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Direktor: Prof. Dr. med. Martin Reincke

A histology-based four protein array for postoperative outcome prediction in clear cell renal cell carcinoma

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Shangqing Song

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Berichterstatter : Prof. Dr. med. Hans-Joachim Anders

Mitberichterstatter : Priv. Doz. Dr. Alexander Buchner

Mitberichterstatter : Prof. Dr. Robert-Dirk Zaak

Mitbetreuung durch den promovierten Mitarbeiter: Dr. med. Marc Weidenbusch

Dekan: Prof. Dr. med. dent. Reinhard Hickel

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Declaration

I hereby declare that all of the present work embodied in this thesis was carried out by me from 11/2014 until 08/2016 under the supervision of Prof. Dr. Hans Joachim Anders, Nephrologisches Zentrum, Medizinische Klinik und Poliklinik IV, Innenstadt Klinikum der Universität München, Prof. Dr. med. Christian G. Stief, Urologische Klinik und Poliklinik, Campus Grosshadern der Universität München, and Dr.med. Marc Weidenbusch, Nephrologisches Zentrum, Medizinische Klinik und Poliklinik IV, Innenstadt Klinikum der Universität München. This work has not been submitted in part or full to any other university or institute for any degree or diploma.

Date: …… Signature: ……..

Place: Munich, Germany (Shangqing Song)

Zusammenfassung

Nierenzellkarzinome (NZKs) zählen weltweit zu den häufigen Krebsformen und sind bei Männern der neunthäufigste, bei Frauen der vierzehnhäufigste bösartige Tumor. Die klarzellige Histologie (kzNZK) ist der häufigste Subtyp und macht circa 70-75% aller Fälle aus. Die Ätiopathogenese des kzNZK ist unvollständig verstanden, gleichwohl verschiedene Risikofaktoren bekannt sind. Die Identifikation neuer klinischer und pathologischer Marker zur Diagnosestellung, Prognoseabschätzung und Therapiestratifizierung ist ein entscheidendes Ziel, um mittelfristig die inbesondere bei höheren Tumorstadien schlechten klinischen Outcomes des kzNZK zu verbessern. Interleukin-22 (IL-22) ist Mitglied der IL-10 Zytokinfamilie und wird von verschiedenen Lymphozytensubsets produziert. IL-22 spielt eine wichtige Rolle für die Homöostase mehrerer Organe und verhindert Gewebeschäden durch Invasion von Pathogenen und die vergesellschaftete Entzündungsreaktion. Allerdings spielt IL-22 auch eine Rolle in der Pathogenese verschiedener Karzinome. Die biologischen Effekte von IL-22 werden hierbei durch Bindung an den heterodimeren Transmembran-Rezeptorkomplex aus IL22RA1 und IL10R2 vermittelt. Die Rolle von IL-22 und seinem Rezeptor bei klarzelligem kzNZK ist bisher unbekannt.

In dieser Arbeit wurden daher zunächst die Effekte der IL-22- und IL22RA1-RNA-Expression auf das Gesamt (OS)- und das Tumor-freie (DFS) Überleben von kzNZK-Patienten der The Cancer Genome Atlas (TCGA)– Kohorte untersucht.

Anschließend wurden zusätzlich Gewebeproben von 60 Patienten mit nicht metastasiertem kzNZK, die an der LMU behandelt wurden, immunhistochemisch auf die Expression von IL-22, IL-22RA1, IL-22BP, Ki-67 und Caspase-3 untersucht. Diese Ergebnisse wurden, zusammen mit klinisch-pathologischen Daten, ebenfalls mit dem OS und DFS der Patienten verglichen.

