Regulation of Growth Factor Receptors by Klotho in Human Renal Cell Carcinoma

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TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

ABBREVATIONS

AKT	v-akt Murine thymoma viral oncogene homolog
CCRCC	Clear cell renal cell carcinoma
EGFR	Epidermal growth factor receptor
ERK1/2	Extracellular signal-regulated kinases1/2
FFPE	Formalin fixed paraffin embedded
FGF	Fibroblast growth factor
GFRs	Growth factor receptors
GLUT-4	Glucose transporter type 4
GRB2	Growth factor receptor-bound protein 2
HEK293	Human embryonic kidney293
HER1~4	Human epidermal growth factor receptor1~4
HIF	Hypoxia-inducible factor
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
IHC	Immunohistochemistry

IR	Insulin receptor
IRS	Insulin receptor substrate
LY294002	2-(4-morpholinyl)-8-phenylchromone
MAD2	Mitotic arrest deficient 2
MAPK	Mitogen-activated protein kinase
mRCC	Metastatic renal cell carcinoma
mTOR	Mammalian target of rapamycin
PDGF	Platelet-derived growth factor
PDGFR-α	Platelet-derived growth factor receptor-α
PDK1	Phosphoinositide-dependent kinase-1
PI(3,4,5)P3, PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
$PI(4,5)P_2$, PIP2	Phosphatidylinositol 4,5-bisphosphate
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
RCC	Renal cell carcinoma
Ser473	Serine473
SH2	Src Homology 2

SH3	SRC Homology 3
SOS	Son of sevenless
Thr308	Threonine 308
TMA	Tissue microarray
VEGF	Vascular endothelial growth factor
VEGFR-1	Vascular endothelial growth factor receptor-1
VEGFR-2	Vascular endothelial growth factor receptor-2
VHL	Von Hippel-Lindau
WMN	Wortmannin

ABSTRACT

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Background: Klotho has been known to be an aging-suppressor gene and predominantly expressed in renal tubules. Some studies revealed that Klotho expression was related to favorable behavior of melanoma, renal cell carcinoma, breast, and lung cancers. In addition, it has been reported that Klotho is a tumor suppressor and modulator of growth factor receptors (GFRs) signaling in human breast cancer. Clear cell renal cell carcinoma (CCRCC) is the most common kidney malignancy, originating from renal tubules, which are the source of Klotho. However, expression and function of Klotho in the

tumorigenesis of renal cancer remain elusive. Therefore, we examined the expression of Klotho and GFRs in CCRCC and validated their prognostic significance. Furthermore, we investigated the molecular mechanism explaining the relationship between Klotho and GFRs in the tumorigenesis of CCRCC.

Materials and methods: Immunohistochemical (IHC) staining for Klotho and GFRs was performed on 126 formalin-fixed paraffin-embedded CCRCC tissue samples. Western blot analysis was used to check the expression of Klotho, insulin receptor (IR), insulin-like growth factor (IGF-1) receptor (IGF-1R), and vascular endothelial growth factor receptor (VEGFR)-1 in 18 fresh tissues of these cases. We also examined phosphorylation of insulin, IGF-1, epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) induced downstream pathways before and after treatment of Klotho in two clear cell renal cell carcinoma cell lines (ACHN and Caki1). The results were compared with various clinico-pathological prognostic factors of CCRCC and with patient survival.

Results: Higher Klotho expression was significantly correlated with favorable prognostic factors of CCRCC. Klotho positive patients had significantly better survival than Klotho negative patients. Among the GFRs, higher expression of IR and VEGFR-1 were related to favorable prognostic factors of CCRCC. In contrast, higher expression of IGF-1R and EGFR

correlated with unfavorable prognostic factors of CCRCC. Interestingly, Klotho inhibited IGF-1- and EGF-induced AKT activation in CCRCC cell lines.

Conclusion: Klotho inhibition of IGF-1 and EGF pathways suggests that Klotho might be a potential tumor suppressor in CCRCC. Therefore, Klotho is critical for the development of CCRCC and is valuable for therapeutic target of CCRCC.

Key Words: Klotho, Clear Cell Renal Cell Carcinoma, Growth Factor Receptors, Insulin-like Growth Factor-1, Insulin Receptor, Tumorigenesis, Prognosis

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I. INTRODUCTION

Renal cell carcinoma (RCC) is the most common renal tumor and accounts for 2-3% of all malignancy in adults.[1] The incidence and mortality of renal cancer has been increasing worldwide, which is probably due to increased prevalence of risk factors as well as an improvement of diagnosis.[2] According to the cancer statistics in Korea, the incidence of RCC in Korea has shown a steady increase, a trend that has also been seen worldwide.[3] In 2010, kidney cancer statistics showed that 3,598 new cases, 797 deaths, and about 20,203 patients were living with the cancer in Korea.[3] Most patients who have localized tumor can be cured by surgery alone. However, one third of patients are diagnosed with

metastatic RCC (mRCC) and an additional 20-40% of patients develop metastases after curative surgery.[4]

Clear cell RCC (CCRCC) is the most common subtype of RCC. The inactivation of tumor suppressor gene Von Hippel-Lindau (*VHL*) promotes the tumorigenesis of renal cancer growth.[5] Inherited *VHL* mutation accounts for 2% of kidney cancers and somatic *VHL* mutations occurs approximately 70% of sporadic CCRCC.[6, 7] The VHL protein (pVHL), a product of *VHL*, targets hypoxia-inducible factor (HIF) under normoxic condition.[8]

HIF is a transcription factor, composed of two subunits: the oxygen-sensitive α subunits (HIF1α, HIF2α, and HIF3α) and the constitutively expressed HIF1β subunit.[9] Under normal oxygen tension (normoxia), α subunits of HIF are prolyl-hydroxylated, poly-ubiquitylated and destroyed by proteasome. During oxygen deprivation (hypoxia), α subunits are not prolyl-hydroxylated, escaping from recognition by pVHL, evading degradation, and heterodimerizing with HIF1β. The HIF complex enters the nucleus and regulates the expression of hundreds of target genes, including basic fibroblast growth factor (bFGF), epidermal growth factor receptor (EGFR), platelet-derived growth factor (PDGF), transforming growth factor alpha (TGF-α), and vascular endothelial growth factor (VEGF).[10-15]

Growth factor receptors (GFRs) signaling regulates cellular growth, proliferation, metabolism, and survival in all kind of malignancy, including

RCC.[16, 17] We describe here a structure, function and role of growth factor receptors in the renal tumorigenesis one by one. First of all, the insulin/IGF signaling system is composed of three ligands, IGF-1, IGF-2, and insulin, and at least four receptors including IGF-1 receptor (IGF-1R), IGF-2 receptor (IGF-2R), insulin receptor (IR), and the hybrid receptors of IGF and insulin.[18] The receptor family members are heterotetrameric protein consisting of two extracellular α subunits and two transmembrane β subunits. The binding of ligand to the subunits of receptor stimulates the intrinsic tyrosine kinase activity of the β subunits.[19] IR and IGF-1R are close structural homologs. Amino acid sequence similarity ranges from 40 to 85% in different domains, with the highest degree of homology being found in the tyrosine kinase domain.[20, 21] Overexpression of IGF-1R is associated with poor prognosis in various human malignancies including renal, breast, prostate, and ovarian cancers.[22-24] However, the expression of IR and its potential prognostic significance have not been elucidated in CCRCC.

Second, as another growth factor, *VEGF* (also referred to as VEGFA) belongs to a gene family that consists of placental growth factor (*PLGF*), *VEGFB, VEGFC,* and *VEGFD.*[25] The *VEGF* gene is composed of eight exons and is differentially spliced to encode four major isoforms, including $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$.[26] VEGF exerts its biological effect mainly through interaction with its two different receptors, VEGF receptor (VEGFR) -1 (Flt-1) and VEGFR-2 (Flk-1), selectively expressed on vascular endothelial cells.[27] Therapeutic targeting directed against VEGF and VEGFR-2 has been successful

for mRCC treatment.[28] The importance of VEGF and VEGFR-1 in regulating tumor angiogenesis in CCRCC has been reported previously.[29, 30] However, the expression level of VEGF and VEGFR-1 and their potential prognostic significance in comparison with clinico-pathological parameters of CCRCC has not been elucidated yet.

Third, EGFR is classified into a family of four closely related cell membrane receptors: EGFR (HER1; ErbB1), HER2 (ErbB1), HER3 (ErbB3), and HER4 (ErbB4).[31] EGFR consists of an extracellular region with two ligand-binding domains, an extracellular juxta-membrane region, a hydrophobic trans-membrane domain, a cytoplasmic tyrosine kinase domain and c-terminal tyrosine residues.[31] Upon ligand binding, receptors homo- or hetero-dimerize, trans-phosphorylate the c-terminal tyrosine residues and activate downstream effectors and biological responses. Previous studies have shown that high expression of EGFR plays important roles in tumor initiation and progression of RCC, since up regulation of EGFR has been associated with high grade and worse prognosis.[32, 33] Therefore, these studies have been suggested that EGFR might be a target for novel anti-cancer therapy in RCC.

Lastly, PDGF family has been extended, and 5 different isoforms have been characterized (PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD), which exert their biologic function by binding to 3 different tyrosine kinase receptors (αα receptor, ββ receptor, and αβ receptor), of which PDGF-αα receptor has the

broadest specificity.[34] It has been reported that high PDGF-αα receptor expression in CCRCC was associated with adverse outcome, thus it was suggested as a potential target in RCC therapy.[35]

GFRs share same downstream signaling pathways. As phosphatidylinositol-4,5-bisphasphate 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) are two major downstream effector pathways of activated GFRs that promote both cellular proliferation and resistance to apoptosis, failure to turn off the activated GFRs can drive oncogenesis. We describe here the classic chain of events occurring upon activation PI3K and MAPK pathways (Fig. 1).

Step 1: Receptor activation. Canonical activation starts with the binding of extracellular growth factors, such as EGF, IGF-1 or insulin, to cognate cell surface tyrosine kinase receptors. Upon binding, the receptor dimerizes, becomes autophoshorylated and recruits adaptor protein, including insulin receptor substrate (IRS) 1 and IRS2.

Step 2: PI3K activation. PI3K constitutes a large family of kinases involved in multiple physiological aspects. The PI3K class mostly implicated in cancer is class IA. PI3KIA containing a catalytic subunit (p110) encoded by PI3K3CA and a regulatory subunits (p85), is recruited to phosphorylated receptor tyrosine kinase, thus being activated. Interestingly, PI3K can be also activated directly by RAS protein. The substrate of PI3K is phosphatidylinositol 4,5-

bisphosphate ($PI(4,5)P_2$, PIP2). PI3K phosphorylates PIP2 to generate phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$, PIP3). PIP3 is a second messenger that promotes the translocation of v-akt Murine thymoma viral oncogene homolog (AKT) to the cell membrane. Phosphatase and Tensin homologue deleted on chromosome Ten (PTEN) is the most well studied negative regulator of the AKT pathway.[36] PTEN is a major lipid phosphatase that dephosphorylates PIP3 and PIP2 to inhibit AKT activation and thereby suppresses tumor formation by restraining PI3K/AKT signaling.[37] Loss of PTEN function leads to an elevated concentration of PIP3 substrate, and consequently constitutive activation of downstream components of PI3K pathway, including AKT and mammalian target of rapamycin (mTOR) kinases.[37]

Step 3: AKT activation. AKT contains specific domain (pleckstrin homology (PH) domain) that binds to PIP3 generated by PI3K. Binding of PIP3 to AKT triggers AKT translocation to the membrane and induces a conformational change enabling AKT phosphorylation and thus its activation by another kinase, phosphoinositide-dependent kinase (PDK)-1. PDK1 phosphorylates AKT at threonine308 (Thr308) and serine473 (Ser473). Upon activation, AKT dissociates from the membrane and translocates to the cytoplasm and nucleus, where it phosphorylates multiple proteins involved in translation, metabolism, proliferation, survival, and angiogenesis.

Similar to AKT signaling, binding of ligand to the GFRs activates tyrosine kinase activity of the cytoplasmic domain of receptors. The GFRs are phosphorylated on tyrosine residues. Docking proteins such as growth factor receptor-bound protein 2 (GRB2) contain an SH2 domain that binds to the phosphotyrosine residues of the activated receptor.[38] GRB2 binds to the guaninenucleatide exchange factor son of sevenless (SOS) by way of the two SH3 domains of GRB2. When the GRB2-SOS complex docks to phosphorylate GFRs, SOS becomes activated, promoting activation of RAS kinase.[39,40] Activated RAS activates the protein kinase activity of RAF kinase.[40] RAF kinase phosphorylates and activates MEK. MEK phosphorylates and activates MAPK. MAPK was originally called extracellular signal-regulated kinase (ERK).

In clinical practice, sorafenib, sunitnib, bevacizumab, temsirolimus, everolimus, pazopanib, and axitinib that block the VEGF pathway and the mTOR pathways are logical therapeutic targets for the treatment of mRCC.[28] The development of these targeted agents has substantially improved survival of patients with mRCC to over 2 years.[41] Although tumor shrinkage is achieved to some extent in a large proportion of RCC patients, complete remission is uncommon.[28] Treatment of locally advanced and/or mRCC is still complicated due to the lack of specific therapeutic targets and inadequate methods to assess certain drug efficacies.[42] Therefore, novel and more effective molecular markers should be studied and developed for the treatment with the purpose of prolonging survival of advanced and/or mRCC patients.

Klotho gene, first identified in mice, encoding a single-pass membrane protein, shares homology with the β-glucosidase. Furthermore, Klotho deficiency exhibits human aging and age-related diseases, including arteriosclerosis, osteoporosis, pulmonary emphysema, infertility, and skin atrophy etc.[43] Human Klotho gene is located on chromosome 13q12, which encodes two distinct proteins, membrane Klotho and secreted Klotho. Its extracellular domain composed of two internal repeats, KL1 and KL2, can be cleaved, shed into the serum and acts as a circulating hormone.[44, 45] Klotho is a type I transmembrane protein localized at the plasma membrane and in the cytoplasm while the cleaved extracellular domain is secreted into the blood and cerebrospinal fluid.[46] Klotho protein was detected in certain organ and/or tissues, majority in distal convoluted tubules of kidney and choroid plexus of brain, and minority in placenta, prostate, and small intestine.[43, 44] Several functions of Klotho have been described to date, such as an obligatory co-receptor for fibroblast growth factor (FGF) 23 in endocrine regulation of phosphate homeostasis,[47] an inhibition of insulin and IGF-1 signaling which is associated with anti-aging properties[48] and increased resistance to oxidative stress.[49]

It was theorized that Klotho had tumor suppressive role in various human cancers, including breast,[50] pancreas,[51] stomach,[52] and lung.[53] However, little is known about the expression of Klotho in RCC,[54] and the molecular and pathological roles of Klotho in CCRCC have not been clearly understood. Furthermore, for a better understanding in tumorigenesis and molecular

mechanism of RCC, it had been postulated that additional studies were required. The present study was designed not only to find a new prognostic marker for patients with CCRCC but also to address the question of whether the detection of Klotho can give rise to a new therapeutic strategy for patients with RCC based on the inhibition of the GFRs signaling. Therefore, we studied the expression of Klotho and GFRs in CCRCC and validated their prognostic significance by IHC staining and correlated with clinicopathologic data in 126 patients with CCRCC. In addition, we investigated the molecular mechanism explaining the crosstalk between Klotho and GFRs in the tumorigenesis of CCRCC.

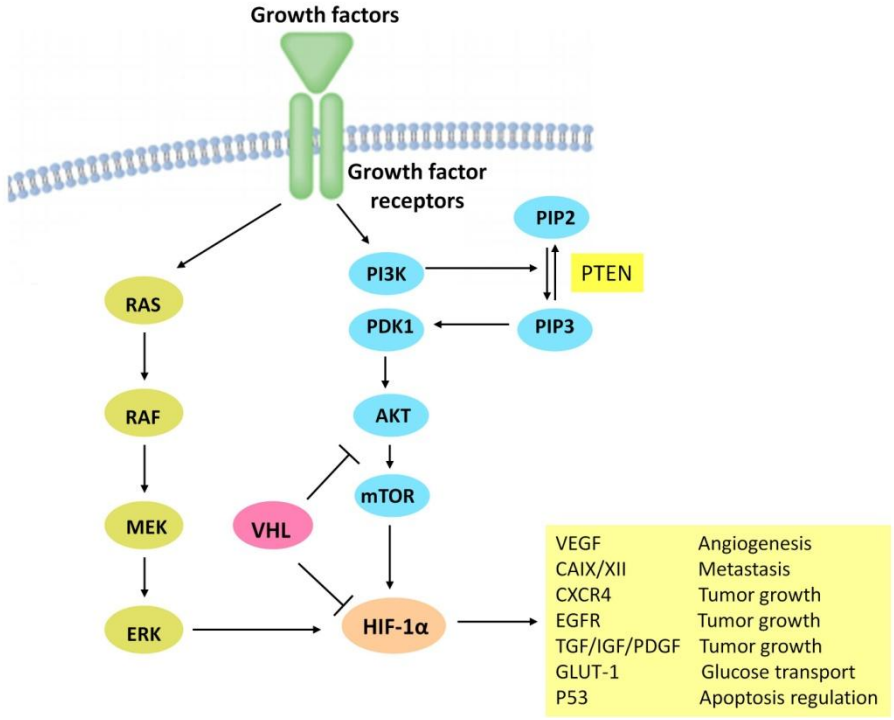

Fig. 1. Important pathways involved in CCRCC tumorigenesis. Abbreviations: CAIX, carbonic anhydrase IX; EGFR, epidermal growth factor receptor; GLUT1, glucose transporter 1; HIF, hypoxia-inducible factor; IGF, insulin-like growth factor; mTOR, mammalian target of rapamycin, PDGF, platelet-derived growth factor; PDK1, phosphoinositide-dependent kinase-1; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome ten; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau. Adopted from Finley DS et al.[16]

II. MATERIALS AND METHODS

1.1. Patients and Tissue Samples

Samples from 126 cases of CCRCC were collected from patients who underwent radical nephrectomy at the Yonsei University Wonju Severance Christian Hospital from 2001 to 2011. Formalin-fixed paraffin-embedded (FFPE) tissues from all cases included in the study were used for IHC staining. Eighteen fresh tissue samples of CCRCC were available for western blot analysis. We collected all clinical data and follow up information of all 126 patients. The characteristics and clinico-pathological information of the patients were represented in Table 1. The patients were predominantly men with the mean age of 57.4±10.5 years. The mean tumor size was 5.3±2.7 cm. Twenty-two patients (17.5%) had a history of diabetes. One hundred twenty-three patients had follow up information. Of the patients with follow up information, 9 (7.1%) had tumor recurrence. Until the last follow up, 12 patients (9.5%) had died due to the tumor.

