PCR of 5-LO mRNA in the indicated RCC cell lines. ß-actin mRNA was used as an internal control for RNA quantity. **C.** Total RNA from a subset of 15 CC-RCCs was isolated using TRIzol. Then RNA was reverse-transcribed into cDNA and PCR was used to amplify 5-LO mRNA. PCR primers were designed based on the sequence of human 5-LO cDNA. The final PCR product was separated on 2% agarose gel and sequenced. RT-PCR product of 5-LO was identical to that reported in the Gene Bank.

smaller starting copy number results in a higher CT value. We used ß-actin mRNA as an internal control. The relative amounts of tissue 5-LO mRNA, standardized against the amount of ß-actin mRNA, were expressed as $\Delta CT = CT_{5\text{-}LO} - CT_{\beta\text{-}actin}$. As observed for 5-LO protein levels, there was a significant difference between tumour 5-LO mRNA and paired normal tissue 5-LO mRNA (P=0.02, signed *Ranks* test; ΔCT value in tumours range from 9.53 to 12.48 with a median of 11.13; ΔCT value in paired normal tissues range from 11.25 to 14.65 with a median of 13.15). The ratio of normalized 5-LO mRNA copies in tumours to normalized 5-LO mRNA copies in paired normal tissue was then calculated as $2^{-\Delta\pi CT}$, where $\Delta\Delta CT = \Delta CT_{Tumor} - \Delta CT_{Normal}$. The relative amount of 5-LO mRNA in tumours was greater than in paired normal tissue in 20 of 30 CC-RCCs (66.6%) (Fig. 2A). Real-time quantitative reverse transcription-PCR of 5-LO mRNA in RCC cell lines also supported the result observed in RCC tissues (Fig. 2B).

To further confirm that 5-LO mRNA in tumours is identical to the 5-LO cDNA sequence reported in the Gene Bank, a PCR product was sequenced in a subset of 15 CC-RCCs. The result showed that the sequence of all PCR products is identical to that reported in the Gene Bank (Fig. 2C).

Relationships between 5-LO expression and clinicopathological features

As determined by immunoblotting, all tumours were categorized into those that showed 5-LO protein levels indistinguishable from that of the normal, matched kidney tissue ("no increased" group), and those that overexpressed 5-LO protein relative to their normal adjacent kidney tissue ("increased" group). These two categories were used to assess whether the 5-LO protein levels correlated with cancer grade (Table 2). The relative 5-LO concentration in the samples (reported as "score") were higher in the high-grade cases, with a significant difference between grades 1 and 2 and grades 1 and 3, although no significant differences in intensity were noted between grades 2 and 3. We further evaluated the relationship between the relative 5-LO protein levels and tumour stage (Table 2). No significant differences were observed between each stage of RCC.

Table 2. 5-LO protein expression in relation to clinicopathological characteristics of renal cell carcinoma.

Clinicopathological features	Relative 5-LO protein levels			Score	*Statistical significance*
	Cases	"no increased"	"increased"		
Histological grade	58				
Grade 1	26	12	14	1.33±0.32	
Grade 2	28	9	19	2.08±0.65	*P<0.01* vs grade 1
Grade 3	4	0	4	2.61±0.75	*P<0.01* vs grade 1
Tumour pathological stage	58				
Low stage (stages I and II)	41	17	24	1.67±0.65	
High stage (stages III and IV)	17	4	13	2.28 ±± 0.40	*P<0.05* vs low stage

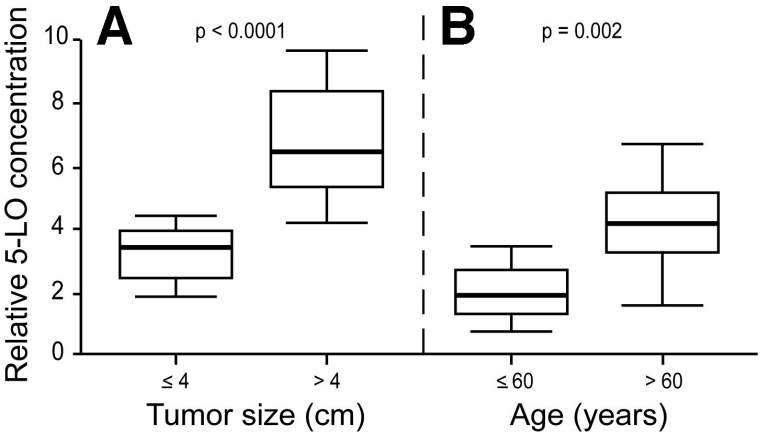


Fig. 3. Box plots of the relative protein expression of 5-LO in 34 CC-RCCs and tumor stage or grade **(A)** as well as in different tumor sizes or age of patients with CC-RCCs **(B)**. The tumor 5-LO levels were significantly between tumours ≤4 cm and >4 cm (P<0.0001) and between age of patients ≤60 and >60 years (P=0.002).