Die Überexpression von IL-22 und IL22RA1 war in der TCGA-Kohorte mit einem signifikant kürzeren OS und DFS assoziiert. Immunhistochemisch konnten in der LMU-Kohorte jeweils in einem Subset der Patienten IL-22, IL-22RA1, Ki-67, and caspase-3 positive Zellen nachgewiesen werden, während die IL-22BP Färbung in allen Fällen negativ blieb. Statistische Analyzen zeigten, dass die Protein-Expression von IL-22 mit dem Fuhrman-Grad, dem initialen T-Stadium, späterem Rezidiv sowie dem Gesamtüberleben der kzNZK-Patienten korrelierten. Die Protein-Expression von IL22RA1 hingegen korrelierte mit Fuhrman-Grad, späterem Rezidiv sowie dem Gesamtüberleben. Weiterhin zeigte sich in der Kaplan-Meier Analyze ein signifikant kürzeres OS und DFS für Patienten mit Überexpression von IL-22, IL22RA1 und Ki-67, während die Überexpression von Caspase-3 nur mit einem kürzeren DFS assoziiert war. Die IL22RA1 Expression konnte darüber hinaus in einer multivariaten Cox-Regression als unabhängiger Prediktor für OS und DFS identifiziert werden.

Zusammenfassend zeigt diese Arbeit eine Rolle der IL-22/IL22RA1-Achse für die Prognoseabschätzung beim kzNZK und bestätigt zusätzlich den Wert der Ki-67 Färbung in Hinblick auf diese Fragestellung.

Summary

Renal cell carcinoma is the 9th and the 14th most common cancer in men and women worldwide, respectively. Clear cell histology is the most common subtype of renal cell carcinoma, which accounts for about 70-75% of all cases. The pathogenesis of renal cell carcinoma is not well clarified and various factors are reported to be associated with kidney cancer. Identifying novel clinical and pathological features associated with the diagnosis, prognosis and targeted therapies of ccRCC patients is an essential task. Interleukin-22 (IL-22) belongs to the IL-10 family of cytokine and is produced by several subsets of lymphocytes. IL-22 plays an important role in sustaining the integrity of several organs and it prevents injury due to invading pathogens and the subsequent inflammatory response. Moreover, IL-22 has been reported to be associated with cancers in several organs. IL-22 mediates its biological effect by binding to the heterodimeric transmembrane complex of IL-22RA1 and IL-10R2.

In the present study, the expression pattern of IL-22 and IL-22RA1 and their association with the survival of ccRCC patients from The Cancer Genome Atlas were analyzed. Then 60 tumor samples from non-metastatic ccRCC patients were selected for IL-22, IL-22RA1, IL-22BP, Ki-67, Caspase-3 immunohistochemical staining. Afterwards, staining evaluation was performed, and the patients' clinicopathological

characteristics were analyzed and the correlations between immunohistochemical variables and patients' outcome were assessed. From the TCGA analysis results, patients without alterations in IL-22 or IL-22RA1 had a significantly good overall survival and disease-free survival compared to patients with alteration in IL-22 or IL-22RA1. Immunohistochemical staining detected IL-22, IL-22RA1, Ki-67, and caspase-3 positive tumor cells, but no IL-22BP positive cell was detected. Statistical analysis showed that IL-22 expression was correlated with Fuhrman grade, primary T stage, relapse and vital status of ccRCC patients. And IL-22RA1 expression was associated with Fuhrman grade, relapse, and vital status of ccRCC patients. Moreover, Kaplan-Meier analysis revealed that ccRCC patients with high expression of IL-22 had a poor overall survival and disease-free survival compared to patients with low expression of IL-22. This was true also for IL-22RA1 and Ki-67. However, ccRCC patients with high expression of caspase-3 only showed a significantly poor disease-free survival, but not overall survival. In addition, multivariable Cox proportional hazards regression model showed that tumor stage, presence of necrosis in tumor, and IL-22RA1 probably are independent predictors for overall survival of ccRCC patients, and Fuhrman grade, tumor stage, presence of venous thrombus and IL-22RA1 are independent predictors of disease-free survival of ccRCC patients. Several conclusions can be drawn: the IL-22-IL-22RA1 axis is correlated with several clinicopathological characteristics such as Fuhrman grade, primary T stage, relapse and vital status of ccRCC patients; ccRCC patients with lower expression level of IL-22, IL-22RA1, Ki-67 tend to have a longer survival time; IL-22RA1 but not IL-22,

is an independent predictive factor for prognosis of ccRCC patients.