Table 1. Summary of clinical data

Prognostic factors	Number of cases (%)
Gender	
Male	94 (74.6)
Female	32 (25.4)
Age (yrs)	
Mean±SD*	57.4±10.5
Diabetes history	
Yes	22 (17.5)
No	104 (82.5)
Tumor size (cm)	
Mean±SD*	5.3±2.7
Tumor recurrence	
Present	9 (7.1)
Absent	103 (81.7)
Health status	
Alive	111(88.1)
Died	12 (9.5)

*SD; standard deviation

1.2. Pathologic Evaluation of Clear Cell Renal Cell Carcinoma (CCRCC)

All the slides of CCRCC included in this study were reviewed by two expert pathologists with the pathologic reports and clinical records. The pathological report includes main diagnosis, Fuhrman nuclear grade, TNM stage, presence of tumor necrosis, sarcomatoid and rhabdoid features, vascular, perirenal fat, renal pelvis, and renal sinus fat invasions and cystic changes, those are well-known prognostic factors of CCRCC. The Fuhrman nuclear grading system, an independent prognostic factor based on nuclear and nucleolar morphology, is divided into grades 1 to 4.[55] Grade 1 refers to small round uniform nuclei, approximately 10 µm, inconspicuous or absent nucleoli (Fig. 2A); In grade 2, nuclei slightly irregular, approximately 15 µm, nucleoli visible at high power (400x) (Fig. 2B). Grade 3 refers to nuclei obviously irregular, approximately 20 µm, prominent nucleoli visible at low power (100x) (Fig. 2C) and grade 4 is similar to grade 3 but with bizarre, often multilobed nuclei and clumped chromatin (Fig. 2D). In this study, Fuhrman nuclear grade was grouped into low (grade 1+2) and high (grade 3+4) grades.

Another reliable prognostic factor is TNM staging system and accounting the tumor size and the extent of the tumor. TNM stage was reclassified according to the seventh edition of AJCC cancer staging manual.[56] Stage I tumor refers to tumor 7 cm or less in greatest dimension, limited to the kidney while in stage II, tumor more than 7 cm in greatest dimension, limited to the kidney. In stage III,

tumor extends into major veins or perinephric tissues but not into the ipsilateral adrenal gland and not beyond Gerota fascia and in stage IV, tumor invades beyond Gerota fascia (including contiguous extension into the ipsilateral adrenal gland). Other pathologic factors that give poor prognosis include the presence of tumor necrosis, sarcomatoid and rhabdoid features, vascular, perirenal fat, renal pelvis, and renal sinus fat invasions.[57] By contrast, presence of cystic change in CCRCC is known as a favorable prognostic factor.[58]

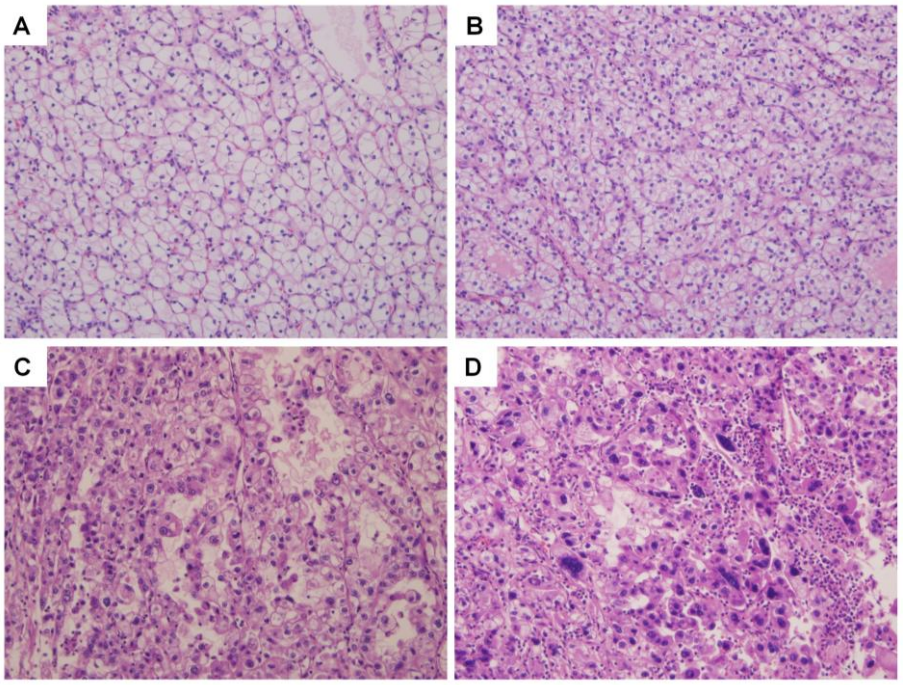

Fig. 2. Fuhrman nuclear grades of CCRCC. Grade 1: Nuclei are round and barely larger than red cells; nucleoli are inconspicuous or absent (A). Grade 2: Nuclei are slightly larger irregular and about twice as big as red cells; nucleoli are visible at high power (B). Grade 3: Nuclei are irregular, at least 20 µm and larger, and large prominent nucleoli (C). Grade 4: Nuclei are very large, irregular and multilobed, with clumped chromatin and large prominent nucleoli (D).

1.3. Pathologic Prognostic Factors of CCRCC Patients

Absent of sarcomatoid and rhabdoid features were observed in 120 and 116 cases, respectively. For tumor necrosis, 100 cases reveal no necrosis, while tumor necrosis was identified in 26 cases. Furthermore, absence of the invasions was detected in 110 cases, 117 cases, 121 cases, and 112 cases in renal fat, renal pelvis, renal sinus fat, and vascular invasion, respectively. Presence of cystic change was found in 34 cases while 92 cases reveal absence of cystic change. Fuhrman nuclear grades in the tumors were as following; grade 1(n=15), grade 2 (n=57), grade 3 (n=42), and grade 4 (n=12). Meanwhile, the pathologic T stage of the tumor were as following; T1 (n=90), T2 (n=18), T3 (n=18), and T4 (n=0). Moreover, TNM stage of the tumors were following; stage I (n=87), stage II (n=13), stage III (n=22), and stage IV (n=4). These pathologic prognostic factors are summarized in Table 2.

Table 2. Summary of pathological prognostic factors of CCRCC

Prognostic factors	Number of cases (%)
Sarcomatoid feature	
Absent	120 (95.2)
Present	6 (4.8)
Tumor necrosis	
Absent	100 (79.4)
Present	26 (20.6)
Rhabdoid feature	
Absent	116 (92.0)
Present	10 (8.0)
Perirenal fat invasion	
Absent	110 (87.3)
Present	16 (12.7)
Renal pelvis invasion	
Absent	117 (92.9)
Present	9 (7.1)
Vascular invasion	
Absent	112 (88.9)
Present	14 (11.1)
Renal sinus fat invasion	
Absent	121 (96.0)
Present	5 (4.0)
Cystic change	
Absent	92 (73.0)
Present	34 (27.0)
Fuhrman nuclear grade	
1	15 (11.9)
2	57 (45.2)
3	42 (33.4)
4	12 (9.5)
Pathologic T stage	
1	90 (71.4)
2	18 (14.3)
3	18 (14.3)
4	0 (0.0)
TNM stage	
I	87 (69.0)
II	13 (10.3)
III	22 (17.5)
IV	4 (3.2)

1.4. Tissue Microarray (TMA) Preparation

To reduce the number of slides for IHC staining and to decrease bias we used TMA technique. A representative tumor site without necrosis, hemorrhage, or artifact was marked in all paraffin blocks. The selected tumor area was harvested using a 5 mm Quick-ray tip-punch (Unitma, Seoul, Korea), placed on a 20 pore TMA mold (Unitma), and re-embedded in paraffin. 4 µm sections of TMA blocks were cut and attached onto coated slides.

1.5. Immunohistochemical (IHC) Staining

Staining was performed using an automatic immunostaining machine, Ventana Benchmark XT (Roche Diagnostics, Basel, Switzerland) automatic immunostaining machine. The sections were deparaffinized in xylene, rehydrated in graded alcohols, and subjected to pretreatment with CC1 (Roche Diagnostics). The sections were washed with reaction buffer followed by incubation with primary antibodies against Klotho, IR-β, IGF-1, IGF-1R, VEGF, VEGFR-1, PDGF, PDGFR-α (Abcam, Cambridge, MA, USA) and EGFR (Invitrogen, Carlsbad, CA, USA), in dilution of 1:200, 1:100, 1:100, 1:100, 1:100, 1:50, 1:100, 1:200, and 1:100, respectively, for 60 minutes at 42^{o}C. Bound antibody was detected with the Ultra View Universal DAB kit (Roche Diagnostics) and sections were counterstained with hemotoxylin (Roche Diagnostics) according to the manufacturer's instructions. Positive and negative control stains were also performed.

1.6. Quantification of IHC

Modified Allred scoring system was used to evaluate positivity, with staining intensity and distribution being scored separately.[59] The staining intensity was scored as 0 points (negative), 1 point (weak), 2 points (intermediate), or 3 points (strong) and the distribution of positive-stained cells was assessed as 0 point (negative), 1 point (<1%), 2 points (1-10%), 3 points (11-33%), 4 points (34-67%), or 5 points (>67%). The total staining score was calculated as the sum of two parameters. Total staining scores from 0 to 2 points were considered negative, while scores from 3 to 8 points were considered positive (Table 3). To overcome the limitations of this quantification method, we also compared the mean staining score as continuous variables in each group.

Table 3. Quantification method of IHC staining (modified Allred test)

Intensity score (IS)		**Proportion score (PS)**	
0	None	0	None
1	Weak	1	-1/100
2	Intermediate	2	>1/100 to 1/10
3	Strong	3	>1/10 to 1/3
		4	>1/3 to 2/3
		5	>2/3 to 1

Total score (TS) = IS + PS; TS 0-2: Negative, TS 3-8: Positive.

1.7. Cell Culture

Renal cell carcinoma cell lines (ACHN and Caki 1) were cultured under high glucose DMEM medium (HyClone, Logan, UT, USA) with 10% FBS (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin at 37℃ in humidified atmosphere. The cells were treated with insulin (Sigma-Aldrich, Louis, MO, USA), IGF-1 (a gift from Makoto Kuro-O), EGF (Roche Diagnostics, Mannheim, Germany), and PDGF (Gemini Bio Products, Sacramento, CA, USA), respectively. Wortmannin (WMN) (Sigma-Aldrich, Louis, MO, USA) and 2-(4-morpholinyl)-8-phenylchromone (LY294002) (Calbiochem, San Diego, CA, USA) were used as inhibitors of PI3K signaling pathway.

1.8. Western Blot Analysis

2.8.1. Western Blot Analysis for Human Tissue Samples

Western blot analysis was performed on 18 cases of CCRCC with adjacent normal kidney parenchyma. The fresh tissues were lysed using 2 ml of PRO-PREP lysis buffer (iNtRon Biotechnology, Daejeon, Korea), and then ground for 15-20 seconds on ice using a homogenizer (ProScience, Woburn, MA, USA). The lysates were centrifuged at 13,000 rpm for 15 minutes; the supernatants were collected and protein concentration was measured using the Bradford method. Ten ug of protein was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to immobilon-P membranes (Millipore, Billerica, MA, USA), using an

electrophoretic transfer system (Bio-Rad, Hercules, CA, USA) at 100 V for one and a half hours. The membranes were blocked with 5% skim milk in TBS-T buffer for an hour. The blocked membranes were washed with TBS-T buffer and incubated overnight at 4℃ in primary antibodies (Abcam), including αKlotho, IR-β, IGF-1R, VEGFR-1, and β-actin, diluted 1:1000, 1:1000, 1:2000, 1:2000, and 1:5000, respectively. The membranes were then incubated for an hour at room temperature in Anti-rabbit IgG-HRP and Anti-Mouse IgG-HRP secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA).

Bound antibodies were detected using Luminata TM Forte Western HRP Substrate (Millipore) and the Biospectrum Imaging System (UVP, Upland, CA, USA). Band densities on immunoblots were measured with ImageJ software (available at http://rsb.info.nih.gov/ij/index.html).

2.8.2. Western Blot Analysis for Cell Lines

Protein extraction and western blotting were conducted as previously described in 2.8.1. Primary antibodies, including AKT, AKT (Thr308), AKT (Ser473), ERK1/2 (Cell Signaling Technology, Danvers, MA, USA), and β-actin (Abcam) were used, with dilution of 1:2000 and 1:5000, respectively. Secondary antibodies, including Anti-rabbit IgG-HRP and Anti-Mouse IgG-HRP (Santa Cruz Biotechnology, Dallas, TX, USA) were used, with dilution of 1:2000 and 1:4000, respectively.

1.9. Ethics Approval

This study has been approved by the Institutional Ethics Committee of Yonsei University Wonju College of Medicine (YWMR-12-0-014) and has been followed the principles outlined in the Declaration of Helsinki.

1.10. Statistical Analysis

Statistical analysis was performed using PASW, version 20.0 (SPSS Inc., Chicago, IL, USA). Student's *t*-test and χ^2 test were used to compare the categorical and continuous variables. The period of overall survival was measured from the date of surgery to the date of death due to the tumor. Tumor recurrence was defined as the presence of clinically diagnosed or pathologically confirmed metastases. Survival analysis was performed using the Cox regression method after normalizing the following parameters: sex, age, Fuhrman nuclear grade, and pathologic T stage. A value of $p<0.05$ was considered statistically significant.

III. RESULTS

3.1. IHC Staining in Clinical Samples

3.1.1. Klotho Expression

A. Pattern and Distribution of Klotho Expression in Non-tumor and Tumor Tissues

IHC staining showed that Klotho expression was detected in both non-tumor renal parenchymal tissues and tumor tissues. Klotho protein was expressed in the cytoplasm and/or membrane of adjacent normal parenchymal tissues, including podocytes of glomeruli, tubular epithelium, endothelial cells of blood vessels, and lymphocytes (Fig. 3A). Klotho expression was observed in the cytoplasm and/or membrane in 107 (84.9%) cases of CCRCC (Fig. 3B).

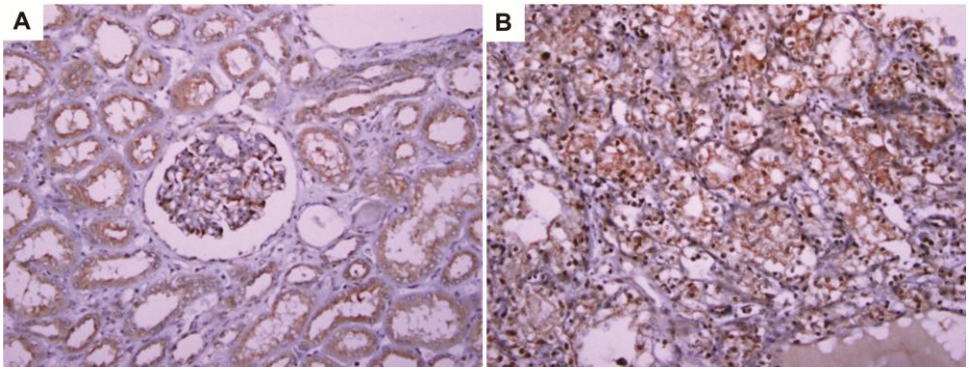

Fig. 3. Pattern and distribution of Klotho expression in non-tumor and tumor tissues. IHC staining shows that Klotho was observed in the cytoplasm and/or membrane of both non-tumor renal parenchymal tissue (A) and tumor tissue (B).

B. Correlation of Klotho and Prognostic Factors of CCRCC

Klotho was positive in 91% of cases without tumor necrosis and 61.5% of cases with tumor necrosis. This difference in Klotho expression was statistically significant (*p=0.000*). Furthermore, Klotho was positive in 91.7% of cases with a low (1+2) Fuhrman nuclear grade and 75.9% of cases with a high (3+4) Fuhrman nuclear grade, which was statistically significant (*p=0.015*). With respect to the vascular invasion, Klotho positivity was seen in 87.5% of cases without vascular invasion and 64.3% of cases with vascular invasion, and this difference was statistically significant (*p=0.038*). Klotho was positive in 97.1% of cases with cystic change and 80.4% of cases without cystic change. This difference in Klotho expression was statistically significant (*p=0.023*). Although Klotho expression seemed to be higher in cases without sarcomatoid or rhabdoid feature, perirenal fat, renal pelvis, and renal sinus fat invasion, these differences were not statistically significant. Klotho was expressed in 92.2% of cases with pathologic T stage 1 and 66.7% of stage 2-4 cases, which was a statistically significant difference (*p=0.000*). Moreover, Klotho positivity was seen in 92% of cases with TNM stage I and 69.2% of cases with TNM stage II-IV, which was also a statistically significant difference (*p=0.001*). These findings are summarized in Table 4.

The mean staining score of each group was compared to overcome the potential limitations with respect to quantification in IHC assays. The mean

staining score of Klotho was 4.80±1.83 in cases without tumor necrosis and 3.58±1.96 in cases with tumor necrosis, which was statistically significant (*p=0.003*). In addition, a statistically significant difference (*p=0.001*) was observed between the mean staining scores of low and high Fuhrman nuclear grades (5.01±1.89 vs. 3.93±1.79). For vascular invasion, the mean staining score was 4.67±1.85 in cases without vascular invasion and 3.57±2.24 in cases with vascular invasion, which was a statistically significant difference (*p=0.043*). Although the mean staining score of Klotho seemed to be higher in cases without sarcomatoid or rhabdoid feature, perirenal fat, renal pelvis, and renal sinus fat invasions, these differences were not statistically significant. For cystic change, the mean staining score was 4.35±1.91 in cases with cystic change and 5.09±1.85 in cases without cystic change, which was not statistically significant. A statistically significant difference (*p=0.000*) was observed between the mean staining scores of pathologic T stage 1 and T stages 2-4 (4.94±1.79 vs. 3.56±1.89). Furthermore, a significant difference (*p=0.000*) was seen between the mean staining scores of TNM stage I and TNM stages II-IV (4.95±1.80 vs. 3.64±1.87). These findings are detailed in Table 5.

Table 4. Correlation of Klotho expression and clinico-pathological parameters of CCRCC

Parameters	Klotho expression		p-value
	No. of positive cases (%)	No. of negative cases (%)	
Sarcomatoid feature			
Absent	103 (85.8)	17 (14.2)	0.200
Present	4 (66.7)	2 (33.3)	
Tumor necrosis			
Absent	91 (91.0)	9 (9.0)	0.000
Present	16 (61.5)	10 (38.5)	
Rhabdoid feature			
Absent	99 (85.2)	17 (14.7)	0.646
Present	8 (80.0)	2 (20.0)	
Fuhrman grade			
Low (1+2)	66 (91.7)	6 (8.3)	0.015
High (3+4)	41 (75.9)	13 (24.1)	
Perirenal fat invasion			
Absent	95 (86.4)	15 (13.6)	0.262
Present	12 (75.0)	4 (25.0)	
Renal pelvis invasion			
Absent	100 (85.5)	17 (14.5)	0.624
Present	7 (77.8)	2 (22.2)	
Vascular invasion			
Absent	98 (87.5)	14 (12.5)	0.038
Present	9 (64.3)	5 (37.5)	
Renal sinus fat invasion			
Absent	102 (84.3)	19 (15.7)	1.000
Present	5 (100.0)	0 (0.0)	
Cystic change			
Present	33 (97.1)	1 (2.9)	0.023
Absent	74 (80.4)	18 (19.6)	
Pathologic T stage			
1	83 (92.2)	7 (7.8)	0.001
2-4	24 (66.7)	12 (33.3)	
TNM stage			
I	80 (92.0)	7 (8.0)	0.001
II-IV	27 (69.2)	12 (30.8)	

χ^2 test.