However, when we divided RCC into two groups on the basis of tumour stage, the intensity of 5-LO in high-stage RCC (stages III and IV) was significantly higher than that in the low stage. Accordingly, there was a significant correlation between the relative 5-LO protein concentration and tumour size (median threshold ≤ 4 vs >4 cm in diameter) (Fig. 3). Also, there was a significant correlation between the relative 5-LO protein levels and age among patients with CC-RCC (median threshold ≤ 60 vs >60 years) (Fig. 3). Similar results were observed when we utilized the values of normalized 5-LO mRNA instead of 5-LO protein levels (data not shown).

Relationships between 5-LO expression, VHL status and VEGF expression

We also investigated whether the relative immunointensity of 5-LO correlated with the relative VHL and VEGF protein levels in CC-RCCs. These relationships were examined in a subset of 34 CC-RCCs. Figures 4A and 4B, show representative pVHL and VEGF protein expression in RCC and corresponding non-malignant kidney cortex. A reduction in pVHL protein levels was detected in 27 of 34 (79%) CC-RCCs; whereas increased VEGF protein expression was detected in 25 of 34 (73%) tumors. Although a reduction of pVHL protein was detected only in 27 tumors, 20 of these tumors (74%) exhibited increased 5-LO protein levels. The χ^2 test revealed that the overexpression of 5-LO was more frequent in pVHL–reduced tumors than in VHL–increased tumors (P=0.03; Table 3).

We next examined whether the 5-LO pathway could interact with the pVHL status in the induction of VEGF in CC-RCCs. VEGF protein was significantly higher in CC-RCCs than paired normal kidney tissue ($P \leq 0.0001$, signed *Ranks* test) (Fig. 4C), and there was a significant relationship between the relative 5-LO protein concentration and increased VEGF protein levels (P=0.02; Table 4).

Because the effect of 5-LO protein expression on VEGF may be mediated, at least in part, at the transcriptional level (Romano et al., 2001), VEGF mRNA expression was determined in a subset of 25 CC-RCCs. Expression of VEGF mRNA was significantly higher in CC-RCCs than in paired normal tissue ($P \leq 0.0001$, signed *Ranks* test) (Fig. 4D). We observed a

Table 3. Relationships between the relative 5-LO protein concentration and the presence of pVHL in a total of 34 CC-RCCs.

		5-LO expression		
	No. (%)	no increased (%)	increased (%)	*P*
pVHL expression				
Negative	27 (79%)	7 (26%)	20 (74%)	0.03
Positive	7 (21%)	3 (43%)	4 (57%)	

Table 4. Relationship of the relative 5-LO protein levels to VEGF protein in a total of 34 CC-RCCs.

		5-LO expression		
	No. (%)	no increased (%)	increased (%)	*P*
VEGF expression				
increased	25 (74%)	5 (20%)	20 (80%)	0.02
no increased	9 (26%)	5 (56%)	4 (44%)	