1. Introduction

1.1 Renal cell carcinoma

1.1.1 Epidemiology

Currently, renal cell carcinoma is the 9th and the 14th most common cancer in men and women worldwide respectively. In the year 2012, the estimated amount of deaths from renal cancer was 143,000, which ranked the 16th most common cause of death from metastatic disease worldwide. (Znaor, Lortet-Tieulent et al. 2015) Among all human malignant diseases, 2-3% are renal cell carcinomas (RCC), which account for approximately 90% of all primary malignancies of the kidney. Despite the advent of improved diagnostic methods, particularly imaging techniques, around 20-30% of all patients have metastatic disease at the time of diagnosis. (Rini, Campbell et al. 2009) (Ljungberg, Campbell et al. 2011) The incidence of renal cell carcinoma varies in different areas of the world, and is higher in developed countries compared to developing countries. Men seem more likely to suffer from renal cancer than women, with the male to female ratio being 1.5:1. (Levi, Ferlay et al. 2008) Incidences of renal cancer increase with age and reach a plateau in the 7th decade of life (Chow and Devesa 2008).

1.1.2 Classification

Renal cell carcinoma is not a single disease, but rather a combination of related, yet different malignancies of various histological subtypes that arise from the renal tubule cell (Larkin, Goh et al. 2012), and different histologic subtypes are believed to originate from different parts of the nephron (Shuch, Amin et al. 2015). Clear cell renal cell carcinoma (ccRCC) accounts for about 70-75% of kidney cancer, and therefore is the most common subtype of this cancer. Papillary RCC is the second most common subtype of kidney cancer, accounting for around 10-16% of all RCC cases. Papillary RCC can further be divided into 2 different subtypes, papillary type 1 and papillary type 2 (Srigley and Delahunt 2009). The third most common subtype of renal cell carcinoma is chromophobe RCC, and accounts for 5% of all RCC cases (Shuch, Amin et al. 2015). Collecting duct RCC is considered to arise from the kidney medulla and only accounts for <0.5% cases of RCC (Fleming and Lewi 1986). Medullary RCC is an extremely rare subtype with a poor survival, many patients die within months after diagnosis. It is recognised as a tumor arising from calyceal epithelium or papillae and possibly be initiated by chronic medullary hypoxia caused by hemoglobinopathy. (Davis, Mostofi et al. 1995) (Swartz, Karth et al. 2002) In addition, there are several other RCC forms associated with specific genes, like “microphthalmia transcription factor family”-translocation RCC as well as hereditary renal cell carcinoma syndromes. (Shuch, Amin et al. 2015)

1.1.3 Pathogenesis

The pathophysiology linking risk factors with RCC is still not completely understood. Etiologic factors involve many aspects, among which specific lifestyle is likely to play an important role in the development of RCC. Cigarette smoking, both active or passive, is an established risk factor for kidney cancer, and the risk compared to non-smokers increases about 50% in male and 20% in female smokers (Hunt, van der Hel et al. 2005). Excessive body weight is also a potential risk factor, with RCC being a cancer type robustly associated with increased Body-mass index (BMI), probably because the adipose tissue can provide an abundant energy source for malignant lesions of the kidney to escape the immune system, to grow and to form metastases (Gati, Kouidhi et al. 2014). Rohrmann et al. studied the relationship between consumption of meat and fish with the risk of RCC and they showed an association between red and processed meat consumption and the risk of kidney cancer in women (Rohrmann, Linseisen et al. 2015). In addition, a pooled analysis of several cohort studies showed a negative correlation between kidney cancer and diets abundant in fruits and vegetables (Lee, Mannisto et al. 2009). A cohort study from Sweden indicated that the risk for RCC development increased with elevation of blood pressure and decreased with reducing elevated blood pressure (Chow, Gridley et al. 2000), suggesting hypertension is another risk factor for RCC. In spite of the risk factors mentioned above, still many RCC patients do not have any identifiable risk factor. In addition, patients with end stage renal disease (ESRD) requiring dialysis are

at risk for developing kidney cancer (Maisonneuve, Agodoa et al. 1999).