Table 5. Correlation of mean staining score of Klotho expression and clinico-pathological parameters of CCRCC

Parameters	Klotho expression	
	Mean ± SD*	p-value
Sarcomatoid feature		
Absent	4.58±1.92	0.476
Present	4.00±1.90	
Tumor necrosis		
Absent	4.80±1.83	0.003
Present	3.58±1.96	
Rhabdoid feature		
Absent	4.59±1.95	0.348
Present	4.00±1.41	
Fuhrman nuclear grade		
Low (1+2)	5.01±1.89	0.001
High (3+4)	3.93±1.79	
Perirenal fat invasion		
Absent	4.65±1.91	0.134
Present	3.88±1.86	
Renal pelvis invasion		
Absent	4.62±1.91	0.153
Present	3.67±1.94	
Vascular invasion		
Absent	4.67±1.85	0.043
Present	3.57±2.24	
Renal sinus fat invasion		
Absent	4.56±1.93	0.681
Present	4.20±1.64	
Cystic change		
Present	4.35±1.91	0.054
Absent	5.09±1.85	
Pathologic T stage		
1	4.94±1.79	0.000
2-4	3.56±1.89	
TNM stage		
I	4.95±1.80	0.000
II-IV	3.64±1.87	

Student's *t*-test. *SD; standard deviation.

C. Prognostic Significance and Clinical Outcome of Klotho in CCRCC Patients

Klotho positive patients had a significantly better survival rate than Klotho negative patients (*p=0.049*) (Fig. 4A). However, there was no significant difference between Klotho positive and Klotho negative patients in recurrence rate (p=0.423) (Fig. 4B).

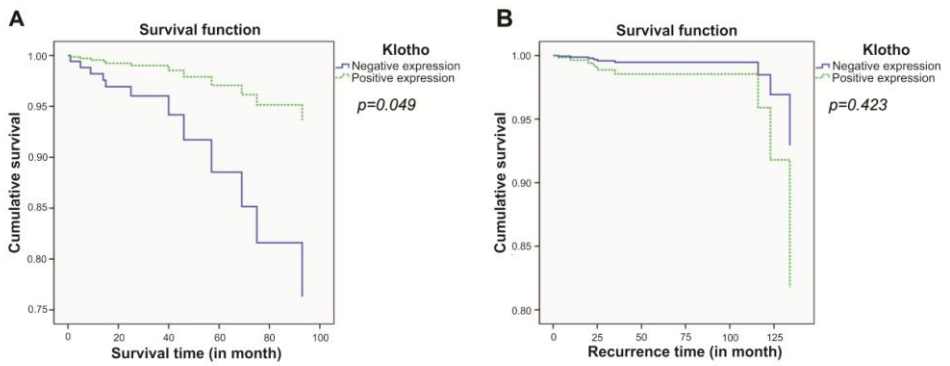

Fig. 4. Association between Klotho expression level and clinical outcome in CCRCC. Survival analysis shows a significant difference between the Klotho positive and Klotho negative patients in survival rate (A). There was no significant difference between Klotho positive and Klotho negative patients in recurrence rate. Survival analysis was determined using the Cox regression method after normalizing the following parameters: sex, age, Fuhrman nuclear grade, and pathologic T stage.

3.1.2. Insulin Receptor (IR) Expression

A. Pattern and Distribution of IR Expression in Non-tumor and Tumor Tissues

IHC staining showed that IR was expressed in both non-tumor renal parenchymal tissues and tumor tissues. IR protein was expressed in the nucleus of tumor tissues, whereas IR protein was observed in nucleus and/or cytoplasm of adjacent normal parenchymal tissues, including podocytes of glomeruli, tubular epithelium, and endothelial cells of blood vessels (Fig. 5A and B). IR expression was detected in 109 (87.9) cases of CCRCC.

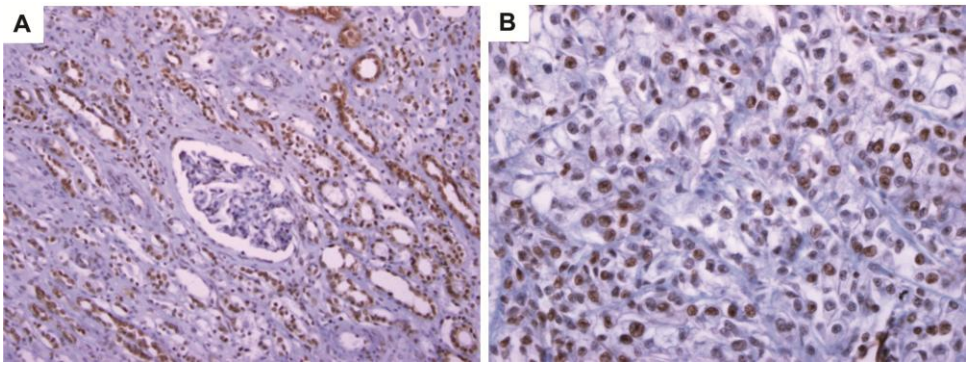

Fig. 5. Pattern and distribution of IR expression in non-tumor and tumor tissues. IHC staining shows that IR was expressed in the nucleus and/or cytoplasm of non-tumor renal parenchymal tissue (A) and in the nucleus of tumor tissue (B).

B. Correlation of IR and Prognostic Factors of CCRCC

IR was positive in 76.5% of patients without diabetes and 68.2% of patients with diabetes. IR was positive in 90.1% of cases with a low (1+2) Fuhrman nuclear grade and 85.9% of cases with a high (3+4) Fuhrman nuclear grade. With respect to the cystic change, IR was positive in 93.9% of cases with cystic change and 85.7% of cases without cystic change. Although the results above were not statistically significant, we observed a trend showing higher IR expression was associated to favorable prognostic factors. Moreover, IR expression seemed to be higher in cases without tumor necrosis, rhabdoid feature, perirenal fat invasion, and vascular invasion and lower in cases without sarcomatoid feature, renal pelvis, and renal sinus fat invasions, these differences were not statistically significant. For pathologic T stage, IR was expressed in 91.0% of cases with stage 1 and 80.0% of cases with stage 2-4, which was not a statistically significant difference. IR was expressed in 80.2% of cases with TNM stage I and 63.2% of cases with TNM stage II-IV. IR expression seemed to be higher in low TNM stage tumors when compared to high TNM stage tumors, although this was not statistically significant. These findings are detailed in Table 6.

The mean staining score of each group was compared to overcome the potential limitations with respect to quantification in IHC assays. With respect to Fuhrman nuclear grade, a statistically significant difference ($p=0.002$) was seen

between the two groups with mean staining scores of 5.21±1.98 and 4.17±1.65 for low and high grades, respectively. The mean staining score of IR was 5.45±1.79 in cases with cystic change and 4.52±1.90 in cases without cystic change, which was a statistically significant difference (*p=0.014*). Although the mean staining score of IR seemed to be higher in cases without tumor necrosis, rhabdoid feature, perirenal fat and vascular invasions and lower in cases with sarcomatoid feature, renal pelvis and renal sinus fat invasions, these difference were not statistically significant. For pathologic T stage, the mean staining score was 4.97±1.93 in cases with stage 1 and 4.26±1.79 in cases with stages 2-4. Although higher IR expression was correlated with lower pathologic T stage, it was not significant. However, a statistically significant difference (*p=0.042*) was seen between the mean staining scores of TNM stage I and TNM stages II-IV (4.99±1.95 vs. 4.26±1.74). These findings are summarized in Table 7.

Table 6. Correlation of IR expression and clinico-pathological parameters of CCRCC

Parameters	Insulin receptor expression		p-value
	No. of positive cases (%)	No. of negative cases (%)	
Diabetes			
Absent	89 (87.3)	13 (12.7)	0.479
Present	20 (90.9)	2 (9.1)	
Sarcomatoid feature			
Absent	104 (87.4)	15 (12.6)	0.519
Present	5 (100.0)	0 (0.0)	
Tumor necrosis			
Absent	88 (88.9)	11 (11.1)	0.355
Present	21 (84.0)	4 (16.0)	
Rhabdoid feature			
Absent	101 (88.6)	13 (11.4)	0.347
Present	8 (80.0)	2 (20.0)	
Fuhrman nuclear grade			
Low (1+2)	64 (90.1)	7 (9.9)	0.271
High (3+4)	45 (84.9)	8 (15.1)	
Perirenal fat invasion			
Absent	98 (89.9)	11 (10.1)	0.085
Present	11 (73.3)	4 (26.7)	
Renal pelvis invasion			
Absent	100 (87.0)	15 (13.0)	0.300
Present	9 (100.0)	0 (0.0)	
Vascular invasion			
Absent	99 (89.2)	12 (10.8)	0.193
Present	10 (76.9)	3 (23.1)	
Renal sinus fat invasion			
Absent	104 (87.4)	15 (12.6)	0.519
Present	5 (100.0)	0 (0.0)	
Cystic change			
Present	31 (93.9)	2 (6.1)	0.178
Absent	78 (85.7)	13 (14.3)	
Pathologic T stage			
1	81 (91.0)	8 (9.0)	0.086
2-4	28 (80.0)	7 (20.0)	
TNM stage			
I	78 (90.7)	8 (9.3)	0.129
II-IV	31 (81.6)	7 (18.4)	

χ^2 test.

Table 7. Correlation of mean staining score of IR expression and the clinico-pathological parameters of CCRCC

Parameters	Insulin receptor expression	
	Mean ± SD*	p-value
Sarcomatoid feature		
Absent	4.76±1.93	0.753
Present	5.00±1.58	
Tumor necrosis		
Absent	4.90±1.93	0.123
Present	4.24±1.76	
Rhabdoid feature		
Absent	4.81±1.91	0.434
Present	4.30±1.89	
Fuhrman nuclear grade		
Low (1+2)	5.21±1.98	0.002
High (3+4)	4.17±1.65	
Perirenal fat invasion		
Absent	4.87±1.90	0.098
Present	4.00±1.93	
Renal pelvis invasion		
Absent	4.77±1.95	0.980
Present	4.78±1.39	
Vascular invasion		
Absent	4.86±1.93	0.090
Present	4.00±1.58	
Renal sinus fat invasion		
Absent	4.76±1.93	0.956
Present	4.80±1.30	
Cystic change		
Present	5.45±1.79	0.014
Absent	4.52±1.90	
Pathologic T stage		
1	4.97±1.93	0.056
2-4	4.26±1.79	
TNM stage		
I	4.99±1.95	0.042
II-IV	4.26±1.74	

Student's *t*-test. *SD; standard deviation.

C. Prognostic Significance and Clinical Outcome of IR in CCRCC Patients

Survival analysis was performed after normalizing following parameters: sex, age, Fuhrman nuclear grade, and pathologic T stage. There was slight difference between IR positive and IR negative patients in both survival and recurrence rate; however, the difference was not statistically significant (Fig.6 A and B).

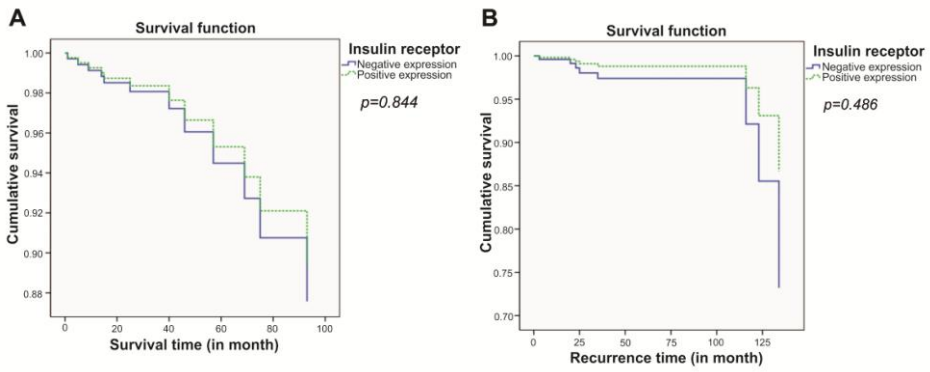

Fig. 6. Association between IR expression level and clinical outcome in CCRCC. Survival analysis shows no significant difference between the IR positive and IR negative patients in both survival (A) and recurrence rate (B). Survival analysis was determined using the Cox regression method.

3.1.3. Insulin-like Growth Factor (IGF)-1 Expression

A. Pattern and Distribution of IGF-1 Expression in Non-tumor and Tumor Tissues

IHC staining showed that IGF-1 was expressed in both non-tumor renal parenchymal tissues and tumor tissues. IGF-1 expression was observed in the nucleus of podocytes of glomeruli and in the cytoplasm and/or membrane of tubular epithelium and endothelial cells of blood vessels (Fig. 7A). Moreover, IGF-1 was expressed in 75 (59.5%) cases of CCRCC, showing positivity in the nucleus and cytoplasm (Fig. 7B).

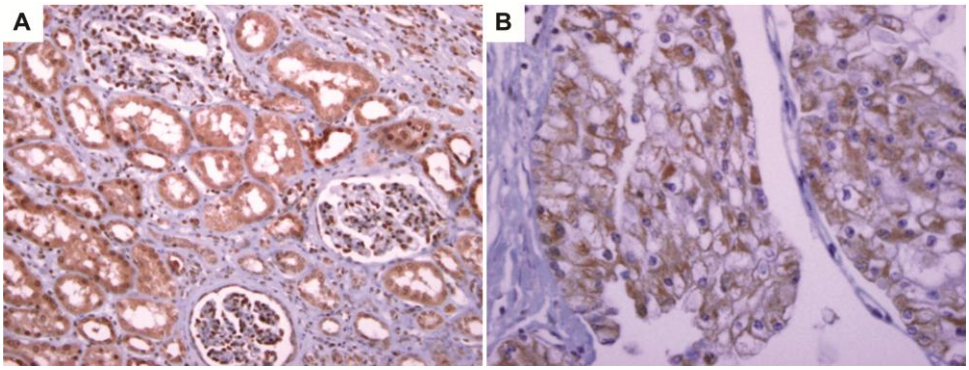

Fig. 7. Pattern and distribution of IGF-1 expression in non-tumor and tumor tissues. IHC staining shows biphasic IGF-1 was observed in non-tumor tissue, showing positivity in the nucleus of podocytes and in the cytoplasm and/or membrane of tubular epithelium and endothelial cells (A). IGF-1 was expressed in the nucleus and/or cytoplasm of tumor cells (B).

B. Correlation of IGF-1 and Prognostic Factors of CCRCC

IGF-1 was positive in 53.7% of patients with diabetes and 63.3% of patients without diabetes, which was not statistically significant. IGF-1 was positive 62.5% of cases with a low (1+2) Fuhrman nuclear grade and 55.6% of cases with a high (3+4) Fuhrman nuclear grade; however, the difference was not statistically significant. Furthermore, IGF-1 expression seemed to be higher in cases without sarcomatoid or rhabdoid feature, tumor necrosis, perirenal fat, renal pelvis, renal sinus fat, and vascular invasions and lower in cases with cystic change, although these differences were not statistically significant. IGF-1 was positive in 65.6% of cases with pathologic T stage 1 and 44.4% of cases with stage 2-4, which was statistically significant (*p=0.029*). IGF-1 was expressed in 64.4% of cases of TNM stage I and 48.7% of cases with TNM stages II-IV. Albeit higher IR expression seemed to be correlated with lower TNM stage, it was not a significant difference. These findings are detailed in Table 8.

The mean staining score of IGF-1 was 3.00±1.95 in cases with low Fuhrman nuclear grade and 2.83±2.09 in cases with high Fuhrman nuclear grade, which was not statistically significant. With respect to vascular invasion, a statistically significant difference (*p=0.016*) was seen between the two groups with mean staining scores of 3.08±1.96 and 1.71±1.98, respectively. For cystic change, the mean staining score was 2.68±2.02 in cases with cystic change and 3.59±1.84 in cases lacking cystic change, which was also statistically significant

(*p=0.024*). Although the mean staining score of IGF-1 seemed to be higher in cases without sarcomatoid or rhabdoid features, perirenal fat, renal pelvis, and renal sinus fat invasions, these differences were not statistically significant. The mean staining score was 3.11±1.92 in cases with pathologic T stage 1 and 2.47±1.74 in cases with stages 2-4. Although higher IR expression seemed to be associated with lower pathologic T stage, it was not a statistically significant difference. The mean staining score was 3.08±1.95 in cases with TNM stage I and 2.59±1.65 in cases with TNM stages II-IV, which was not statistically significant. These findings are summarized in Table 9.

Table 8. Correlation of IGF-1 expression and clinico-pathological parameters of CCRCC

Parameters	IGF-1 expression		p-value
	No. of positive cases (%)	No. of negative cases (%)	
Diabetes			
Absent	61 (58.7)	43 (41.3)	0.665
Present	14 (63.3)	8 (36.4)	
Sarcomatoid feature			
Absent	73 (60.8)	47 (39.2)	0.180
Present	2 (33.3)	4 (66.7)	
Tumor necrosis			
Absent	62 (62.0)	38 (38.0)	0.267
Present	13 (50.0)	13 (50.0)	
Rhabdoid feature			
Absent	71 (61.2)	45 (38.8)	0.190
Present	4 (40.0)	6 (60.0)	
Fuhrman grade			
Low (1+2)	45 (62.5)	27 (37.5)	0.432
High (3+4)	30 (55.6)	24 (44.4)	
Perirenal fat invasion			
Absent	69 (62.7)	41 (37.3)	0.055
Present	6 (37.2)	10 (62.5)	
Renal pelvis invasion			
Absent	71 (60.7)	46 (39.3)	0.339
Present	4 (44.4)	5 (55.6)	
Vascular invasion			
Absent	70 (62.5)	42 (37.5)	0.054
Present	5 (35.7)	9 (64.3)	
Renal sinus fat invasion			
Absent	73 (60.3)	48 (39.7)	0.364
Present	2 (40.0)	3 (60.0)	
Cystic change			
Absent	51 (44.6)	41 (44.6)	0.124
Present	24 (70.6)	10 (29.4)	
Pathologic T stage			
1	59 (65.6)	31 (34.4)	0.029
2-4	16 (44.4)	20 (55.6)	
TNM stage			
I	56 (64.4)	31 (35.6)	0.098
II-IV	19 (48.7)	20 (51.3)	

χ^2 test.