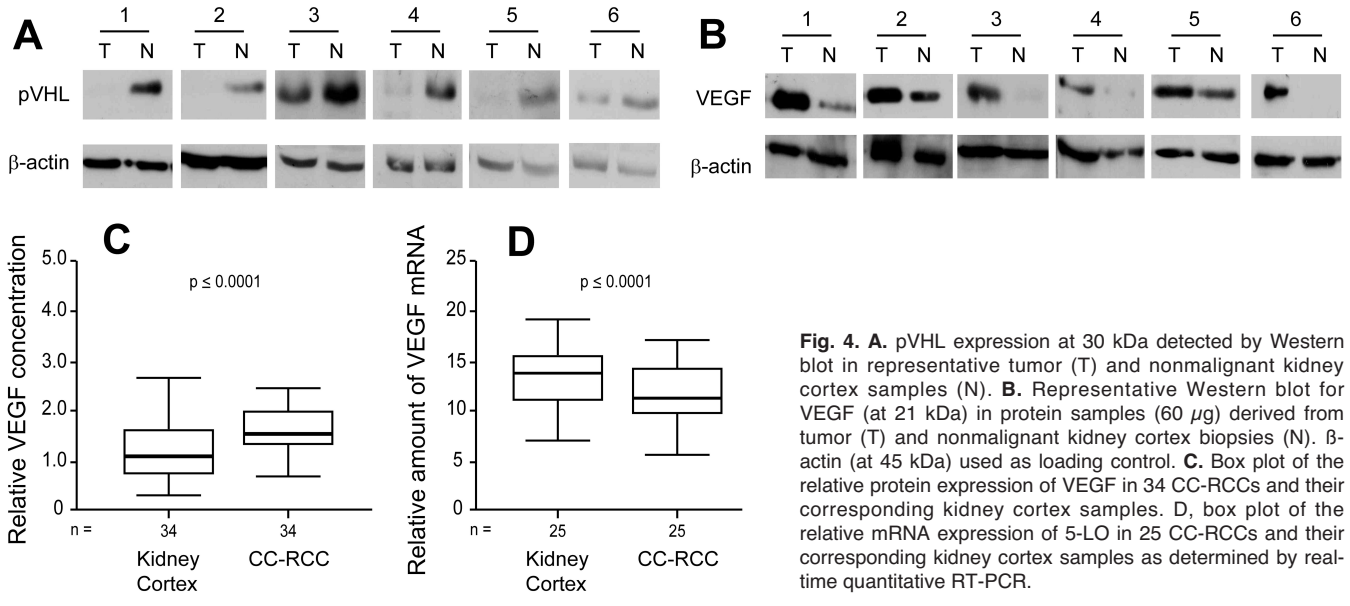


Fig. 4. A. pVHL expression at 30 kDa detected by Western blot in representative tumor (T) and nonmalignant kidney cortex samples (N). **B.** Representative Western blot for VEGF (at 21 kDa) in protein samples (60 μg) derived from tumor (T) and nonmalignant kidney cortex biopsies (N). ß-actin (at 45 kDa) used as loading control. **C.** Box plot of the relative protein expression of VEGF in 34 CC-RCCs and their corresponding kidney cortex samples. D, box plot of the relative mRNA expression of 5-LO in 25 CC-RCCs and their corresponding kidney cortex samples as determined by real-time quantitative RT-PCR.

trend between increased tumor 5-LO protein and VEGF mRNA, although this correlation only provided an R^2 value of 0.33 (P=0.093) and would not be considered statistically significant.

Evidence for induction of VEGF expression by 5-LO metabolites

Because 5-LO activity may be a potent inductor for VEGF expression in different cancer cells, including mesothelioma cells (Romano et al., 2001), we studied the inducibility of VEGF expression in RCC cell lines by exogenous exposure to 5(S)-HETE under serum-free conditions. All four RCC cell lines and the human mesothelioma cell line NCI-H28 (positive control) were treated with various concentrations of 5(S)-HETE. The exogenous addition of 5(S)-HETE increased VEGF mRNA expression 3–5-fold above control (untreated) in all cell lines tested (Fig. 5A). Other derivatives of 5-LO pathway such as 5(R)-HETE, 5(±)-HETE, or 5oxoETE had no significant effect on the cell lines at the concentrations we tested. The RCC cell lines were also evaluated for VEGF mRNA expression after exposure to leukotriene (LT)D_4, and concentrations of 1 and 10 nM increased VEGF mRNA expression 2.3-4.6-fold over control (data not shown). In contrast, no significant effects were observed with other arachidonic acid metabolites, including LTB_4, 12(S)-HETE, or 15(S)-HETE under the same experimental conditions (data not shown). Accordingly, 5(S)-HETE and LTD_4 dose-dependently increased VEGF release by Caki-1 and Caki-2 cells, with a significant induction after 48 h (Fig. 5B).

Loss of VHL function leads to high levels of VEGF in renal carcinoma cells (Maxwell et al., 1999). To assess the contribution of 5-LO pathway to constitutive VEGF expression, we used the renal carcinoma cell line 786-O, which have no pVHL expression (Maxwell et al., 1999). We observed that the 786-O cells had consistently higher basal levels of 5-LO and VEGF compared with pVHL-*positive* cells, such as Caki-1 cells (Fig. 6A,B). This led us to hypothesize that 5-LO may be an important co-regulator of VEGF expression upon loss of VHL function. Using siRNA techniques to silence 5-LO expression (Fig. 6A), we analyzed the effects of transient expression of 5-LO siRNA in pVHL-*null* 786-O cells.