Approximately 2-3% of renal cell carcinomas are familial (Lipworth, Tarone et al. 2006). The risk to suffer RCC is nearly two-fold increased for a first grade relative of a kidney cancer patient (Ljungberg, Campbell et al. 2011), suggesting one ore more genetic risk factors. A two-stage genome-wide association study of RCC by a research group led by International Agency for Research on Cancer and the US National Cancer Institute showed that two genetic loci (2p21 [EPAS1] and 11q13.3 [no characterized genes]) were associated with RCC susceptibility (Purdue, Johansson et al. 2011). It provides evidence that genetics can affect susceptibility to renal cell carcinoma.

1.1.4 Molecular pathways of clear cell renal cell carcinoma

The prognosis associated with RCC differs among subtypes, but there is also considerable outcome variability within the same subtype. The clinical outcomes of ccRCC are influenced by several prognostic factors including tumor stage, Fuhrman grade, as well as the presence of necrosis (Frank, Blute et al. 2002, Delahunt, McKenney et al. 2013).

So far, several biomarkers have been considered as potential prognostic markers. However, none of them have been adopted in clinical care. The molecular

mechanisms contributing to the development of RCC have not been understood completely. CSNK2A1 (Casein Kinase 2 Alpha 1), SPP1 (Secreted Phosphoprotein 1) as well as DEFB1 (Defensin Beta 1) are three new identified genes, there is significant association between high expression and a poor outcome in ccRCC patients (Rabjerg, Bjerregaard et al. 2016). ccRCC originates from proximal convoluted tubules in nephrons and is associated with a mutation and/or inactivation of the von Hippel-Lindau (VHL) gene and activation of the Raf-MAPK (mitogen-activated protein kinase)-ERK (extracellular signal-regulated kinases) pathway (Wong, Ojo et al. 2015). The current therapies include agents that target the vascular endothelial growth factor (VEGF) pathway or mammalian target of rapamycin (mTOR) (Singer, Gupta et al. 2013). The VHL-hypoxia-inducible factor (HIF) pathway also plays an important role in ccRCC (Latif, Tory et al. 1993). In up to 41% of ccRCC tumors there are mutations in the Polybromo 1(PBRM1) gene, which is located on 3p21, making PBRM1 one of the most commonly mutated genes in ccRCC, ranking second after VHL (Varela, Tarpey et al. 2011).. Several studies including The Cancer Genome Atlas (TCGA) project (found BAP1 (BRCA1 associated protein 1) alterations in 8-10% of ccRCC (Guo, Gui et al. 2012, Cancer Genome Atlas Research 2013). Mutations in BAP1 and PBRM1 are associated with pathology characters such as tumor grade and sarcomatoid variants, biology as well as prognosis (Su, Singer et al. 2015). Mutations in PTEN (Phosphatase and tensin homolog) and mTOR were present in 4% and 6% of ccRCC, respectively(Cancer Genome Atlas Research 2013). Novel targeted immunotherapy such as PD-1 (programmed death 1) and PD-L1 (programmed death

ligand 1) targeted agents have been developed. Previous studies revealed that PD-L1 expression is correlated with a poor outcome in RCC mostly because of its immunosuppressive function (Thompson, Gillett et al. 2004, Thompson, Kuntz et al. 2006, Choueiri, Fishman et al. 2016). PD-1 from T cell binding to PD-L1 from renal cancer cell blocks the immune response such as inhibiting cytokine release and antitumor T cells activity (Harshman, Drake et al. 2014). Figure 1 summarizes the pathways in renal cell carcinoma.