Table 9. Correlation of mean staining score of IGF-1 expression and clinico-pathological parameters of CCRCC

Parameters	IGF-1 expression	
	Mean ± SD*	p-value
Sarcomatoid feature		
Absent	3.00±2.00	0.74
Present	1.50±1.76	
Tumor necrosis		
Absent	3.01±1.89	0.373
Present	2.61±2.42	
Rhabdoid feature		
Absent	2.98±2.03	0.303
Present	2.30±1.59	
Fuhrman nuclear grade		
Low (1+2)	3.00±1.95	0.646
High (3+4)	2.83±2.09	
Perirenal fat invasion		
Absent	3.04±2.00	0.114
Present	2.19±1.94	
Renal pelvis invasion		
Absent	2.98±2.01	0.274
Present	2.22±1.92	
Vascular invasion		
Absent	3.08±1.96	0.016
Present	1.71±1.98	
Renal sinus fat invasion		
Absent	2.95±1.99	0.550
Present	2.40±2.51	
Cystic change		
Present	2.68±2.02	0.024
Absent	3.59±1.84	
Pathologic T stage		
1	3.11±1.92	0.106
2-4	2.47±1.74	
TNM stage		
I	3.08±1.95	0.205
II-IV	2.59±1.65	

Student's *t*-test. *SD; standard deviation.

C. Prognostic Significance and Clinical Outcome of IGF-1 in CCRCC Patients

Survival analysis was performed after normalizing following parameters: sex, age, Fuhrman nuclear grade, and pathologic T stage. There was no significant difference between IGF-1 positive group and IGF-1 negative group in both survival (p=0.780) and recurrence rate (p=0.455) (Fig. 8A and B).

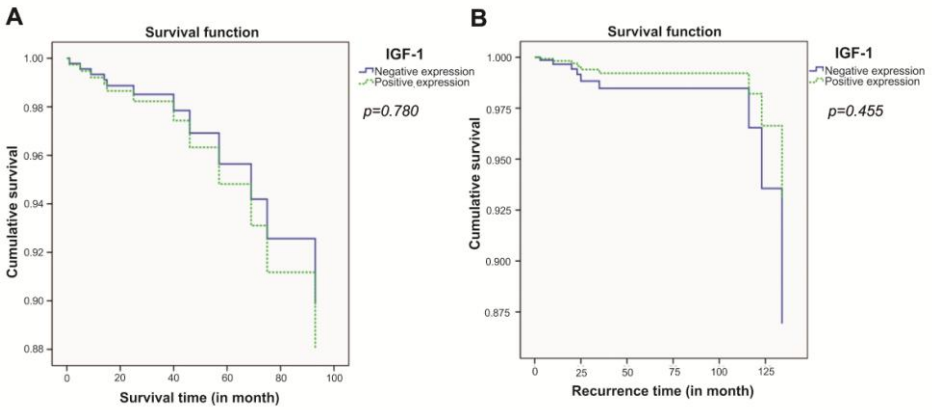

Fig. 8. Association between IGF-1 expression level and clinical outcome in CCRCC. Survival analysis shows no significant difference between the IGF-1 positive and IGF-1 negative patients in both survival (A) and recurrence rate (B). Survival analysis was determined using the Cox regression method.

3.1.4. IGF-1Receptor (IGF-1R) Expression

A. Pattern and Distribution of IGF-1R Expression in Non-tumor and Tumor Tissues

IHC staining showed that IGF-1R was expressed strongly in the nucleus of both non-tumor renal parenchymal tissues, including podocytes of glomeruli, tubular epithelium, endothelial cells, and lymphocytes (Fig. 9A) and tumor cells (Fig. 9B). IGF-1R expression was observed in 121 (96%) cases of CCRCC.

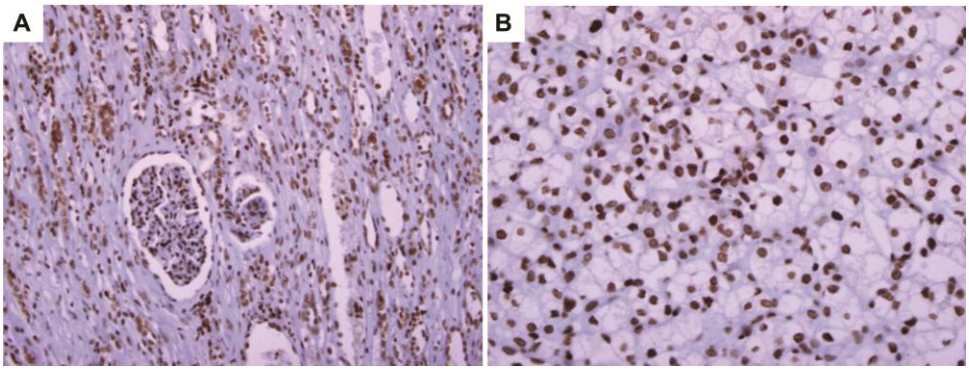

Fig. 9. Pattern and distribution of IGF-1R expression in non-tumor and tumor tissue. IHC staining shows IGF-1R was expressed in the nucleus of both non-tumor renal parenchymal tissue (A) and tumor tissue (B).

B. Correlation of IGF-1R and Prognostic Factors of CCRCC

IGF-1R was positive in 96.2% of patients with diabetes and 95.5% of patients without diabetes, though this was not statistically significant. IGF-1R was positive in 95.8% of cases with a low (1+2) Fuhrman nuclear grade and 96.3% of cases with a high (3+4) Fuhrman nuclear grade. For perirenal fat invasion, IGF-1R was positive in 96.4% of cases without perirenal fat invasion and 93.8% of cases with perirenal fat invasion. Despite the fact that IGF-1R expression seemed to be lower in cases without sarcomatoid or rhabdoid feature, tumor necrosis, renal pelvis, renal sinus fat, and vascular invasions, these differences were not statistically significant. With respect to the cystic change, IGF-1R positivity was seen in 44.6% of cases without cystic change and 44.6% of cases with cystic change. This difference in IGF-1R expression was not significant. In addition, IGF-1R was expressed in 95.6% of cases of pathologic T stage 1 and 97.2% of cases with pathologic T stage 2-4. IGF-1R was expressed in 95.4% of cases of TNM stage I and 97.4% of cases with TNM stages II-IV. IGF-1R expression seemed to be lower in lower pathologic T stage and TNM stage tumors as compared to higher stage tumors, although these were not statistically significant. These findings are detailed in Table 10.

The mean staining score of IGF-1R was 6.65±1.86 in cases with low Fuhrman nuclear grade and 6.48±1.93 in cases with high Fuhrman nuclear grade, which was not statistically significant. With respect to renal pelvis invasion, a

statistically significant difference (*p=0.0041*) was seen between the mean staining scores in presence and absence of pelvis invasion (6.49±1.87 vs. 7.78±0.44). For cystic change, the mean staining score was 6.59±1.96 in cases with cystic change and 6.56±1.46 in cases without cystic change, which was not a significant difference. Although the mean staining score of IGF-1R seemed to be lower in cases without sarcomatoid or rhabdoid feature, tumor necrosis, renal sinus fat, and vascular invasions, these differences were not statistically significant. In contrast, the mean staining score was 6.58±1.80 in cases without perirenal fat invasion and 6.56±2.10 in cases with perirenal fat invasion, but it was not statistically significant. Moreover, the mean staining score was 6.49±1.90 in cases with pathologic T stage 1 and 6.81±1.67 in cases with stages 2-4. The mean staining score was 6.47±1.91 in cases with TNM stage I and 6.82±1.65 in cases with TNM stages II-IV. IGF-1R expression seemed to be lower in low pathologic T stage and TNM stage tumors as compared to high stage tumors, even though these differences were not statistically significant. These findings are summarized in Table 11.

Table 10. Correlation of IGF-1R expression and clinico-pathological parameters of CCRCC

Parameters	IGF-1R expression		p-value
	No. of positive cases (%)	No. of negative cases (%)	
Diabetes			
Absent	100 (96.2)	4 (3.8)	0.879
Present	21 (95.5)	1 (4.5)	
Sarcomatoid feature			
Absent	115 (95.8)	5 (4.2)	0.610
Present	6 (100.0)	0 (0.0)	
Tumor necrosis			
Absent	96 (96.0)	4 (4.0)	0.971
Present	25 (96.2)	1 (3.8)	
Rhabdoid feature			
Absent	111 (95.7)	5 (4.3)	0.503
Present	10 (100.0)	0 (0.0)	
Fuhrman grade			
Low (1+2)	69 (95.8)	3 (4.2)	0.895
High (3+4)	52 (96.3)	2 (3.7)	
Perirenal fat invasion			
Absent	106 (96.4)	4 (3.6)	0.617
Present	15 (93.8)	1 (6.3)	
Renal pelvis invasion			
Absent	112 (95.7)	5 (4.3)	0.527
Present	9 (100.0)	0 (0.0)	
Vascular invasion			
Absent	107 (95.5)	5 (4.5)	0.420
Present	14 (100.0)	0 (0.0)	
Renal sinus fat invasion			
Absent	116 (95.9)	5 (4.1)	0.643
Present	5 (100.0)	0 (0.0)	
Cystic change			
Absent	87 (94.6)	5 (5.4)	0.165
Present	34 (100.0)	0 (0.0)	
Pathologic T stage			
1	86 (95.6)	4 (4.4)	0.665
2-4	35 (97.2)	1 (2.8)	
TNM stage			
I	83 (95.4)	4 (4.6)	0.589
II-IV	38 (97.4)	1 (2.6)	

χ^2 test.

Table 11. Correlation of mean staining score of IGF-1R expression and clinico-pathological parameters of CCRCC

Parameters	IGF-1R expression	
	Mean ± SD*	p-value
Sarcomatoid feature		
Absent	6.55±1.86	0.424
Present	7.17±1.17	
Tumor necrosis		
Absent	6.57±1.80	0.911
Present	6.62±1.98	
Rhabdoid feature		
Absent	6.55±1.86	0.567
Present	6.90±1.52	
Fuhrman nuclear grade		
Low (1+2)	6.65±1.77	0.606
High (3+4)	6.48±1.93	
Perirenal fat invasion		
Absent	6.58±1.80	0.969
Present	6.56±2.10	
Renal pelvis invasion		
Absent	6.49±1.87	0.041
Present	7.78±0.44	
Vascular invasion		
Absent	6.53±1.90	0.365
Present	7.00±1.11	
Renal sinus fat invasion		
Absent	6.57±1.86	0.785
Present	6.80±1.11	
Cystic change		
Present	6.59±1.96	0.939
Absent	6.56±1.46	
Pathologic T stage		
1	6.49±1.90	0.384
2-4	6.81±1.67	
TNM stage		
I	6.47±1.91	0.325
II-IV	6.82±1.65	

Student's *t*-test. *SD; standard deviation

C. Prognostic Significance and Clinical Outcome of IGF-1R in CCRCC Patients

Survival analysis was performed after normalizing following parameters: sex, age, Fuhrman nuclear grade, and pathologic T stage. There was slight difference between IGF-1R positive patients and IGF-1R negative patients in survival rate (p=0.273), however, the difference was not statistically significant (Fig. 10A). In addition, there was no statistically significant difference between IGF-1R positive patients and IGF-1R negative patients in recurrence rate (p=0.995) (Fig. 10B).

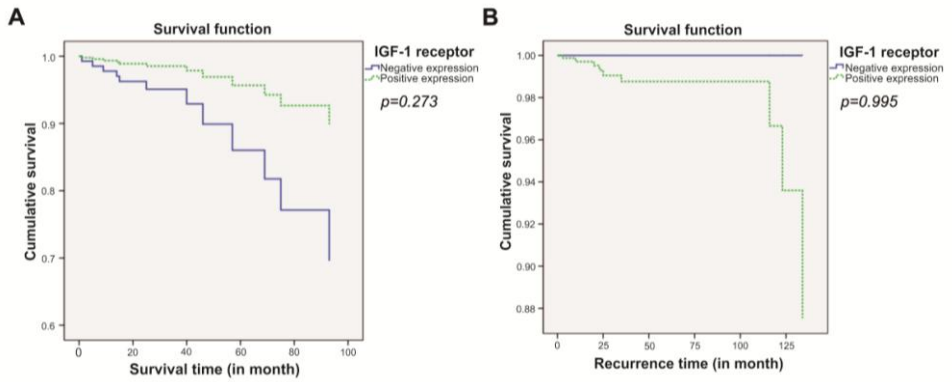

Fig. 10. Association between IGF-1R expression level and clinical outcome in CCRCC. Survival analysis shows no significant difference between the IGF-1R positive and IGF-1R negative patients in both survival (A) and recurrence rate (B). Survival analysis was determined using the Cox regression method.

3.1.5. Vascular Endothelial Growth Factor (VEGF) Expression

A. Pattern and Distribution of VEGF Expression in Non-tumor and Tumor Tissues

VEGF was observed in the cytoplasm and/or membrane of both non-tumor renal parenchymal tissues, including podocytes of glomeruli, tubular epithelium, and endothelial cells of blood vessels (Fig. 11A) and in tumor cells (Fig. 11B). The expression of VEGF was detected in 70 (55.6%) cases of CCRCC.

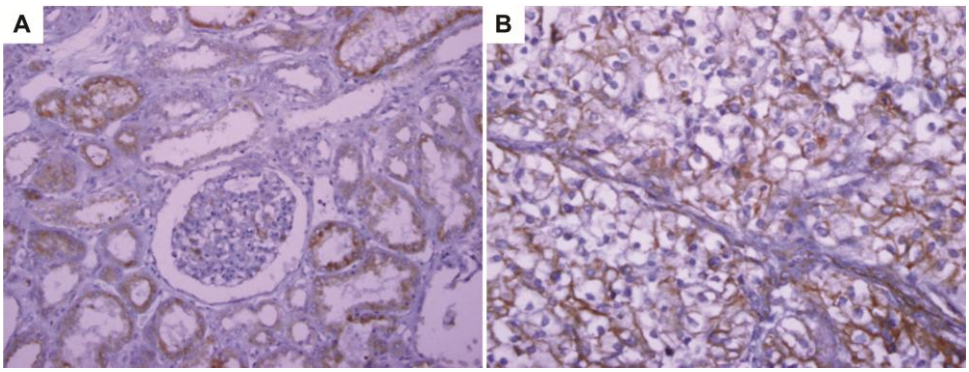

Fig. 11. Pattern and distribution of VEGF expression in non-tumor and tumor tissue. IHC staining shows VEGF was expressed in the cytoplasm and/or membrane of both non-tumor renal parenchymal tissue (A) and tumor tissue (B).

B. Correlation of VEGF and Prognostic Factors of CCRCC

VEGF was positive 55.6% of cases with a low (1+2) Fuhrman nuclear grade and 55.6% of cases with a high (3+4) Fuhrman nuclear grade, but it was not statistically significant. Despite the fact that VEGF expression seemed to be higher in cases with sarcomatoid or rhabdoid features, tumor necrosis, cystic change, renal pelvis and renal sinus fat invasions and in cases without perirenal fat and vascular invasions, these differences were not statistically significant. Furthermore, VEGF was expressed in 60% of cases with pathologic T stage 1 and 44.4% of cases with pathologic T stage 2-4. This difference in VEGF expression was not statistically significant. VEGF was expressed in 59.8% of cases of TNM stage I and 46.2% of cases with TNM stages II-IV. VEGF expression seemed to be higher in low TNM stage tumors as compared to high stage tumors, although this was not statistically significant. These findings are detailed in Table 12.

The mean staining score of VEGF was 2.60±2.21 and 2.52±1.96 in cases with low and high Fuhrman nuclear grades, respectively. This difference in VEGF was not statistically significant. For cystic change, mean staining score was 2.67±2.12 in cases with cystic change and 2.26±2.06 in cases without cystic change, but these differences were not statistically significant. Although the mean staining score of VEGF seemed to be higher in cases with sarcomatoid or rhabdoid feature, perirenal fat, renal pelvis and renal sinus fat invasions, and in cases without vascular invasion, respectively, these differences were not

statistically significant. Furthermore, the mean staining score of VEGF was 2.61±1.88 in cases with pathologic T stage 1 and 2.44±2.60 in cases with pathologic T stage 2-4. For TNM stage, the mean staining score was 2.59±1.90 in cases with TNM stage I and 2.51±2.52 in cases with TNM stage II-IV. However VEGF expression seemed to be higher in lower pathologic T stage and TNM stage tumors as compared to higher stage tumors, these were not statistically significant. These findings are detailed in Table 13.

Table 12. Correlation of VEGF expression and clinico-pathological parameters of CCRCC

Parameters	VEGF expression		p-value
	No. of positive cases (%)	No. of negative cases (%)	
Sarcomatoid feature			
Absent	65 (54.2)	55 (45.8)	0.161
Present	5 (83.3)	1 (16.7)	
Tumor necrosis			
Absent	54 (54.0)	46 (46.0)	0.491
Present	16 (61.5)	10 (38.5)	
Rhabdoid feature			
Absent	62 (53.4)	54 (46.6)	0.105
Present	8 (80.0)	2 (20.0)	
Fuhrman nuclear grade			
Low (1+2)	40 (55.6)	32 (44.4)	1.000
High (3+4)	30 (55.6)	24 (44.4)	
Perirenal fat invasion			
Absent	62 (56.4)	48 (43.6)	0.632
Present	8 (50.0)	8 (50.0)	
Renal pelvis invasion			
Absent	63 (53.8)	54 (46.2)	0.164
Present	7 (77.8)	2 (22.2)	
Vascular invasion			
Absent	64 (57.1)	48 (42.9)	0.310
Present	6 (42.9)	8 (57.1)	
Renal sinus fat invasion			
Absent	66 (54.5)	55 (45.5)	0.262
Present	4 (80.0)	1 (20.0)	
Cystic change			
Present	53 (57.6)	39 (42.4)	0.446
Absent	17 (50.0)	17 (50.0)	
Pathologic T stage			
1	54 (60.0)	36 (40.0)	0.112
2-4	16 (44.4)	20 (55.6)	
TNM stage			
I	52 (59.8)	35 (40.2)	0.155
II-IV	18 (46.2)	21 (53.8)	

χ^2 test.

Table 13. Correlation of mean staining score of VEGF expression and clinico-pathological parameters of CCRCC

Parameters	VEGF expression	
	Mean ± SD*	p-value
Sarcomatoid feature		
Absent	2.50±2.10	0.130
Present	3.83±1.94	
Tumor necrosis		
Absent	2.43±2.03	0.163
Present	3.08±2.35	
Rhabdoid feature		
Absent	2.47±2.08	0.105
Present	3.60±2.17	
Fuhrman nuclear grade		
Low (1+2)	2.60±2.21	0.836
High (3+4)	2.52±1.96	
Perirenal fat invasion		
Absent	2.54±2.09	0.706
Present	2.75±2.27	
Renal pelvis invasion		
Absent	2.52±2.12	0.420
Present	3.11±1.90	
Vascular invasion		
Absent	2.59±2.07	0.699
Present	2.36±2.41	
Renal sinus fat invasion		
Absent	2.51±2.06	0.181
Present	3.80±2.95	
Cystic change		
Present	2.67±2.12	0.334
Absent	2.26±2.06	
Pathologic T stage		
1	2.61±1.88	0.690
2-4	2.44±2.60	
TNM stage		
I	2.59±1.90	0.857
II-IV	2.51±2.52	

Student's *t*-test. *SD; standard deviation.