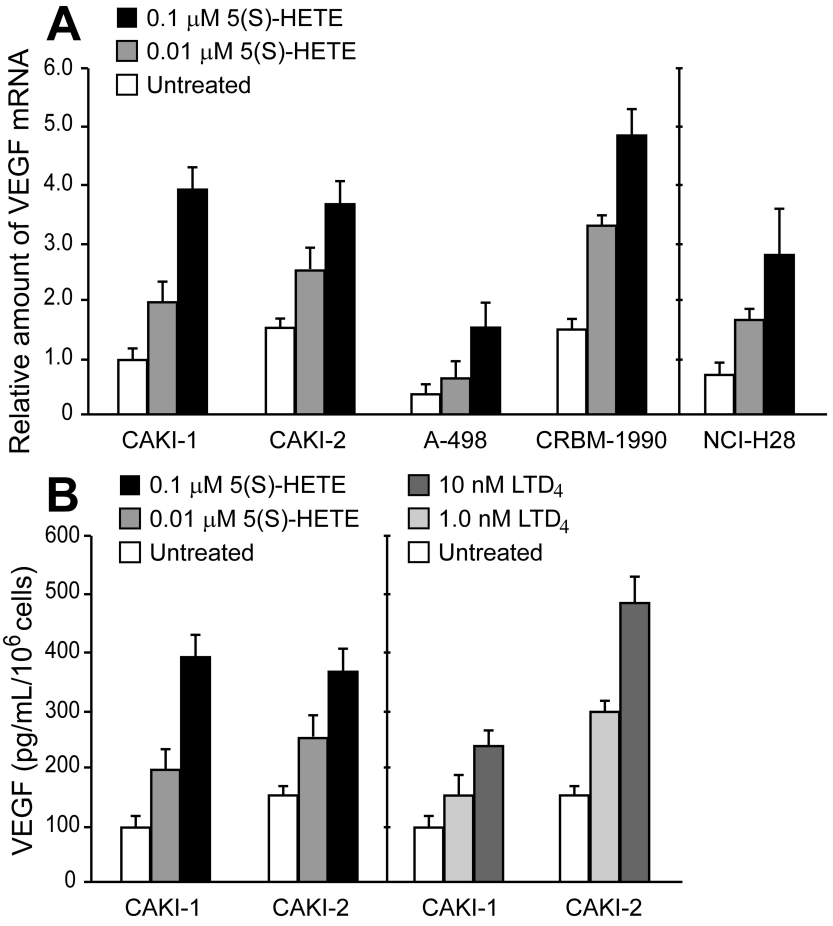


Fig. 5. A. To show ability of VEGF stimulation by 5-LO derivatives, we incubated the cell lines CAKI-1, CAKI-2, A-498, and CRBM-1990 for 24 h with 5(S)-HETE at various concentrations. Quantitative determination of VEGF mRNA expression in RCC cell lines was then obtained by real-time RT-PCR. **B.** CAKI-1 and CAKI-2 cell lines were incubated for 48 h with indicated concentration of 5(S)-HETE and LTD_4. Secreted VEGF was determined in conditioned medium by ELISA and normalized to cell number. *Columns*, mean of triplicate values of one representative experiment; *bars*, SE. Experiments were repeated twice. The differences between cells exposed to 5-LO metabolites compared with untreated (control) were statistically significant ($P<0.05$) as determined by *t* test for all of the metabolites shown.