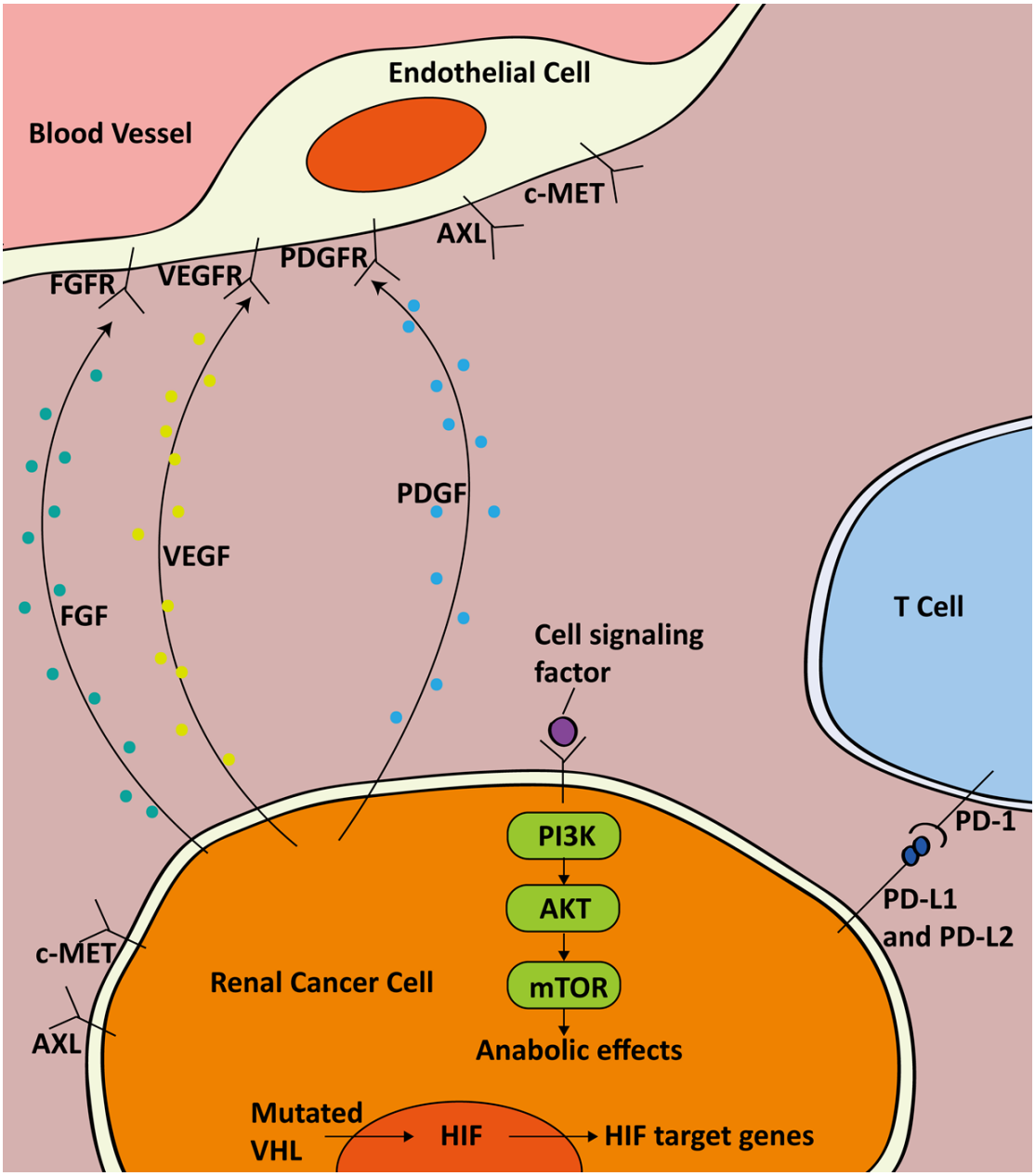

Figure 1 Pathways in renal cell carcinoma. An endothelial cell, a renal cancer cell and a T cell are show. Accumulated HIF translocates into the nucleus of renal cancer cell after the mutation of VHL, following by activation of HIF target genes including VEGF, VEGFR, FGF (fibroblast growth factor), FGFR (FGF receptor), PDFG (platelet derived growth factor) and PDFGR (PDFG receptor). PD-1 binding to PD-L1 blocks the immune response between T cell and cancer cell. Cell signaling factor activates PI3K-AKT-mTOR pathway, leading to anabolic effects in renal cancer cell. (Choueiri and Motzer 2017)

Ki-67 is a well-established marker of tumor cell proliferation. The association

between Ki-67 staining indices and outcome of cancer is a continuous function, the higher the score, the greater the risk of biochemical or clinical failure (Pollack, DeSilvio et al. 2004). One of the largest studies indicated that patients with higher expression of Ki-67 were more likely to die because of RCC and that Ki-67 had a significant relationship with adverse pathological features of RCC (Tollefson, Thompson et al. 2007).