C. Prognostic Significance and Clinical Outcome of VEGF in CCRCC Patients

Survival analysis was performed after normalizing the following parameters: sex, age, Fuhrman nuclear grade, and pathologic T stage. There was no significant difference between VEGF positive patients and VEGF negative patients in both survival (p=0.253) and recurrence rate (p=0.460) (Fig. 12 A and B).

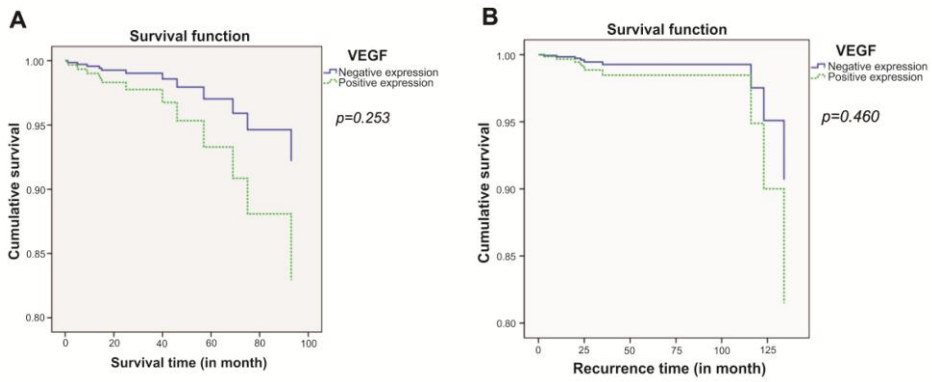

Fig. 12. Association between VEGF expression level and clinical outcome in CCRCC. Survival analysis show no significant difference between the VEGF positive and VEGF negative patients in both survival (A) and recurrence rate (B). Survival analysis was determined using the Cox regression method.

3.1.6. VEGF Receptor (VEGFR)-1 Expression

A. Pattern and Distribution of VEGFR-1 Expression in Non-tumor and Tumor Tissues

IHC staining showed that VEGFR-1 was expressed in the cytoplasm and/or membrane of both non-tumor renal parenchymal tissues, including podocytes of glomeruli, tubular epithelium, and endothelial cells of blood vessels (Fig. 13A) and in the tumor cells (Fig. 13B). VEGFR-1 expression was observed in 59 (46.8%) cases of CCRCC.

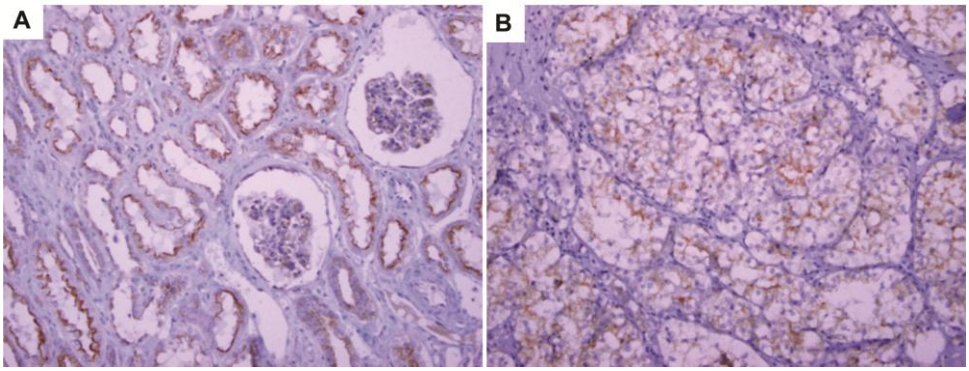

Fig. 13. Pattern and distribution of VEGFR-1 expression in non-tumor and tumor tissue. IHC staining shows VEGFR-1 was expressed in the cytoplasm and/or membrane of both non-tumor renal parenchymal tissue (A) and tumor tissue (B).

C. Correlation of VEGFR-1 and Prognostic Factors of CCRCC

VEGFR-1 was positive in 56.9% of cases with a low (1+2) Fuhrman nuclear grade and 33.3% of cases with a high (3+4) Fuhrman nuclear grade, which was statistically significant (*p=0.009*). Furthermore, VEGFR-1 was positive in 49.6% of cases lacking renal pelvis invasion and 11.1% of cases with renal pelvis invasion. This difference in VEGFR-1 expression was statistically significant (*p=0.036*). With respect to the rhabdoid component, VEGFR-1 positivity was observed in 49.1% of cases without rhabdoid feature and 20% of cases with rhabdoid feature, but the difference was not statistically significant. Although VEGFR-1 expression seemed to be higher in cases without sarcomatoid feature, tumor necrosis, perirenal fat, renal sinus fat, and vascular invasion, and lower in cases with cystic change, these differences were not statistically significant. Moreover, 50% of cases with pathologic T stage 1 were VEGFR-1 positive, as were 38.9% of cases with pathologic T stage 2-4 cases, which was not significant. VEGFR-1 was expressed in 50.6% of cases with TNM stage I and 38.5% of cases with TNM stages II-IV. VEGFR-1 expression seemed to be higher in low TNM stage tumors as compared to high TNM stage tumors, although this was not a statistically significant finding. These findings are summarized in Table 14.

A statistically significant difference was observed between the mean staining scores of low and high Fuhrman nuclear grade (2.63±2.18 vs. 1.56±2.04)

(*p=0.006*). For renal pelvis invasion, the mean staining score was 2.26±2.20 in cases without renal pelvis invasion and 0.89±1.45 in cases with renal pelvis invasion, but the difference was not significant. Although the mean staining score of VEGFR-1 seemed to be higher in cases without sarcomatoid or rhabdoid feature, tumor necrosis, perirenal fat, renal sinus fat, and vascular invasions, and lower in cases with cystic change, these differences were not statistically significant. With respect to pathologic T stage, the mean staining score was 2.36±2.18 in cases with stage 1 and 1.69±2.12 in cases with stages 2-4. Although higher VEGFR-1 expression was correlated with lower pathologic T stage, this was not significant. Furthermore, the mean staining score of VEGFR-1 was 2.38±2.21 and 1.69±2.07 in cases with TNM stage I and TNM stages II-IV, respectively. The difference was not statistically significant. These findings are detailed in Table 15.

Table 14. Correlation of VEGFR-1 expression and clinico-pathological parameters of CCRCC

Parameters	VEGFR-1 expression		p-value
	No. of positive cases (%)	No. of negative cases (%)	
Sarcomatoid feature			
Absent	57 (47.5)	63 (52.5)	0.402
Present	2 (33.3)	4 (66.7)	
Tumor necrosis			
Absent	49 (49.0)	51 (51.0)	0.337
Present	10 (38.5)	16 (61.5)	
Rhabdoid feature			
Absent	57 (49.1)	59 (50.9)	0.072
Present	2 (20.0)	8 (80.0)	
Fuhrman nuclear grade			
Low (1+2)	41 (56.9)	31 (43.1)	0.009
High (3+4)	18 (33.3)	36 (66.7)	
Perirenal fat invasion			
Absent	53 (48.2)	57 (51.8)	0.424
Present	6 (37.5)	10 (62.5)	
Renal pelvis invasion			
Absent	58 (49.6)	59 (50.4)	0.036
Present	1 (11.1)	8 (88.9)	
Vascular invasion			
Absent	53 (47.3)	59 (52.7)	0.752
Present	6 (42.9)	8 (57.1)	
Renal sinus fat invasion			
Absent	57 (47.1)	64 (52.9)	0.560
Present	2 (40.0)	3 (60.0)	
Cystic change			
Present	18 (52.9)	16 (47.1)	0.403
Absent	41 (44.6)	51 (55.4)	
Pathologic T stage			
1	45 (50.0)	45 (50.0)	0.176
2-4	14 (38.9)	22 (61.1)	
TNM stage			
I	44 (50.6)	43 (49.4)	0.143
II-IV	15 (38.5)	24 (61.5)	

χ^2 test.

Table 15. Correlation of mean staining score of VEGFR-1 expression and clinico-pathological parameters of CCRCC

Parameters	VEGFR-1 expression	
	Mean ± SD*	p-value
Sarcomatoid feature		
Absent	2.21±2.20	0.339
Present	1.33±1.51	
Tumor necrosis		
Absent	2.30±2.20	0.179
Present	1.65±2.08	
Rhabdoid feature		
Absent	2.26±2.21	0.107
Present	1.10±1.52	
Fuhrman nuclear grade		
Low (1+2)	2.63±2.18	0.006
High (3+4)	1.56±2.04	
Perirenal fat invasion		
Absent	2.27±2.19	0.153
Present	1.44±2.03	
Renal pelvis invasion		
Absent	2.26±2.20	0.068
Present	0.89±1.45	
Vascular invasion		
Absent	2.28±2.23	0.109
Present	1.29±1.54	
Renal sinus fat invasion		
Absent	2.21±2.19	0.313
Present	1.20±1.64	
Cystic change		
Present	2.59±2.41	0.188
Absent	2.01±2.08	
Pathologic T stage		
1	2.36±2.18	0.124
2-4	1.69±2.12	
TNM stage		
I	2.38±2.21	0.102
II-IV	1.69±2.07	

Student's *t*-test. *SD; standard deviation.

C. Prognostic Significance and Clinical Outcome of VEGFR-1 in CCRCC Patients

Survival analysis was performed after adjusting for sex, age, Fuhrman nuclear grade, and pathologic T stage. There was no significant difference between VEGFR-1 positive patients and VEGFR-1 negative patients in both survival (p=0.064) and recurrence rate (p=0.249) (Fig. 14A and B).

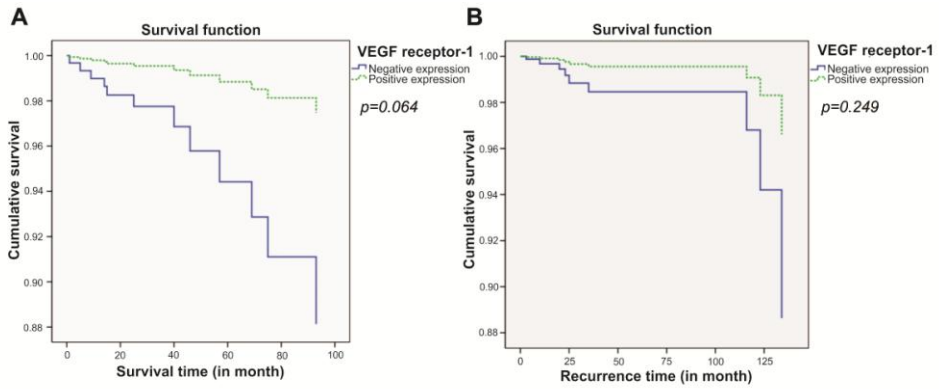

Fig. 14. Association between VEGFR-1 expression level and clinical outcome in CCRCC. Survival analysis shows no significant difference between the VEGFR-1 positive and VEGFR-1 negative patients in both survival (A) and recurrence rate (B). Survival analysis was determined using the Cox regression method.

3.1.7. Platelet-derived Growth Factor (PDGF) Expression

A. Pattern and Distribution of PDGF Expression in Non-tumor and Tumor Tissues

IHC staining showed that PDGF expression was observed in the cytoplasm of distal tubular epithelium (Fig. 15A). Furthermore, nuclear positivity of PDGF was detected in 98 (77.8%) cases of CCRCC (Fig. 15B).

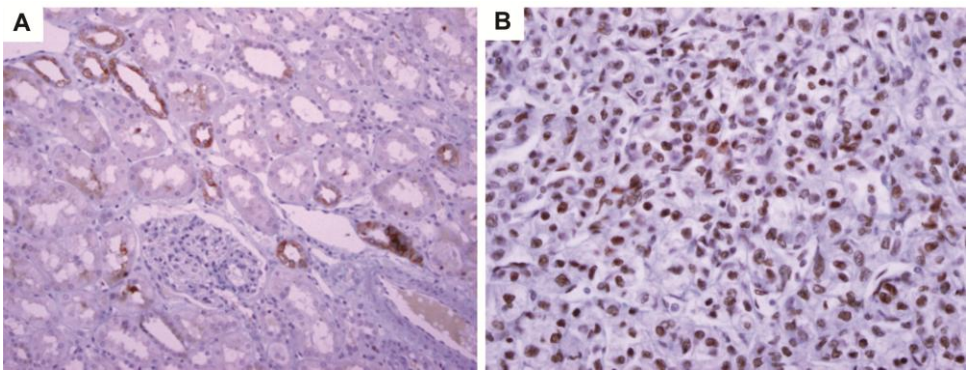

Fig. 15. Pattern and distribution of PDGF expression in non-tumor and tumor tissue. IHC staining shows PDGF was expressed in the cytoplasm and/or membrane of normal tubular epithelium (A) and in the nucleus of tumor cells (B).

B. Correlation of PDGF and Prognostic Factors of CCRCC

PDGF was positive 81.9% of cases with a low (1+2) Fuhrman nuclear grade and 72.2% of cases with a high (3+4) Fuhrman nuclear grade. With respect to the cystic change, PDGF positivity was seen in 76.1% of cases without cystic change and 82.4% of cases with cystic change. Moreover, PDGF expression seemed to be higher in cases without sarcomatoid or rhabdoid feature, tumor necrosis, perirenal fat, renal pelvis, renal sinus fat and vascular invasions, these differences were not statistically significant. PDGF expression seemed be to higher in lower pathologic T stage tumors (81.1%) as compared to higher stage tumors (69.4%). Moreover, PDGF was expressed in 80.5% of cases with TNM stage I and 71.8% of cases with TNM stages II-IV. Although the results above were not statistically significant, we observed a trend, showing higher PDGF expression was related to favorable prognostic factors. These findings are detailed in Table 16.

The mean staining score of PDGF was 3.31±1.75 in cases with low Fuhrman nuclear grade and 3.26±1.88 in cases with high Fuhrman nuclear grade. With respect to the cystic change, the mean staining score was 3.50±1.92 in cases with cystic change and 3.35±1.47 in cases without cystic change. Although the mean staining score of PDGF seemed to be higher in cases without sarcomatoid feature, tumor necrosis, perirenal fat, renal sinus fat and vascular invasions, and in cases with rhabdoid feature, the differences were not statistically significant.

The mean staining score was 3.59±2.44 in cases with pathologic T stage 1 and 1.83±2.08 in cases with stages 2-4, but the difference was not statistically significant. Besides the mean staining score was 3.56±1.76 in cases with TNM stage I and 3.23±1.93 in cases with TNM stages II-IV, which was also not significant difference. These findings are summarized in Table 17.

Table 16. Correlation of PDGF expression and clinico-pathological parameters of CCRCC

Characteristics	PDGF expression		p-value
	No. of positive cases (%)	No. of negative cases (%)	
Sarcomatoid feature			
Absent	94 (78.3)	26 (21.7)	0.502
Present	4 (66.7)	2 (33.3)	
Tumor necrosis			
Absent	81 (81.0)	19 (19.0)	0.088
Present	17 (65.4)	9 (34.6)	
Rhabdoid feature			
Absent	92 (79.3)	24 (20.7)	0.159
Present	6 (60.0)	4 (40.0)	
Fuhrman grade			
Low (1+2)	59 (81.9)	13 (18.1)	0.194
High (3+4)	39 (72.2)	15 (27.8)	
Perirenal fat invasion			
Absent	87 (79.1)	23 (20.9)	0.353
Present	11 (68.7)	5 (31.3)	
Renal pelvis invasion			
Absent	93 (79.5)	24 (20.5)	0.096
Present	5 (55.6)	4 (44.4)	
Vascular invasion			
Absent	89 (79.5)	23 (20.5)	0.198
Present	9 (64.3)	1 (35.7)	
Renal sinus fat invasion			
Absent	95 (78.5)	26 (21.5)	0.329
Present	3 (60.0)	2 (40.0)	
Cystic change			
Absent	70 (76.1)	22 (23.9)	0.453
Present	28 (82.4)	6 (17.6)	
Pathologic T stage			
1	73 (81.1)	17(18.9)	0.155
2-4	25 (69.4)	11 (30.6)	
TNM stage			
I	70 (80.5)	17 (19.5)	0.279
II-IV	28 (71.8)	11 (28.2)	

χ^2 test.

Table 17. Correlation of mean staining score of PDGF expression and clinico-pathological parameters of CCRCC

Parameters	PDGF expression	
	Mean ± SD*	p-value
Sarcomatoid feature		
Absent	3.48±1.84	0.525
Present	3.00±1.09	
Tumor necrosis		
Absent	3.52±1.77	0.470
Present	3.23±1.99	
Rhabdoid feature		
Absent	3.46±1.78	0.943
Present	3.50±2.28	
Fuhrman nuclear grade		
Low (1+2)	3.61±1.75	0.282
High (3+4)	3.26±1.88	
Perirenal fat invasion		
Absent	3.49±1.80	0.624
Present	3.25±1.92	
Renal pelvis invasion		
Absent	3.50±1.81	0.328
Present	2.89±1.76	
Vascular invasion		
Absent	3.55±1.81	0.102
Present	2.71±1.73	
Renal sinus fat invasion		
Absent	3.50±1.81	0.280
Present	2.60±1.82	
Cystic change		
Present	3.50±1.92	0.687
Absent	3.35±1.47	
Pathologic T stage		
1	3.59±2.44	0.209
2-4	1.83±2.08	
TNM stage		
I	3.56±1.76	0.342
II-IV	3.23±1.93	

Student's *t*-test. *SD; standard deviation.

C. Prognostic Significance and Clinical Outcome of PDGF in CCRCC Patients

Survival analysis was performed after normalizing the following parameters: sex, age, Fuhrman nuclear grade, and pathologic T stage. Although, there was slight difference between PDGF positive group and PDGF negative group in survival (p=0.312), it was not statistically significant (Fig. 16A). In addition, there was no significant difference between PDGF positive group and PDGF negative group in recurrence rate (p=0.476) (Fig. 16B).

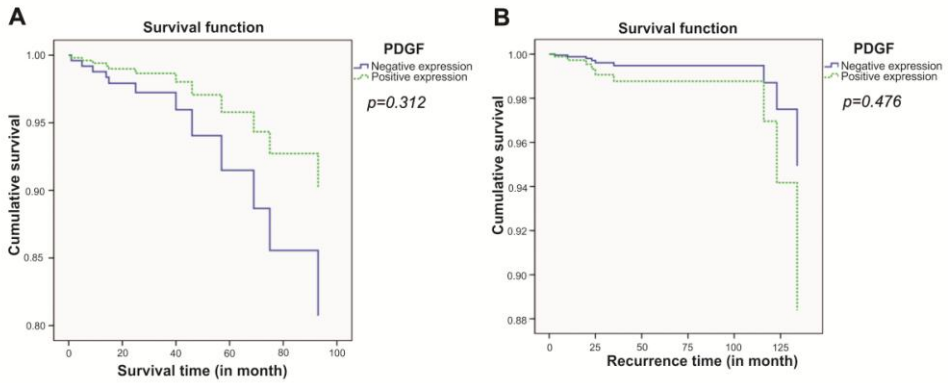

Fig. 16. Association between PDGF expression level and clinical outcome in CCRCC. Survival analysis show no significant difference between the PDGF positive and PDGF negative groups in both survival (A) and recurrence rate (B). Survival analysis was determined using the Cox regression method.