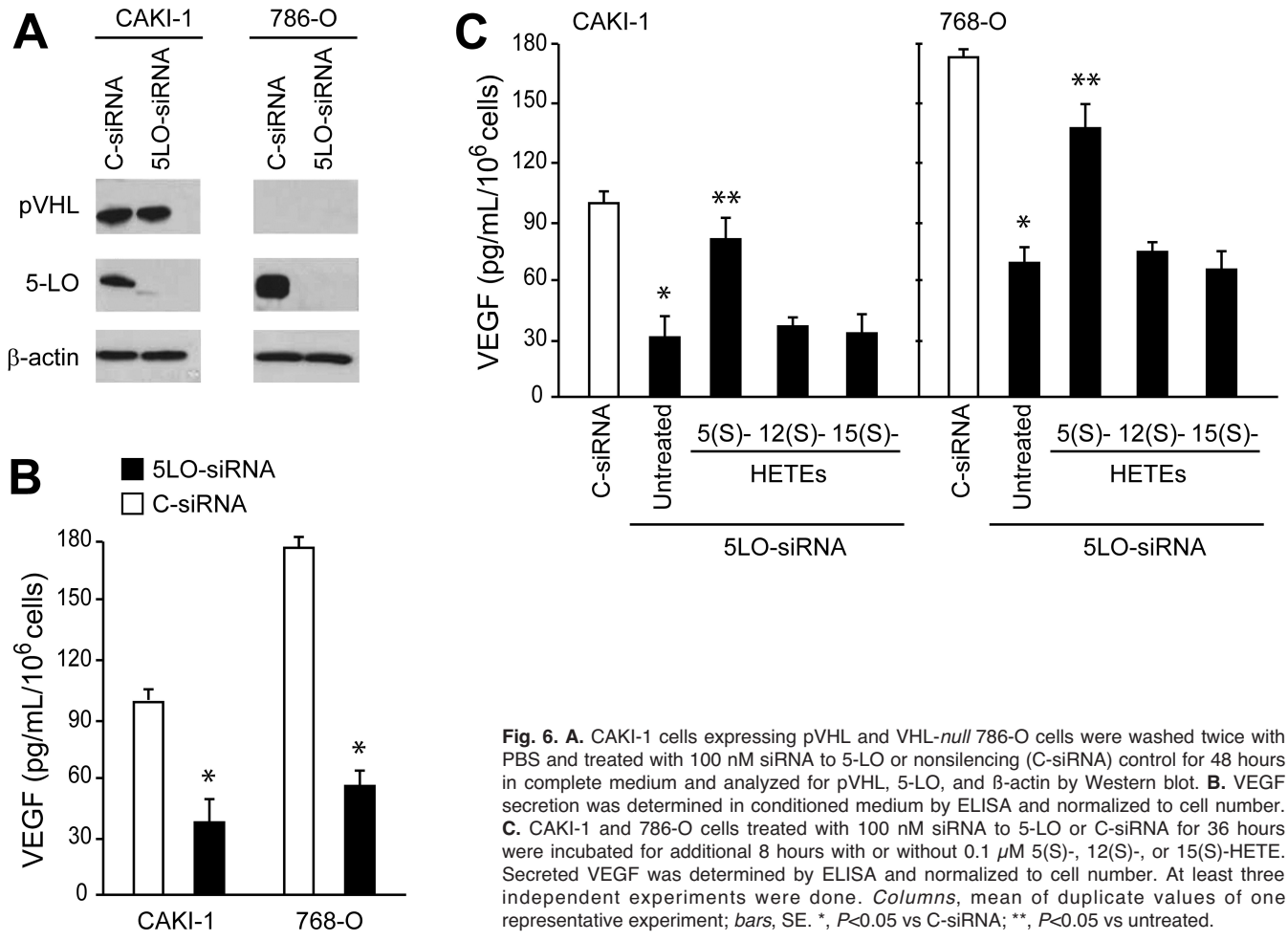


Fig. 6. A. CAKI-1 cells expressing pVHL and VHL-*null* 786-O cells were washed twice with PBS and treated with 100 nM siRNA to 5-LO or nonsilencing (C-siRNA) control for 48 hours in complete medium and analyzed for pVHL, 5-LO, and ß-actin by Western blot. **B.** VEGF secretion was determined in conditioned medium by ELISA and normalized to cell number. **C.** CAKI-1 and 786-O cells treated with 100 nM siRNA to 5-LO or C-siRNA for 36 hours were incubated for additional 8 hours with or without 0.1 μM 5(S)-, 12(S)-, or 15(S)-HETE. Secreted VEGF was determined by ELISA and normalized to cell number. At least three independent experiments were done. *Columns*, mean of duplicate values of one representative experiment; *bars*, SE. *, $P<0.05$ vs C-siRNA; **, $P<0.05$ vs untreated.

We found that VEGF protein expression in 786-O cells was significantly inhibited by 5-LO siRNA (Fig. 6B). Moreover, the 5-LO product 5(S)-HETE (0.1 μM) completely abrogated the 5-LO siRNA effects on VEGF expression in both Caki-1 and 786-O cells and an equimolar concentration of either 12(S)-HETE or 15(S)-HETE were ineffective (Fig. 6C). Therefore, our results suggest that 5-LO pathway contributes to constitutive VEGF gene expression in renal carcinoma cells that have lost VHL function.