Caspase-3 (also known as CPP32, YAMA, and apopain) is an apoptosis-related gene located at 4q35.1 (Yan, He et al. 2013). Caspase-3 acts as an effector in the caspase apoptotic cascade.It can be activated by the mitochondrial or the death receptor apoptotic pathway (Soung, Lee et al. 2004, Chen, Zhao et al. 2008, Kuo, Yu et al. 2011, Onouchi, Suzuki et al. 2013). The normal apoptotic process can be inhibited by the activation of caspase3, and deregulations of apoptotic pathways can increase cell survival in a lot of cancers, and may further support anchorage-independent survival during the process of metastasis (Frisch and Francis 1994, Glinsky and Glinsky 1996, Takaoka, Adachi et al. 1997). However, several cancers showed significantly lower expression of the caspase3 protein compared to the expression in normal tissues (Hosgood, Baris et al. 2008, Kinslow, El-Zein et al. 2008). One study indicated that gene polymorphisms and haplotypes of caspase3 increased the risk for gastric cancer (Li, Liu et al. 2014). Another study provided evidence that caspase3 expression in metastatic lymph nodes can be a potential independent prognostic factor of shorter overall survival in esophageal squamous cell carcinoma patients

with lymph nodes metastasis (Wang, Luo et al. 2014). However, the correlation between caspase3 and outcome of RCC has not been established from the literature.

Advanced ccRCC is still an incurable disease with a poor prognosis. Without doubt there is an increasing need for the identification of biomarkers that may predict ccRCC recurrence and survival.

1.1.5 Therapy of clear cell renal cell carcinoma

Radical nephrectomy and partial nephrectomy are the only curative options for nonmetastatic clear cell renal cell carcinoma, and surgery is also an important option to relieve symptoms from metastatic ccRCC (mccRCC). Surgical resection should be used in combination with systemic therapy for the patients with mccRCC.(Biswas, Kelly et al. 2009) ccRCC is resistant to radiotherapy or chemotherapy, and while high-dose interleukin-2 or interferon-alfa may induce durable responses in a small percentage of mccRCC patients, the therapy is toxic and only suitable for a small patient population (Keizman, Maimon et al. 2015). VEGF-TKIs (Sorafenib, Sunitinib) and mTOR inhibitors (Everolimus, Temsirolimus) are used as first line treatment options for patients with mccRCC. Second line or later options include axitinib, which is an inhibitor of VEGFRs and cabozantinib, an inhibitor of VEGFRs, MET, and AXL (Choueiri and Motzer 2017). Recently, monoclonal antibodies (Nivolumab) targeting and blocking the inhibitory T-cell receptor, PD-1 and its ligand PD-L1 emerged as

promising therapy for mccRCC (Shin, Jeon et al. 2015).

1.2 Interleukin-22

1.2.1 Introduction of IL-22

Interleukin-22 (IL-22) is a member of the IL-10 cytokine family along with IL-19, IL-20, IL-24, IL-26 and IL-28 (α and β). It is produced by several subsets of lymphocytes including CD4+ T helper 17 (Th17) cells (also known as IL-17-producing cells, which are considered as a key cellular source of IL-22) (Ouyang, Rutz et al. 2011) and Th22 cells, CD8+ cytotoxic T cells, natural killer (NK) cells, γδT cells and lymphoid tissue inducer (LTi)-like cells (Lim and Savan 2014). Of the CD4+ T cells, Th17 and Th22 cells are the major sources of IL-22. Th1 cells can also produce IL-22 (Gurney 2004). In humans, IL-22 is mostly produced by Th1, Th17 and Th22 cells. However, murine IL-22 is mainly produced by Th17 cells (Zheng, Danilenko et al. 2007, Duhen, Geiger et al. 2009, Eyerich, Eyerich et al. 2009, Volpe, Touzot et al. 2009). What should be paid attention to is, that the principal cellular sources of IL-22 obviously vary between tissues, so in different kinds of tumors, the regulation of IL-22 production may involve different immune cell populations (Lim and Savan 2014).