3.1.8. PDGF Receptor (PDGFR)-α Expression

A. Pattern and Distribution of PDGFR-α Expression in Non-tumor and Tumor Tissues

IHC staining showed that PDGFR-α was observed in the cytoplasm and/or membrane of both non-tumor renal parenchymal tissues, including podocytes of glomeruli, tubular epithelium, and endothelial cells of blood vessels (Fig. 17A) and tumor cells (Fig. 17B). The expression of PDGFR-α was observed in 122 (96.8%) cases of CCRCC.

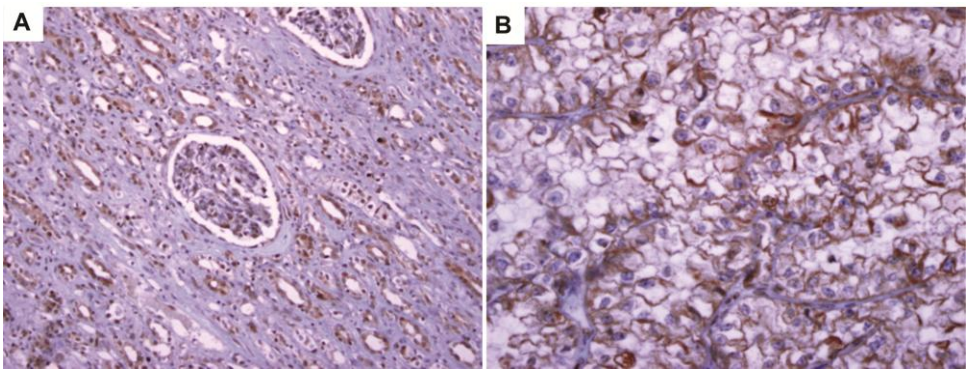

Fig. 17. Pattern and distribution of PDGFR-α expression in non-tumor and tumor tissue. IHC staining shows PDGFR-α was expressed in the cytoplasm and/or membrane of both non-tumor renal parenchymal tissue (A) and tumor tissue (B).

B. Correlation of PDGFR-α and Prognostic Factors of CCRCC

PDGFR-α was positive 97.2% of cases with a low (1+2) Fuhrman nuclear grade and 96.3% of cases with a high (3+4) Fuhrman nuclear grade. Despite the fact that PDGFR-α expression seemed to be higher in cases with sarcomatoid or rhabdoid feature, renal pelvis and renal sinus fat invasions and lower in cases with tumor necrosis, perirenal fat invasion, and cystic change, these differences were not statistically significant. For the pathologic T stage, PDGFR-α was expressed in 97.8% of cases with pathologic T stage 1 and 94.4% of cases with pathologic T stage 2-4, but it was not statistically significant. Moreover, PDGFR-α was expressed in 97.7% of cases with TNM stage I and 94.9% of cases with TNM stages II-IV, which was not statistically significant. These findings are detailed in Table 18.

The mean staining score of PDGFR-α was 5.25±1.39 in cases with low Fuhrman nuclear grade and 5.71±1.57 in cases with high Fuhrman nuclear grade, which was not statistically significant. Similar findings were observed the mean staining score of PDGFR-α seemed to be lower in cases without sarcomatoid or rhabdoid feature, tumor necrosis, and renal pelvis invasion and higher in cases without perirenal fat, renal pelvis, renal sinus fat, and vascular invasions, these differences were not statistically significant. With respect to cystic change, the mean staining score was 5.58±1.57 in cases with cystic change and 5.15±1.39 in cases without cystic change, which was not a significant difference. The mean

staining score was 5.51±1.38 in cases with pathologic T stage 1 and 5.33±1.74 in cases with stages 2-4. In addition, the mean staining score was 5.51±1.40 in cases with TNM stage I and 5.36±1.68 in cases with TNM stages II-IV. Although the mean staining score of PDGFR-α seemed to be higher in cases with low pathologic T stage and TNM stage, which were not statistically significant difference. These findings are summarized in Table 19.

Table 18. Correlation of PDGFR-α expression and clinico-pathological parameters of CCRCC

Parameters	PDGFR-α expression		p-value
	No. of positive cases (%)	No. of negative cases (%)	
Sarcomatoid feature			
Absent	116 (96.7)	4 (3.3)	0.649
Present	6 (100.0)	0 (0.0)	
Tumor necrosis			
Absent	97 (97.0)	3 (3.0)	0.826
Present	25 (96.2)	1 (3.8)	
Rhabdoid feature			
Absent	112 (96.6)	4 (3.4)	0.551
Present	10 (100.0)	0 (0.0)	
Fuhrman nuclear grade			
Low (1+2)	70 (97.2)	2 (2.8)	0.769
High (3+4)	52 (96.3)	2 (3.7)	
Perirenal fat invasion			
Absent	107 (97.3)	3 (2.7)	0.453
Present	15 (93.8)	1 (6.3)	
Renal pelvis invasion			
Absent	113 (96.6)	4 (3.4)	0.573
Present	9 (100.0)	0 (0.0)	
Vascular invasion			
Absent	109 (97.3)	3 (2.7)	0.369
Present	13 (92.9)	1 (7.1)	
Renal sinus fat invasion			
Absent	117 (96.7)	4 (3.3)	0.679
Present	5 (100.0)	0 (0.0)	
Cystic change			
Present	88 (95.7)	4 (4.3)	0.217
Absent	34 (100.0)	0 (0.0)	
Pathologic T stage			
1	88 (97.8)	2 (2.2)	0.335
2-4	34 (94.4)	2(5.6)	
TNM stage			
I	85 (97.7)	2 (2.3)	0.402
II-IV	37 (94.9)	2 (5.1)	

χ^2 test.

Table 19. Correlation of mean staining score of PDGFR-α expression and clinico-pathological parameters of CCRCC

Parameters	PDGFR-α expression	
	Mean ± SD*	p-value
Sarcomatoid feature		
Absent	5.42±1.46	0.243
Present	6.17±1.83	
Tumor necrosis		
Absent	5.33±1.47	0.053
Present	5.96±1.59	
Rhabdoid feature		
Absent	5.41±1.43	0.156
Present	6.10±1.91	
Fuhrman nuclear grade		
Low (1+2)	5.25±1.39	0.066
High (3+4)	5.71±1.57	
Perirenal fat invasion		
Absent	5.51±1.43	0.335
Present	5.13±1.82	
Renal pelvis invasion		
Absent	5.43±1.48	0.371
Present	5.89±1.56	
Vascular invasion		
Absent	5.46±1.47	0.933
Present	5.43±1.65	
Renal sinus fat invasion		
Absent	5.48±1.46	0.481
Present	5.00±2.12	
Cystic change		
Present	5.58±1.57	0.151
Absent	5.15±1.39	
Pathologic T stage		
1	5.51±1.38	0.546
2-4	5.33±1.74	
TNM stage		
I	5.51±1.40	0.610
II-IV	5.36±1.68	

Student's *t*-test. *SD; standard deviation.

C. Prognostic Significance and Clinical Outcome of PDGFR-α in CCRCC Patients

Survival analysis was performed after normalizing the following parameters: sex, age, Fuhrman nuclear grade, and pathologic T stage. There was no significant difference between PDGFR-α positive group and PDGFR-α negative group in both survival (p=0.990) and recurrence rate (p=0.993) (Fig. 18A and B).

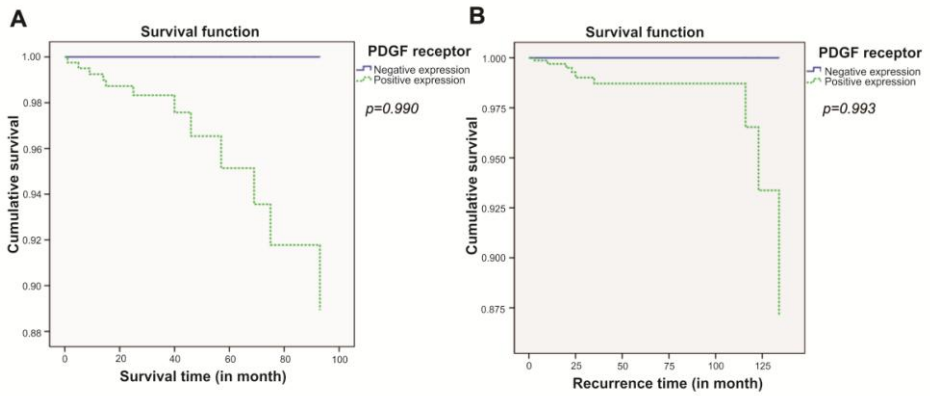

Fig. 18. Association between PDGFR-α expression level and clinical outcome in CCRCC. Survival analysis shows no significant difference between the PDGFR-α positive group and PDGFR-α negative group in both survival (A) and recurrence rate (B). Survival analysis was determined using the Cox regression method.

3.1.9. Epidermal Growth Factor Receptor (EGFR) Expression

A. Pattern and Distribution of EGFR Expression in Non-tumor and Tumor Tissues

IHC staining showed that EGFR was expressed in the cytoplasm and/or membrane of proximal tubular epithelium in the normal renal parenchyma (Fig. 19A). EGFR expression was observed in 107(98.4%) cases of CCRCC, showing positivity in the cytoplasm and/or membrane of tumor cells (Fig. 19B).

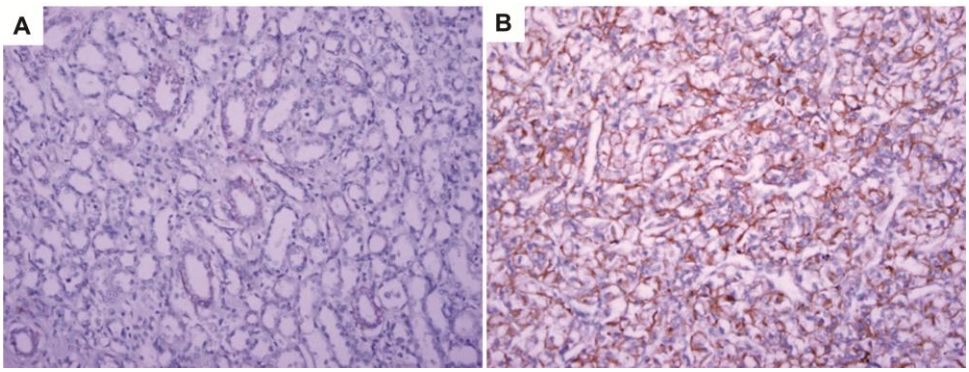

Fig. 19. Pattern and distribution of EGFR expression in non-tumor and tumor tissue. IHC staining shows EGFR was expressed in the cytoplasm and/or membrane in normal tubular epithelium (A) and in tumor cells (B).

B. Correlation of EGFR and Prognostic Factors of CCRCC

EGFR was positive 84.5% of cases with a low (1+2) Fuhrman nuclear grade and 88.7% of cases with a high (3+4) Fuhrman nuclear grade, which was not statistically significant. However EGFR expression seemed to be higher in cases with sarcomatoid or rhabdoid features, tumor necrosis, cystic change, renal pelvis and renal sinus fat invasions and lower in cases with vascular invasion, these differences were not statistically significant. For the pathologic T stage, EGFR was expressed in 85.4% of cases with pathologic T stage 1 and 88.6% of cases with pathologic T stage 2-4, but it was not statistically significant. Furthermore, EGFR was expressed in 87.2% of cases with TNM stage I and 84.2% of cases with TNM stages II-IV, which was not a statistically significant difference. These findings are detailed in Table 20.

The mean staining score of EGFR was 5.32±2.53 in cases without rhabdoid feature and 7.04±1.26 in cases with rhabdoid feature, which was a statistically significant difference (*p=0.012*). Moreover, the mean staining score of EGFR was 5.04±2.39 in cases with low Fuhrman nuclear grade and 6.09±2.56 in cases with high Fuhrman nuclear grade, which was again statistically significant (*p=0.020*). However, the mean staining score of EGFR seemed to be higher in cases with sarcomatoid feature, tumor necrosis, renal pelvis invasion and cystic change and lower in cases with vascular invasion and renal sinus fat invasions, these differences were not statistically significant. The mean staining

score was 5.46±2.52 in cases with pathologic T stage 1 and 5.57±2.52 in cases with stages 2-4. In addition, the mean staining score was 5.55±2.46 in cases with TNM stage I and 5.37±2.65 in cases with TNM stages II-IV, which was not statistically significant. These findings are summarized in Table 21.

Table 20. Correlation of EGFR expression and clinico-pathological parameters of CCRCC

Parameters	EGFR expression		p-value
	No. of positive cases (%)	No. of negative cases (%)	
Sarcomatoid feature			
Absent	102 (85.7)	17 (14.3)	0.472
Present	5 (100.0)	0 (0.0)	
Tumor necrosis			
Absent	85 (85.9)	14 (14.1)	0.538
Present	22 (88.0)	3 (12.0)	
Rhabdoid feature			
Absent	97 (85.1)	17 (14.9)	0.215
Present	10 (100.0)	0 (0.0)	
Fuhrman nuclear grade			
Low (1+2)	60 (84.5)	11 (15.5)	0.346
High (3+4)	47 (88.7)	6 (11.3)	
Renal pelvis invasion			
Absent	99 (86.1)	16 (13.9)	0.643
Present	8 (88.9)	1 (11.1)	
Vascular invasion			
Absent	96 (86.5)	15 (13.5)	0.561
Present	11 (84.6)	2 (15.4)	
Renal sinus fat invasion			
Absent	102 (85.7)	17 (14.3)	0.472
Present	5 (100.0)	0 (0.0)	
Cystic change			
Present	31 (93.9)	2 (6.1)	0.112
Absent	76 (83.5)	15 (16.5)	
Pathologic T stage			
1	76 (85.4)	13 (14.6)	0.444
2-4	31 (88.6)	4 (11.4)	
TNM stage			
I	75 (87.2)	11 (12.8)	0.425
II-IV	32 (84.2)	6 (15.8)	

χ^2 test.

Table 21. Correlation of mean staining score of EGFR expression and clinico-pathological parameters of CCRCC

Parameters	EGFR expression	
	Mean ± SD*	p-value
Sarcomatoid feature		
Absent	5.46±2.53	0.522
Present	6.20±2.05	
Tumor necrosis		
Absent	5.34±2.47	0.192
Present	6.08±2.64	
Rhabdoid feature		
Absent	5.32±2.53	0.012
Present	7.40±1.26	
Fuhrman nuclear grade		
Low (1+2)	5.04±2.39	0.020
High (3+4)	6.09±2.56	
Renal pelvis invasion		
Absent	5.47±2.52	0.725
Present	5.78±2.59	
Vascular invasion		
Absent	5.50±2.47	0.872
Present	5.38±2.93	
Renal sinus fat invasion		
Absent	5.45±2.54	0.412
Present	6.40±1.67	
Cystic change		
Present	5.67±1.96	0.643
Absent	5.43±2.69	
Pathologic T stage		
1	5.46±2.52	0.826
2-4	5.57±2.52	
TNM stage		
I	5.55±2.46	0.718
II-IV	5.37±2.65	

Student's *t*-test. *SD; standard deviation.

C. Prognostic Significance and Clinical Outcome of EGFR in CCRCC Patients

Survival analysis was performed after normalizing the following parameters: sex, age, Fuhrman nuclear grade, and pathologic T stage. There was no significant difference between EGFR positive group and EGFR negative group in both survival (p=0.978) and recurrence rate (p=0.550) (Fig. 20A and B).

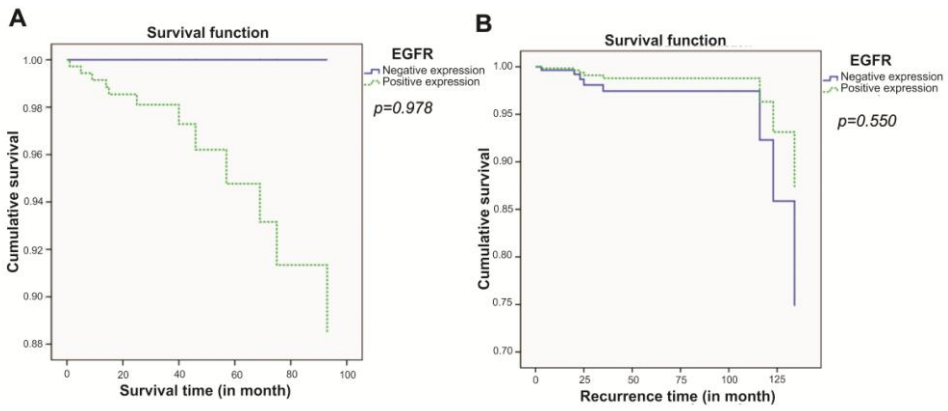

Fig. 20. Association between EGFR expression level and clinical outcome in CCRCC. Survival analysis shows no significant difference between the EGFR positive and EGFR negative groups in both survival (A) and recurrence rate (B). Survival analysis was determined using the Cox regression method.

3.2. Correlation between the Expression of Klotho and GFRs

There was significant linear correlation between the expression of Klotho and IR, IGF-1, IGF-1R, VEGF, VEGFR-1, and PDGFR-α, respectively (p=0.000, p=0.000, p=0.010, p=0.000, p=0.000, & p=0.000). Although, there was no significant linear correlation between the expression of Klotho and PDGF or EGFR, respectively (p=0.379 & p=0.086).

3.3. Western Blot Analysis for Klotho and GFRs in Clinical Samples

3.3.1. Klotho Expression

Klotho has been known to be an aging-suppressor gene and predominantly expressed in distal convoluted tubules of kidney.[43, 44] Here we profiled Klotho expression in paired fresh tissues, normal tissue against tumor tissue from same patients (Fig. 21A). Compared with that of non-tumor tissue, the expression level of Klotho was higher in tumor tissue (Fig. 21B). However, there was no significant difference between the expression levels of Klotho in low and high grade tumors (Fig. 21C). On the other hand, it did not support our IHC staining results, which showed high Klotho expression was significantly correlated with low grade tumors.