Discussion

In this work, we have examined the impact of 5-LO in CC-RCCs. We show that 5-LO is markedly up-regulated in the majority of CC-RCCs in comparison with normal kidney at both transcriptional and translational levels. We also found that the relative expression level of 5-LO was increased in higher grade and higher stage RCC. Thus, we speculate that 5-LO might play an important role in renal cell carcinogenesis, and might be useful as a biomarker in RCC. In addition to neoplastic tissue, immunohistochemistry studies have shown that the distal tubules of the normal kidney expressed 5-LO, although proximal tubular cells, thought to be the origin of RCC, did not. Glomeruli and Bowman's capsules were not stained for 5-LO (data not shown). Hashimoto et al. (2004) reported that COX-2 protein, which was overexpressed in RCC, was expressed in only the distal tubules in normal kidney. Perhaps, in addition to COX-2 protein, overexpression of 5-LO leads to the formation of renal cancers due to an alteration in normal tissue homeostasis. This hypothesis is supported by the finding of elevated levels of leukotrienes in cancer compared with corresponding normal tissues (Reich and Martin, 1996). Therefore, our findings may be important in the understanding of the relationship between the oxidative metabolism of linoleic and arachidonic acid and RCC pathogenesis.

The reason(s) why the 5-LO protein is highly expressed in some nonmalignant kidney cortex remains incompletely understood. Increasing 5-LO expression is

frequently directly related to cellular proliferation and inversely related to cellular differentiation, in particular, terminal differentiation (Shureiqi et al., 2005). Moreover, stem and basal cells, the active and partially differentiated progenitors of skin epithelium, express 5-LO, whereas the differentiated keratinocytes do not (Breton et al., 1996). Metaplastic, dysplastic, and malignant proliferations of epithelium similarly often show *de novo* or overexpression of 5-LO (Catalano et al., 2005). Tumor initiation has in some cases been clearly linked to increasing 5-LO expression, and abundant expression in early lesions may serve as an early detection marker of impending invasive malignancies (Hennig et al., 2005). Therefore, normal 5-LO regulation provides a flexible regulatory plasticity required for normal epithelial development and maintenance, whereas 5-LO dysregulation upsets these delicate regulatory balances, facilitating aberrant cellular proliferation and dedifferentiation.

Furthermore, lipoxygenase activity seems to be involved in tumor progression (Damtew and Spagnuolo, 1997). Wenger et al. (2002) reported that 5-LO inhibitors reduced the incidence, number and size of liver metastases in pancreatic cancer. RCC is usually a hypervascular tumour and metastasizes to lung parenchyma, bone, liver and brain via the venous routes. We found, for the first time, that the relative 5-LO protein levels are often increased in tumors with reduced expression levels of pVHL and increased levels of VEGF, alterations frequently associated with CC-RCCs progression. Our results are consistent with a recent report by Nie et al. (2006) in prostate cancer showing the involvement of lipoxygenase products in the formation of neovessels in more aggressive tumor lesions. Several previous studies showed overexpression of VEGF in CC-RCCs at the protein and mRNA levels and correlations with microvessel density (Jacobsen et al., 2000). In this context, we noted that many, but not all, VEGF-positive RCC sections tend to exhibit highest level of 5-LO. Furthermore, recent studies have shown that the expression of VEGF is associated with VHL gene alterations which in turn are associated with a more aggressive tumor phenotype in CC-RCCs (Minardi et al., 2005). However, other studies show that regulation of angiogenesis and proliferation is not directly influenced by pVHL, as they found no association between VHL alterations and tumor grade, stage, microvessel density, or tumor cells proliferation in RCC (Schraml et al., 2002). In our study using the χ^2 test analysis, we showed that increased 5-LO expression correlated positively with reduced VHL. There are no reports that have examined the relationship between 5-LO expression and pVHL system in human RCC. We speculate that reduced pVHL expression may be associated with increased VEGF via regulation of 5-LO expression. However, the pVHL system, composed of several target proteins, has an independent activity for regulating angiogenic and metastatic potential that is distinct from 5-LO and mainly involved in hypoxia and HIF1α dependent pathway (Maxwell et al., 1999). Whether 5-LO is also a part of this signalling is currently under investigation.