The human IL-22 gene is located on chromosome 12p15, while the mouse and rat genes are located on chromosomes 10 and 7, respectively (Weidenbusch, Rodler et

al. 2015). The human IL-22 protein consists of 146 amino acids and has 80.8% identity with murine IL-22, and IL-22 also has an α-helical secondary structure just as other family members of IL-10 (Wolk and Sabat 2006). IL-22 normally plays a role in sustaining the integrity as well as the barrier function of these organs, preventing injury from either invading pathogens or an inflammatory response (Witte, Witte et al. 2010, Sonnenberg, Fouser et al. 2011, Zenewicz and Flavell 2011). In this process, IL-22 will create pro-inflammatory epithelial resist mechanisms which are crucial for host protection, however, if this process is not well regulated, inflammation will be amplified by IL-22 alone or together with other cytokines, therefore inducing deviant epithelial proliferation as well as differentiation (Rutz, Eidenschenk et al. 2013). There are three different ways in which IL-22 functions in host defense and protection: first, during the invasion of pathogens, IL-22 may help to maintain and rebuild the epithelial barrier after epithelial injury; second, IL-22 may induce host defense antimicrobial protein expression together with other cytokines like IL-17 or tumor necrosis factor- α (TNF-α); third, IL-22 may enhance the expression of inflammatory mediators such as IL-6, IL-1β, granulocyte colony-stimulating factor (G-CSF), lipopolysaccharide (LPS)-binding protein (LBP) and serum amyloid A (SAA) (Wolk, Witte et al. 2006, Boniface, Guignouard et al. 2007, Aujla, Chan et al. 2008, Liang, Nickerson-Nutter et al. 2010).

Additionally, IL-22 also contributes to tissue regeneration and the promotion of wound healing. IL-22 may promote proliferation and survival of the epithelial cell in

many organs such as intestine, liver, thymus and lung (Ki, Park et al. 2010, Dudakov, Hanash et al. 2012, Kong, Feng et al. 2012). IL-22 may also promote liver cell regeneration after hepatic injury. There are anti-apoptotic genes in hepatocytes which can protect hepatocytes from cell death caused by inflammation. Interestingly, expression of these genes can be induced by IL-22 (Radaeva, Sun et al. 2004). Meanwhile, IL-22 has a protective role in some other inflammatory diseases and the development of fibrosis, such as allergic airway inflammation, autoimmune myocarditis, uveitis and pulmonary fibrosis (Chang, Hanawa et al. 2006, Simonian, Wehrmann et al. 2010, Ke, Sun et al. 2011, Nakagome, Imamura et al. 2011)

1.2.2 IL-22 receptor and IL-22 binding protein

The IL-22 receptor (IL-22R) is a member of class II cytokine receptor family. IL-22 plays its role via binding to the heterodimeric transmembrane complex iof IL-22RA1 and IL-10R2 (IL-10RB) (Xin, Namaka et al. 2015). The human gene encoding IL-22RA1 is located at chromosome 1p36.11, whereas the gene encoding IL-10R2 is located on chromosome 21q22.11 (Xie, Aggarwal et al. 2000). IL-22RA1 was found to have restricted expression on cells of epithelial origin in various kinds of non-immune tissues such as the pancreas, skin and colonic mucosa, followed by kidney, lung and liver. (Tachiiri, Imamura et al. 2003, Wolk, Kunz et al. 2004) On the contrary, IL-10R2 is believed to be expressed ubiquitously in immune cells such as T cells, B cells and NK cells, as well as epithelial cells (Wolk, Kunz et al. 2004).