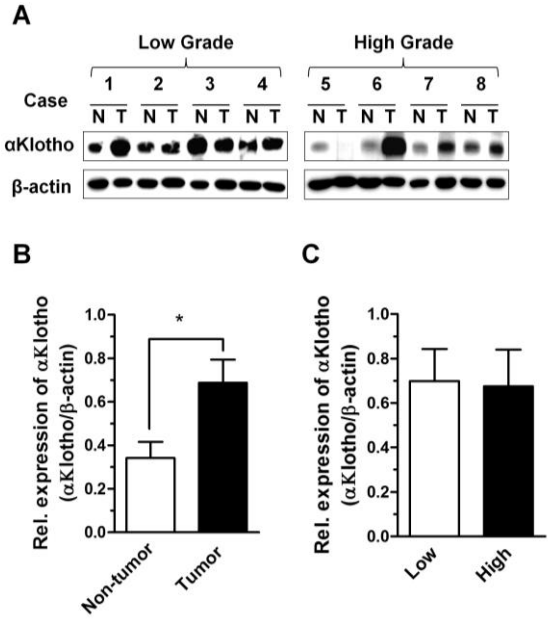

Fig. 21. Klotho expression in paired fresh tissues. (A) Representative immunoblotting of αKlotho. Expression level of αKlotho in paired tissues of normal tissue (N) and tumor tissue (T), including low and high grades, were analyzed with immunoblotting. β-actin served as a protein loading control. (B) Relative (Rel.) expression of αKlotho in non-tumor and tumor tissues. (mean±SEM, n=18). Asterisk denotes $p<0.05$ non-tumor versus tumor. (C) Relative (Rel.) expression of αKlotho in low and high grade tumor tissues.

3.3.2. IGF-1R Expression

IGF-1 signaling plays a major role in cancer cell proliferation and survival. Moreover, IGF-1R is overexpressed in most types of cancer including CCRCC.[60] Therefore, we checked the expression level of IGF-1 in paired fresh tissues, normal tissue against tumor tissue from same patients (Fig. 22A). We found a significant elevation of IGF-1R in tumor tissue than non-tumor tissue (Fig. 22B). In addition, the expression level of IGF-1R was higher in high grade tumors than low grade tumors (Fig. 22C). From these results, we believe that IFG-1R expression in CCRCC is related to poor prognosis, similar to our IHC staining results.

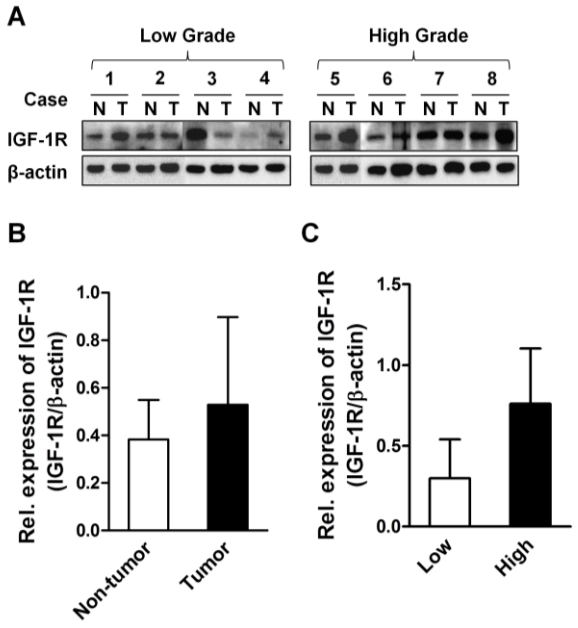

Fig. 22. IGF-1R expression in paired fresh tissues. (A) Representative immunoblotting of IGF-1R. Expression level of IGF-1R in paired tissues of normal tissue (N) and tumor tissue (T), including low and high grades, were analyzed with immunoblotting. β-actin served as a protein loading control. (B) Relative (Rel.) expression of IGF-1R in non-tumor and tumor tissues. (mean±SEM, n=18). Asterisk denotes $p<0.05$ non-tumor versus tumor. (C) Relative (Rel.) expression of IGF-1R in low and high grade tumor tissues.

3.3.3. Insulin Receptor (IR) Expression

IGF-1R expression in CCRCC was associated with poor prognosis. IR and IGF-1R share down-stream signaling cascades.[20] Those observations prompted us to carry out to examine the IR expression in non-tumor renal tissues and CCRCC tissues. The IR was expressed in both carcinoma and normal renal parenchymal tissues (Fig. 23A). There was no difference of total protein amount of IR between non-tumor and tumor tissues in fresh samples (Fig. 23B). Compared with that of high grade tumors, the expression level of IR was slightly elevated in low grade tumors (Fig. 23C). This result may suggest that IR protein was inversely correlated with Fuhrman nuclear grade of CCRCC, which is identical to IHC staining results.

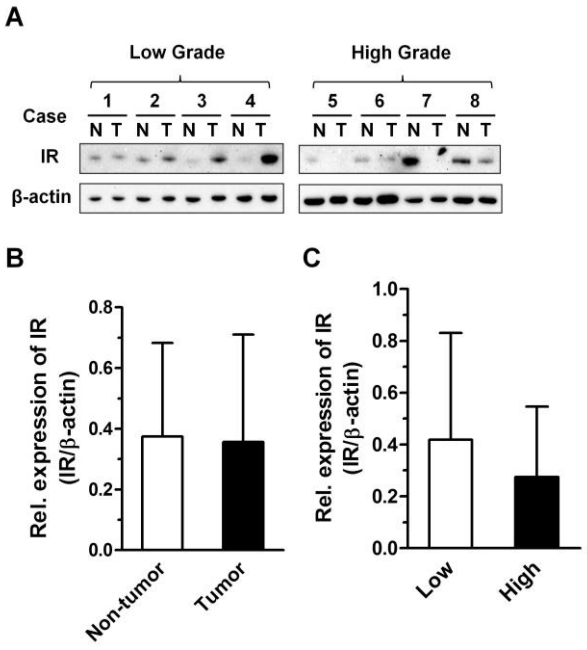

Fig. 23. Insulin receptor (IR) expression in paired fresh tissues. (A) Representative immunoblotting of IR. Expression level of IR in paired tissues of normal tissue (N) and tumor tissue (T), including low and high grades, were analyzed with immunoblotting. β-actin served as a protein loading control. (B) Relative (Rel.) expression of IR in non-tumor and tumor tissues. (C) Relative (Rel.) expression of IR in low and high grade tumor tissues.

3.3.4. VEGFR-1 Expression

Increasing evidence suggests that VEGF/VEGFR-1 signaling is crucial for angiogenesis of CCRCC, which consists of highly vascularized malignant tumors.[61] We checked expression level of VEGFR-1 in paired fresh tissues, normal tissue against tumor tissue from same patients (Fig. 24A). The expression level of VEGFR-1 was significantly elevated in non-tumor tissues compared to tumor tissues (Fig. 24B). In addition, the expression level of VEGFR-1 was slightly higher in low grade tumors than high grade tumors (Fig. 24C). These results support that VEGFR-1 expression in CCRCC is related to favorable prognosis, similar to IHC staining results.

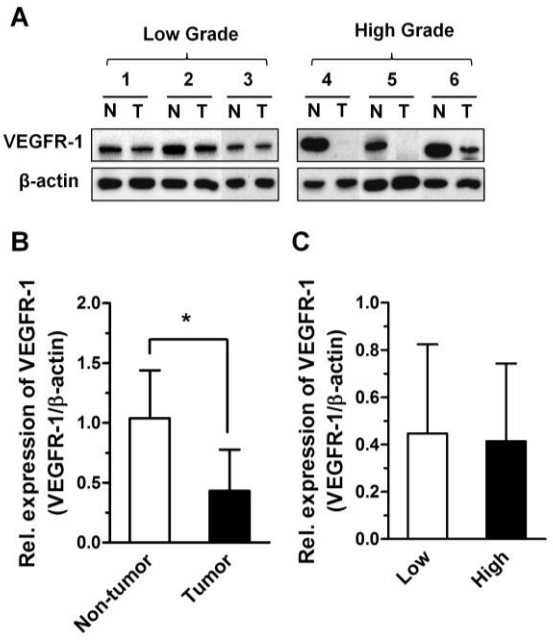

Fig. 24. VEGFR-1 expression in paired fresh tissues. (A) Representative immunoblotting of VEGFR-1. Expression level of VEGFR-1 in paired tissues of normal tissue (N) and tumor tissue (T), including low and high grades, were analyzed with immunoblotting. β-actin served as a protein loading control. (B) Relative (Rel.) expression of VEGFR-1 in non-tumor and tumor tissues. (mean±SEM, n=18). Asterisk denotes $p<0.01$ non-tumor versus tumor. (C) Relative (Rel.) expression of VEGFR-1 in low and high grade tumor tissues.

3.4. Molecular Mechanism Explaining the Crosstalk between Klotho and GFRs in Pathogenesis of CCRCC

Klotho inhibits activation of IGF-1 and EGF induced pathways.[50, 62] Klotho overexpression in breast cancer cell line was associated with reduced phosphorylation of not only IGF-1R, but also its downstream targets.[50] Therefore, we studied the effect of Klotho on insulin, IGF-1, EGF, and PDGF pathways in two RCC cell lines (ACHN and Caki1).

First, IGF-1 stimulation enhanced the phosphorylation of AKT at Thr308 and Ser473 (Fig. 25A-C). However, Klotho stimulation reduced IGF-1 induced activation of AKT, especially at Ser473 in Caki1 cell line (Fig. 25A-C). On the other hand, Klotho does not have significant effect on ERK1/2 pathway (Fig. 25D and E).

Second, we studied the effect of Klotho on insulin induced downstream pathways. Only minor effects of Klotho were noted on insulin induced activation of AKT and ERK1/2 pathways (Fig. 26A-E).

Third, we examined the crosstalk between Klotho and EGF & PDGF. EGF stimulation enhanced the activation of AKT (Fig. 27A-C). However, Klotho expression slightly reduced EGF induced phosphorylation of AKT at Thr308 and Ser473 (Fig. 27A-C). There was no difference before and after treatment with Klotho on EGF induced ERK activation (Fig. 27D and E). Lastly, according to our results, PDGF did not enhance the activation of AKT and ERK

pathways. Besides, there was no significant difference before and after treatment with Klotho on PDGF induced AKT and ERK1/2 activation.

Wortmannin (WMN) and 2-(4-morpholinyl)-8-phenylchromone (LY294002) has been known as PI3K inhibitors.[63] Moreover, PI3K inhibition by WMN and LY294002 eliminate insulin and IGF-1 induced phosphorylation of AKT (Fig. 28A and B). These findings support that insulin and IGF-1 share same downstream signaling, such as PI3K pathway. The inhibitory effect of Klotho on downstream signaling pathways of IGF-1 and EGF might explain the tumor suppressive role of Klotho.

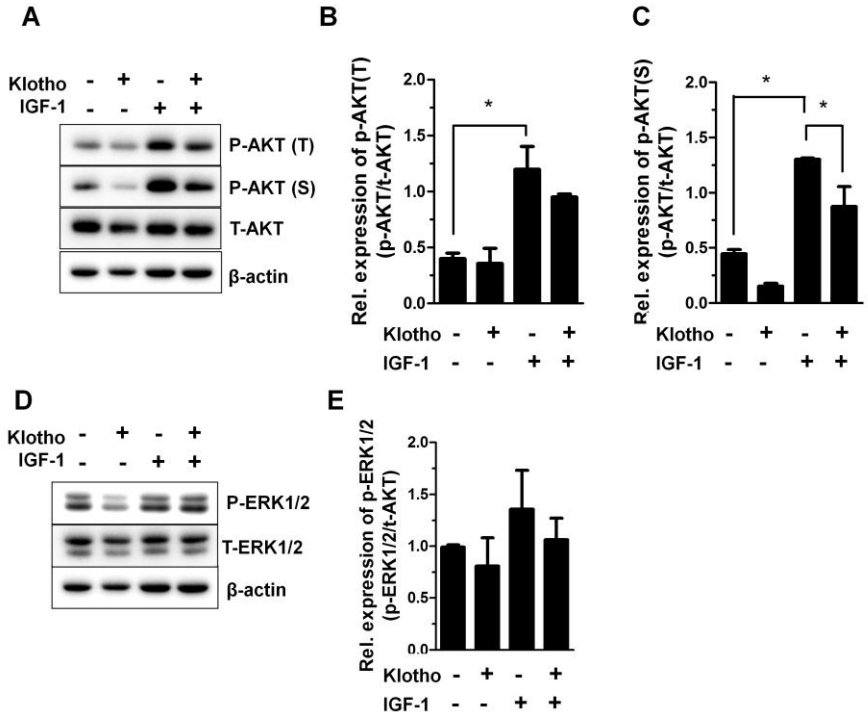

Fig. 25. Klotho inhibits IGF-1 induced AKT activation in Caki1 cell line. Cells were starved for 48 hours in serum-free medium and then treated with Klotho (500 pM). After 50 minutes, the cells were treated with IGF-1 (10 nM) for 10 minutes. Following treatment, cells were harvested and western blot analysis was used to check expression of indicated proteins. Asterisk denotes $p<0.05$. β-actin served as a protein loading control.

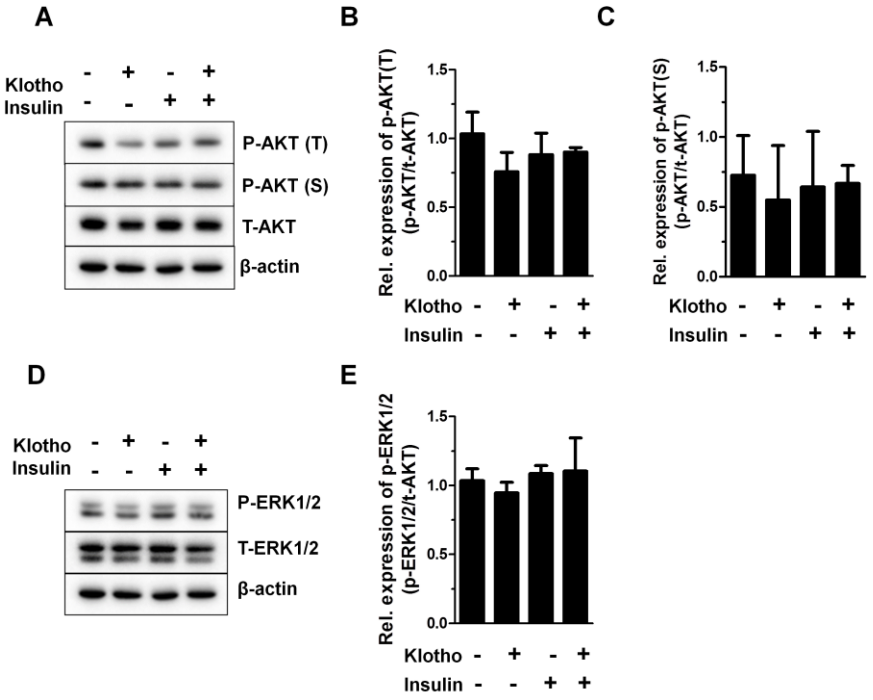

Fig. 26. Effect of Klotho on insulin induced downstream pathways in Caki1 cell line. Cells were starved for 48 hours in serum-free medium and then treated with Klotho (500 pM). After 50 minutes, the cells were treated with insulin (10 nM) for 10 minutes. Following treatment, cells were harvested and western blot analysis was used to check expression of indicated proteins. β-actin served as a protein loading control.

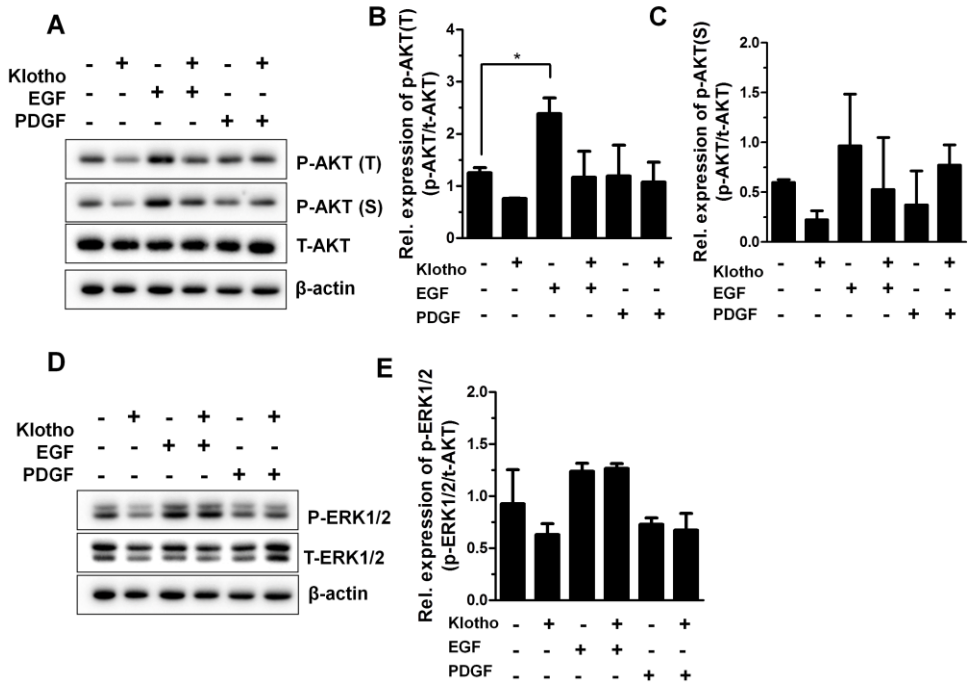

Fig. 27. Klotho inhibits EGF induced AKT activation in Caki1 cell line. Cells were starved for 48 hours in serum-free medium and then treated with Klotho (500 pM). After 50 minutes, the cells were treated with EGF (60 ng/ml) and PDGF (20 ng/ml) for 10 minutes. Following treatment, cells were harvested and western blot analysis was used to check expression of indicated proteins. Asterisk denotes $p<0.05$. β-actin served as a protein loading control.

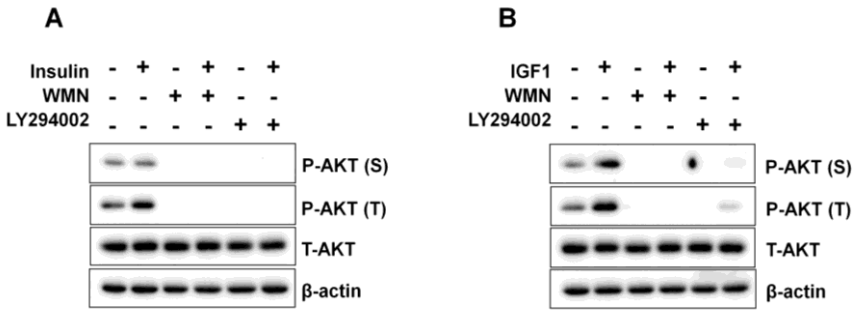

Fig. 28. Effect of PI3K inhibitors on insulin and IGF-1 downstream signaling. Cells were starved for 48 hours in serum-free and then treated with WNM (200 nM) and LY294002 (50 uM), respectively. After 50 minutes, the cells were treated with insulin (10 nM) and IGF-1 (10 nM) for 10 minutes. Following treatment, cells were harvested and western blot analysis was used to check expression of indicated proteins. β-actin served as a protein loading control.