The incidence of CC-RCCs is increasing steadily, with the current worldwide annual mortality estimated to exceed 100,000. Advanced CC-RCC is associated with a poor prognosis. At the time of diagnosis >30% of patients have overt metastasis, and the response to therapy is poor. Thus, there is great interest in identifying the molecular basis of the aggressive biological behaviour of RCC. Our findings showed that the expression of 5-LO might be related to tumour stage and progression of RCC. Recently, selective inhibitors of 5-LO have been developed and these compounds possess anti-cancer properties and appear to be safer than traditional NSAIDs. Further studies are required to evaluate whether 5-LO-targetted therapy using 5-LO inhibitors might prove effective against RCCs.

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References

Avis I.M., Jett M., Boyle T., Vos M.D., Moody T., Treston A.M., Martinez A. and Mulshine J.L. (1996). Growth control of lung cancer by interruption of 5-lipoxygenase-mediated growth factor signalling. J. Clin. Invest. 97, 806-813.

Avnet S., Cenni E., Granchi D., Perut F., Amato I., Battistelli L., Brandi M.L., Giunti A. and Baldini N. (2004). Isolation and characterization of a new cell line from a renal carcinoma bone metastasis. Anticancer Res. 24, 1705-1711.

Breton J., Woolf D., Young P. and Chabot-Fletcher M. (1996). Human keratinocytes lack the components to produce leukotriene B4. J. Invest. Dermatol. 106, 162-167.

Catalano A. and Procopio A. (2005). New aspects on the role of lipoxygenases in cancer progression. Histol. Histopathol. 20, 969-975.

Catalano A., Rodilossi S., Caprari P., Coppola V. and Procopio A. (2005). 5-Lipoxygenase regulates senescence-like growth arrest by promoting ROS-dependent p53 activation. EMBO J. 24, 170-179.

Damtew B. and Spagnuolo P.J. (1997).Tumour cell-endothelial cell interactions: evidence for roles for lipoxygenase products of arachidonic acid in metastasis. Prostagland. Leuko. Ess. Fatty Acids 56, 295-300.

Fleming I.D., Cooper J.S., Henson D.E., Hutter R.V., Kennedy B.J., Murphy G.P., O'Sullivan B., Sobin L.H. and Yarbrau J.W. (1997). AJCC Cancer Staging Manual. 5th ed. Lippincott-Raven. Philadelphia. pp 231-232.

Hashimoto Y., Kondo Y., Kimura G., Matsuzawa I., Sato S., Ishizaki M., Imura N., Akimoto M. and Hara S. (2004). Cyclooxygenase-2 expression and relationship to tumour progression in human renal cell carcinoma. Histopathology 44, 353-359.

Hennig R., Grippo P., Ding X.Z., Rao S.M., Buchler M.W., Friess H., Talamonti M.S., Bell R.H. and Adrian T.E. (2005). 5-Lipoxygenase, a marker for early pancreatic intraepithelial neoplastic lesions. Cancer Res. 65, 6011-6016.

Hong S.H., Avis I., Vos M.D., Martinez A., Treston A.M. and Mulshine J.L. (1999). Relationship of arachidonic acid metabolizing enzyme

expression in epithelial cancer cell lines to the growth effect of selective biochemical inhibitors. Cancer Res. 59, 2223-2228.

Jacobsen J., Rasmuson T., Grankvist K. and Ljungberg B. (2000). Vascular endothelial growth factor as prognostic factor in renal cell carcinoma. J. Urol. 163, 343-347.

Jiang W.G., Douglas-Jones A.G. and Mansel R.E. (2006). Aberrant expression of 5-lipoxygenase-activating protein (5-LOXAP) has prognostic and survival significance in patients with breast cancer. Prostaglandins Leukot. Essent. Fatty Acids 74, 125-134.

Lewis R.A., Austen K.F. and Soberman R.J. (1990). Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases. N. Engl. J. Med. 323, 645-655.

Matsuyama M., Yoshimura R., Mitsuhashi M., Hase T., Tsuchida K., Takemoto Y., Kawahito Y., Sano H. and Nakatani T. (2004a). Expression of lipoxygenase in human prostate cancer and growth reduction by its inhibitors. Int. J. Oncol. 24, 821-827.

Matsuyama M., Yoshimura R., Tsuchida K., Takemoto Y., Segawa Y., Shinnka T., Kawahito Y., Sano H. and Nakatani T. (2004b). Lipoxygenase inhibitors prevent urological cancer cell growth. Int. J. Mol. Med. 13, 665-668.