IV. DISCUSSION

We found that the expression of IR, IGF-1, and IGF-1R were present in 87.9%, 59.5%, and 96.0% of CCRCC by IHC staining, respectively. Higher IR and IGF-1 expression was related to the favorable prognostic factors of CCRCC. In contrast, higher IGF-1R expression was related to the unfavorable prognostic factors of CCRCC. Additionally, the results of western blot analyses showed that the expression of IR was higher in low-grade CCRCC than in high-grade tumors, which was supported by IHC staining results. Also, western blot analyses showed that the expression of IGF-1R was higher in tumor tissue than non-tumor tissue, which was also supported IHC staining. In this study, our survival analysis of IR, IGF-1, and IGF-1R did not show any significant results.

IR exists in two isoforms, A and B, which are formed due to exclusion (isoform A) or inclusion (isoform B) of exon 11 of the IR gene.[19] IR-A is ubiquitously expressed, whereas IR-B is expressed largely in the classically insulin-sensitive tissues, such as adipose tissue, liver, and skeletal muscle. Interestingly, IR-B is also expressed abundantly in the kidney.[64] In the current study, we demonstrated diffuse IR staining in distal tubular epithelium as well as in podocytes of glomeruli. Therefore, we can conclude that the kidney is an insulin sensitive organ. In healthy individuals, blood glucose concentration is maintained by a state of balance between insulin production by specialized pancreatic β cells and insulin-mediated glucose uptake in target tissues, which is promoted by the glucose transporter proteins, such as glucose transporter type 4

(GLUT-4), to the cell surface.[65] Insulin resistance in classic insulin-target organs and the related hyperglycemia and hyperinsulinemia are pathologic hallmarks of metabolic disorders such as obesity and type 2 diabetes.[66] Several studies have reported an association between type 2 diabetes and an increase in the risk of developing various human malignancies, including liver, pancreas, bladder, breast, and colon cancers.[67-72] However, the association between RCC, and type 2 diabetes and/or obesity remains to be understood. According to a meta-analysis on nine cohort studies by Larsson et al.,[68] diabetes is associated with a 42% increased risk of kidney cancer. This association was found to be stronger in women than in men. In contrast, a study by Höfner et al.[73] showed that obesity and type 2 diabetes have no significant effect on cancer-specific and recurrence-free survival in RCC patients who had undergone nephrectomy for localized RCC. In the present study, IR, IGF-1, and IGF-1R expression were not related to the diabetes status of patients with CCRCC supporting the notion that type 2 diabetes and/or obesity may not be associated with CCRCC development.

Insulin/IGF-1 pathway plays a crucial role in most cancer cell development and the overexpression of IGF-1R is one of the important factors of cancer hallmarks. Several studies reported that high IGF-1R expression is associated with better survival in malignancies of breast, lung and soft tissue.[74-76] On the other hand, patients with IGF-1R positive CCRCC had poorer outcomes than patients with IGF-1R negative CCRCC.[22, 77] Our results were also similar; showing the expression of IGF-1R associated with unfavorable prognostic factors,

which was adverse to expression of IR and IGF-1. Reasons for these discrepancies between IGF-1R and IR are not well known. Indeed, although both IR and IGF-1R share major down-stream signaling pathways, there are several specific substrates for each receptor. For instance, pp120, a plasma membrane glycoprotein expressed by hepatocytes and a substrate of the IR tyrosine kinase, mediates the phosphorylation of IR but not the phosphorylation of IGF-1.[78] Similarly, mitotic arrest deficient 2 (MAD2), a cell cycle checkpoint regulator, binds to the C-terminal domain of IR but does not bind to the homologous region in IGF-1R.[79] It is also conceivable that IR and IGF-1R differently regulates downstream targets such as VHL acting on HIF-1α. Hereditary RCC is commonly associated with mutational inactivation of *VHL* gene which plays an important role in tumor growth.[80] VHL protein is E3-ubiquitin ligase and functions as a tumor suppressor by inhibiting HIF-1α which is activated by hypoxia. VHL-mediated HIF-1α regulation is a major pathway involved in RCC biology and tumorigenesis.[16] IGF-1R activates HIF-1α independent of oxygen status by suppressing VHL that induces RCC development. Interestingly, IR signaling and hypoxia share common target genes, but HIF-1α is unique to hypoxia.[81] Whether the regulation of VHL-HIF-1α pathway by IR and IGF-1R has any role in the CCRCC biology and tumorigenesis awaits future investigation. Moreover, dissecting of IGF-1R and IR signaling cascades in RCC may provide clues for treatment or prognosis of CCRCC.

Interestingly, IR and IGF-1R were expressed positive in the nucleus contrary to our expectations. It has been reported that nuclear IGF-1R is detectable in primary RCC cultures, as well as in FFPE tissue from RCC and that this nuclear IGF-1R is associated with an adverse prognosis for CCRCC.[82] Indeed, it has been shown that full-length IGF-1R translocates into the nucleus following activation by its ligands,[82] and SUMOylation mediates this nuclear translocation of IGF-1R.[83] IR can also be translocated to the nucleus to regulate cell proliferation as well as IGF-1R.[84]

We found that VEGF and VEGFR-1 expression were identified in the membrane and/or cytoplasm in 55.6% and 46.8% cases of CCRCC, respectively. Higher VEGFR-1 expression was significantly related to a lower Fuhrman nuclear grade and the absence of renal pelvis invasion. In addition, Western blot analyses showed that expression of VEGFR-1 was significantly higher in adjacent normal tissue than in CCRCC tissue. However, there was no significant different between high and low grade tumors. Therefore, we suggest that high VEGFR-1 expression may be associated with tumorigenesis of CCRCC, although the survival analysis data were not statistically significant.

RCC is a malignant tumor that is characterized by high tumor vascularity and VEGF is the most important angiogenic factor. The importance of VEGF and VEGFR-1 in regulating tumor angiogenesis in CCRCC has been reported previously.[29, 30] One study suggests that knockdown of VEGFR-1 impairs growth

of CCRCC.[61] Ljungberg et al.[30] found that the *VEGF*, *VEGFR-1,* and *VEGFR-2 mRNA* levels were higher in tumors compared to the normal kidney cortex, which is contrary to our results. However, it has been suggested that VEGFR-1 may not be the primary receptor transmitting a mitogenic signal, but rather it is a 'decoy' receptor, able to negatively regulate the activity of VEGF on the vascular endothelium, preventing VEGF from binding to VEGFR-2.[85] The functions and signaling properties of VEGFR-1 can be different depending on the developmental stage of the animal and the cell type.[25]

HIF-1α induces transcription of several factors such as VEGF/VEGFR.[86] Overexpression of HIF-1α is associated with poor prognosis of cervical and breast cancers.[87, 88] In contrast, elevated HIF-1α expression is correlated with better survival in patients with CCRCC, although no association with tumor stage was found.[89] Furthermore, higher *VEGF mRNA* levels are associated with a better prognosis in CCRCC.[30] Similarly, our present study showed that higher VEGFR-1 expression may be correlated with favorable prognostic factors for CCRCC, including the Fuhrman nuclear grading, which showed significant correlation. Further study is required to understand the underlying mechanism of VEGF/ VEGFR-1 signaling pathways in CCRCC.

We found that EGFR was expressed in the membrane and/or cytoplasm in 86.2% cases of CCRCC. Higher EGFR expression was related to high Fuhrman nuclear grade and the majority of unfavorable prognostic factors. Our

results were also similar with previous studies showing higher expression of membranous EGFR frequently detected and had a poorer outcome in cancer cells.[33, 90] However, the prognostic significance of EGFR in RCC remains controversial. Some studies shows that EGFR expression is associated with well differentiated RCC,[91] or regarded strong membranous EGFR IHC staining as an indicator of good prognosis,[92] whereas others showed EGFR expression is associated with high tumor stage/grade and poor prognosis,[93] which was similar with our study, or no significant association.[94] The relatively low expression of EGFR in normal kidney tissue supports the involvement of this biomarker in pathways of carcinogenesis.[90] Recently, EGFR is a well-known novel target therapy for several kinds of malignant tumors. Therefore, anticancer therapies targeting EGFR pathway have shown promising results in clinical trials of RCC.[95]

We found that PDGF and PDGFR-α expression were detected in the membrane and/or cytoplasm in 77.8% and 96.8% cases of CCRCC, respectively. High PDGF expression was related to the most of favorable prognostic factors of CCRCC, which is contrary to previous study.[35] Besides, PDGF did not enhance the activation of AKT and ERK pathways in renal cancer cell lines. Therefore, further study is required to understand the prognostic significance and regulatory mechanism of PDGF and its receptors in RCC.

Klotho exerts multiple functions on the kidney, which includes regulation of vitamin D3 production and modulation of urinary phosphate, calcium, and potassium excretion. Moreover understanding of renal and extrarenal function of Klotho will give novel strategies for both diagnosis and treatment of acute and chronic kidney disease.[96] Therefore, we checked the expression of Klotho in CCRCC. In this study, Klotho was expressed positively in the cytoplasm and/or membrane, using IHC staining, in both tumor and non-tumor. As we know Klotho is abundantly expressed in the distal convoluted tubules of kidney. Higher Klotho expression was significantly related to the presence of cystic change, absence of tumor necrosis and vascular invasion, lower Fuhrman nuclear grade, lower pathologic T stage, and lower TNM stage by IHC staining. Intratumoral Klotho levels negatively correlated with tumor size, TNM stage and nuclear grade in RCC,[54] these findings are similar to our study. However, western blot analysis showed that higher expression of Klotho was noted in tumor tissue than non-tumor renal tissue. This discrepancy may be explained by the fact that we studied a relatively lower number of cases (18 cases) for western blot analysis compared to the number (126 cases) used for IHC staining. Therefore, we suggest that high Klotho expression may be associated with the favorable prognostic factors of CCRCC.

Klotho is reported to have tumor suppressive features during various malignant transformations. At first, Klotho was considered a tumor suppressor and a modulator, inhibiting insulin and IGF-1 pathways and activating fibroblast

growth factor (FGF) pathway in human breast cancer.[50] This inhibitory effect of Klotho on insulin/IGF-1 pathways reported in human lung cancer cell line A549, also involved regulating the expression of the apoptosis-related gene bax/bcl-2.[53] Moreover, patients with Klotho expression had a significantly better survival rate than Klotho negative patients. Yu Zhu et al.[54] concluded that Klotho acts a tumor suppressor by inhibiting PI3K/AKT/GSK3β/Snail signaling in RCC. However, molecular mechanisms for Klotho mediated PI3K/AKT inhibition during RCC development remains to be elucidated. Therefore, we expected that tumor suppressive role of Klotho may be inhibit GFRs induced PI3K/AKT signaling in RCC. As we have described above Klotho is considered a tumor suppressor and a modulator, regulating IGF-1 and FGF pathways in breast, lung, and pancreatic cancers.[50, 51, 53] The AKT and ERK cascades are downstream of several signaling pathways, including insulin, IGF-1, and EGF pathways. In our study, we observed a significant reduction of IGF-1 induced AKT phoshorylation after Klotho treatment in Caki1 cell line, which is similar to previous studies. However, there was no effect on insulin induced AKT activation before and after treatment with soluble Klotho in renal cancer cell lines, which is different from previous studies in breast cancer.[50] This variation may be explained by that the functions and signaling properties of IR can be different depending on an organ and tumor specificity. In addition, IHC staining and western blot analysis support that the difference between the IR and IGF-1R expression in clinical outcome and tumorigenesis of RCC.

Klotho gene is activated by EGF through the ERK signaling pathway in HEK293 human embryonic kidney cells.[62] In this study, western blot analysis shows no correlation between Klotho and EGF mediated ERK signaling pathways in renal cancer cell line. Interestingly, we observed that Klotho expression slightly reduced EGF induced activation of AKT signaling pathway in Caki1 cell line. Therefore, our observations indicate Klotho a tumor suppressor in RCC, by regulating activation of the IGF-1 and EGF pathways in RCC.

However, the prognostic significance of Klotho in malignancy is still controversial, probably depending on the origin of the tumor. Functional loss of Klotho due to epigenetic silencing in late stage of cervical cancer may induce atypical activation of the canonical Wnt pathway in uterine cervical carcinogenesis.[97] The loss of Klotho expression in more metastatic and higher Wnt5a, a number of the non-canonical Wnt pathway, expressing melanoma suggested that Klotho and Wnt pathway exist in a regulatory feedback loop.[98] It is suggested that Klotho is as novel therapeutic intervention for pancreatic cancer as soluble Klotho reduced the growth of pancreatic cancer cells *in vitro* and *in vivo*.[51] In contrast high expression of secreted Klotho was associated with increased risk of epithelial ovarian cancer progression and death.[99] Recently, it has been postulated that Klotho expression confers hepatoma cells with resistance to apoptosis via activation of VEGFR2/RAK1 signaling, resulting to tumor aggressiveness and poor overall survival in hepatoma patients. This shows a novel oncogenic function of Klotho in hepatocarcinogenesis.[100]

Several mechanisms may govern Klotho growth-inhibitory activities. Mechanisms other than inhibition of IGF-1 and EGF pathways may mediate the growth-inhibitory activities of Klotho in CCRCC. One of the positive mechanisms is inhibition of the bFGF pathway. Klotho is an inhibitor of the bFGF pathway in HEK293 cells[50, 101] and in pancreatic cancer cells.[51] Moreover, the modulation of the bFGF pathway by Klotho has also been shown in breast and hepatic cancers,[50, 102] indicating bFGF as an important mediator of Klotho activities.

Klotho can also regulate calcium channels, including transient receptor potential cation channel subfamily V member 5 (TRPV5) through modifying their glycans.[103] TRPV5 expressed distal nephron to mediate renal calcium ion reabsorption.[104] An elevation of intracellular calcium influx is known to be essential for regulating distinct processes involving exocytosis, enzyme activation, gene transcription, cell growth, cell proliferation, and apoptosis.[105] Orai1, a pore-forming subunit of store-operated Ca2+ entry, is highly expressed in CCRCC tissues suggesting that Orai1 is involved in RCC development.[106] Thus, coexpression of Klotho and TRPV5 or Orai1 may hide possible mechanism which regulates tumorigenesis of RCC.

Another possible mechanism is the effect of Klotho on HIF-α induced tumorigenesis of RCC. RCC lacks functional VHL protein that leads to increased HIF-α expression. Both pVHL and HIF-α are important for RCC tumorigenesis.

Therefore, the effect of Klotho on HIF-α or VHL induced tumorigenesis of RCC needs further study. Overexpression of HIF-α was associated with an unfavorable prognosis has been detected in several human malignancies, including cervix and breast cancer.[87, 88] Therefore, additional studies are required to explore the underlying mechanism of Klotho as a tumor suppressor in RCC.

In summary, this study identified Klotho as a potential tumor suppressor and growth inhibitor and modulator of IGF-1 and EGF pathways in CCRCC. The role of Klotho as a novel therapeutic approach for RCC treatment, as well as other malignant diseases, should be explored.

V. CONCLUSION

Klotho expression was detected in both non-tumor renal parenchymal tissues and tumor tissues. Klotho protein was expressed in the cytoplasm and/or membrane of adjacent normal parenchymal tissues, including podocytes of glomeruli, tubular epithelium, endothelial cells of blood vessels, and lymphocytes. Klotho expression was observed in the cytoplasm and/or membrane in 107 out of 126 (84.9%) cases of CCRCC. The expression of Klotho was significantly correlated with favorable prognostic factors, including low Fuhrman nuclear grade and low pathologic and TNM stages.

GFRs were detected in both non-tumor renal parenchymal tissues and tumor tissues. The expression of IR, IGF-1, VEGFR-1, and PDGF are related with favorable prognostic factors of CCRCC. In contrast, the expression of IGF-1R and EGFR are related with unfavorable prognostic factors of CCRCC. There was a significant linear correlation between the expression of Klotho and the most of GFRs in CCRCC.

In this study, Klotho is a tumor suppressor and modulator of IGF-1 and EGF pathways in RCC. Klotho seems to be a potent therapeutic agent against mRCC and other forms of malignancies; however, more studies need to be done to explore the regulatory mechanism through which Klotho exerts its effects. Klotho function in the pathogenesis of other malignant diseases should be explored.

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VII. ABSTRACT IN KOREAN

사람 콩팥세포암종에서 Klotho 에 의한 성장인자 수용체 조절

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연구배경: 노화 억제 유전자로 알려진 Klotho 는 주로 신장의 세뇨관에서 발현된다. Klotho 발현은 흑색종, 콩팥세포암종, 유방암 및 폐암의 예후와 관련이 있으며, 유방암에서 성장인자 수용체(Growth Factor Receptors, GFRs)의 신호전달을 조절하여 종양을 억제하는 것으로 보고되었다. 투명세포 콩팥세포암종(Clear Cell Renal Cell Carcinoma, CCRCC)은 Klotho 가 발현되는 세뇨관 상피세포로부터 유래되며 가장 흔한 콩팥의

악성 종양이다. 그러나 콩팥암종의 종양발생에 있어 Klotho 의 발현과 기능은 명확하지 않다. 따라서 본 연구에서는 콩팥세포암종에서 Klotho 및 성장인자 수용체의 발현과 그 예후에 대한 중요성을 밝히고, 콩팥세포암종의 종양발생에 있어 Klotho 와 성장인자 수용체간의 분자생물학적 기전을 밝히고자 하였다.

재료 및 방법: Klotho 와 성장인자 수용체의 발현을 알아보기 위해 126 예의 포르말린-고정 파라핀-포매(formalin-fixed paraffin-embedded, FFPE) 콩팥세포암종 조직 샘플에서 면역조직화학염색을 시행하였고, 그 중 18 예의 신선 조직과 콩팥암 세포주 (ACHN, Caki-1)에서 웨스턴 블롯을 시행하였다. 실험결과를 환자의 생존률과 CCRCC 의 다양한 임상병리학적 예후인자와 비교하였다.

결과: Klotho 발현이 높은 경우가 CCRCC 의 좋은 예후인자와 상관 관계가 있었고, Klotho 가 발현되는 환자는 그렇지 않은 환자에 비해 생존률이 높았다. 성장인자 수용체 중에서 인슐린수용체와 VEGF 수용체의 발현이

높을 수록 CCRCC 의 좋은 예후인자와의 상관성이 높았다. 대조적으로 IGF-1 수용체와 EGF 수용체의 발현이 높은 경우에는 CCRCC 의 좋지 않은 예후인자와 상관성이 있었다. 또한 Klotho 는 CCRCC 세포주에서 IGF-1 과 EGF 에 의해 유도된 AKT 의 활성화를 억제시켰다.

결론: Klotho 는 CCRCC 의 잠재적인 종양억제 유전자로 사료되며 이 종양억제효과는 IGF-1 과 EGF 신호전달체계를 억제함으로써 매개될 수 있다. 따라서 Klotho 는 CCRCC 발생에 중요한 역할을 하며 CCRCC 의 치료에서 중요한 표적이 될 수 있을 것으로 사료된다.

핵심 단어: 클로토, 투명세포 콩팥세포암종, 성장인자 수용체, 인슐린 유사 성장인자-1, 인슐린 수용체, 종양발생, 예후

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