Matsuyama M., Yoshimura R., Mitsuhashi M., Tsuchida K., Takemoto Y., Kawahito Y., Sano H. and Nakatani T. (2005). 5-Lipoxygenase inhibitors attenuate growth of human renal cell carcinoma and induce apoptosis through arachidonic acid pathway. Oncol. Rep. 14, 73-79.

Maxwell P.H., Wiesener M.S., Chang G.W., Clifford S.C., Vaux E.C., Cockman M.E., Wykoff C.C., Pugh C.W., Maher E.R. and Ratcliffe P.J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygendependent proteolysis. Nature 399, 271-275.

Minardi D., Lucarini G., Mazzucchelli R., Milanese G., Natali D., Galosi A.B., Montironi R., Biagini G. and Muzzonigro G. (2005). Prognostic role of Fuhrman grade and vascular endothelial growth factor in pT1a clear cell carcinoma in partial nephrectomy specimens. J. Urol. 174, 1208-1212.

Na X., Wu G., Ryan C.K., Schoen S.R., di'Santagnese P.A. and Messing E.M. (2003). Overproduction of vascular endothelial growth factor related to von Hippel-Lindau tumor suppressor gene mutations and hypoxia-inducible factor-1 alpha expression in renal cell carcinomas. J. Urol. 170, 588-592.

Natarajan R. and Reddy M.A. (2003). HETEs/EETs in renal glomerular and epithelial cell functions. Curr. Opin. Pharmacol. 3, 198-203.

Nie D., Krishnamoorthy S., Jin R., Tang K., Chen Y., Qiao Y., Zacharek A., Guo Y., Milanini J., Pages G. and Honn K.V. (2006). Mechanisms regulating tumor angiogenesis by 12-lipoxygenase in prostate cancer cells. J. Biol. Chem. 281, 18601-18609.

Öhd J.F., Nielsen C.K., Campbell J., Landberg G., Lööfberg H. and Sjöölander A. (2003). Expression of the leukotriene D4 receptor CysLT1, COX-2, and other cell survival factors in colorectal adenocarcinomas. Gastroenterology 124, 57-70.

Reich R. and Martin G.R. (1996). Identification of arachidonic acid pathways required for the invasive and metastatic activity of malignant tumor cells. Prostaglandins 51, 1-17.

Reuter V.E. and Presti J.C. (2000). Contemporary approach to the classification of renal epithelial tumors. Semin. Oncol. 27, 124-137.

Romano M., Catalano A., Nutini M., D'Urbano E., Crescenzi C., Claria J., Libner R., Davi G. and Procopio A. (2001). 5-lipoxygenase regulates malignant mesothelial cell survival: involvement of vascular endothelial growth factor. FASEB J. 15, 2326-2336.

Schraml P., Struckmann K., Hatz F., Sonnet S., Kully C., Gasser T., SauterG., Mihatsch M.J. and Moch H. (2002). VHL mutations and their correlation with tumour cell proliferation, microvessel density, and patient prognosis in clear cell renal cell carcinoma. J. Pathol. 196, 186-193.

Shureiqi I., Wu Y., Chen D., Yang X.L., Guan B., Morris J.S., Yang P., Newman R.A. and Lippman S.M. (2005). The critical role of 15-lipoxygenase-1 in colorectal epithelial cell terminal differentiation and tumorigenesis. Cancer Res. 65, 11486-11492.

Soberman R.J. and Christmas P. (2003). The organization and consequences of eicosanoid signalling. J. Clin. Invest. 111, 1107-1113.

Turner K.J., Moore J.W., Jones A., Taylor C.F., Cuthbert-Heavens D., Han C., Leek R.D., Gatter K.C., Maxwell P.H., Ratcliffe P.J., Cranston D. and Harris A.L. (2002). Expression of hypoxia-inducible factors in human renal cancer: relationship to angiogenesis and to the von Hippel-Lindau gene mutation. Cancer Res. 62, 2957-2961.

Wenger F.A., Kilian M., Bisevac M., Khodadayan C., von Seebach M., Schimke I., Guski H. and Muller J.M. (2002). Effects of Celebrex and Zyflo on liver metastasis and lipidperoxidation in pancreatic cancer in Syrian hamsters. Clin. Exp. Metastasis 19, 681-687.

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