In addition to the membrane-bound IL-22R, there is a soluble receptor, IL-22RA2, also known as IL-22 binding protein (IL-22BP) (Weiss, Wolk et al. 2004, Sabat 2010, Sabat, Ouyang et al. 2014). The gene encoding human IL-22BP is located at chromosome 6q23.3 and is closeto genes encoding the IFN (Interferon)-γR and IL-20R (Gruenberg, Schoenemeyer et al. 2001, Kotenko, Izotova et al. 2001, Weiss, Wolk et al. 2004). IL-22BP binds to IL-22 with a higher affinity and specificity compared to IL-22RA1 (20-to1000-fold higher) (Wolk, Witte et al. 2007, Jones, Logsdon et al. 2008), acts as a decoy receptor and inhibits the effects of the interaction between IL-22 with its trans-membrane receptor complex (Sabat, Ouyang et al. 2014). Interestingly, IL-22BP specifically binds to IL-22, but not other cytokines of the IL-10 family (Dumoutier, Lejeune et al. 2001, Kotenko, Izotova et al. 2001, Xu, Presnell et al. 2001, Wei, Ho et al. 2003). Therefore IL-22BP has been used as a competitive antagonist of IL-22 signaling in vitro (Lim and Savan 2014). IL-22BP is expressed at a highest level in placenta and mammary gland, at an intermediate level in stomach, thymus, spleen, lung, skin and lymph nodes, and expressed modestly in peripheral blood leucocytes, heart, brain and prostate (Dumoutier, Lejeune et al. 2001, Kotenko, Izotova et al. 2001, Xu, Presnell et al. 2001, Wei, Ho et al. 2003, Weiss, Wolk et al. 2004). (Figure 2)

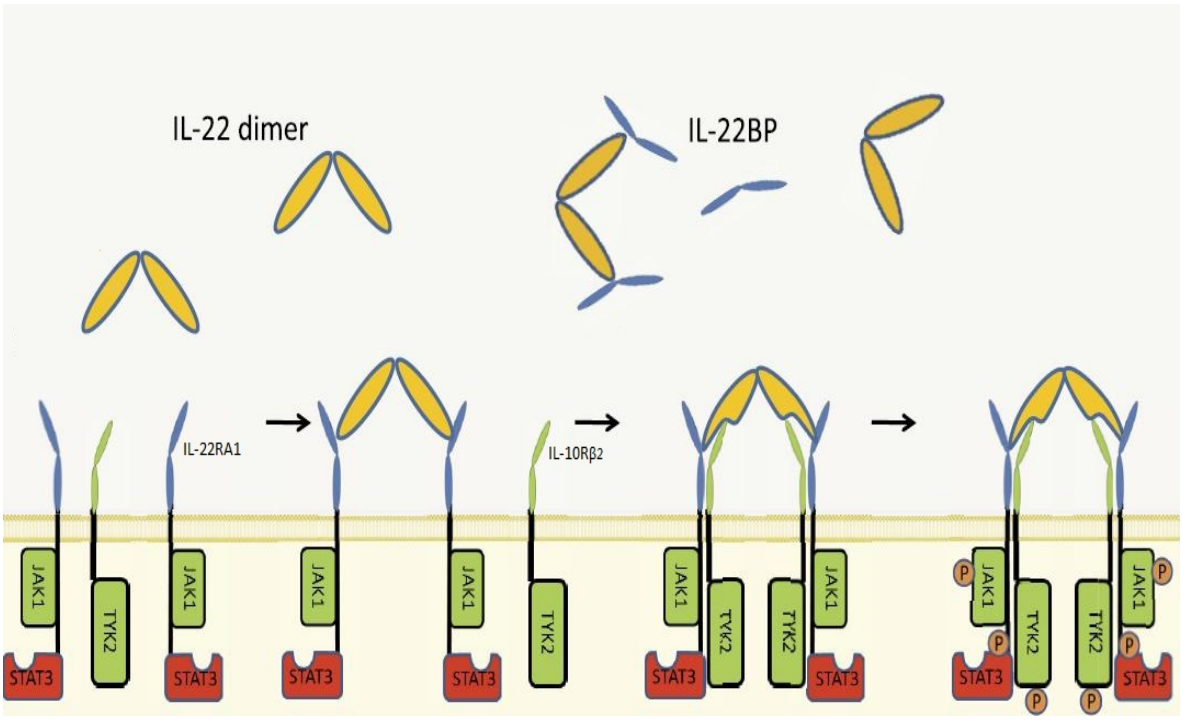

Figure 2 Principles of IL-22 signaling. IL-22 activates cellular responses by combining to receptor IL-22RA1 and IL-10Rβ2. IL-22 binds to IL-22RA1 with high affinity but no affinity for IL-10Rβ2 . After binding to IL-22RA1, the conformation of IL-22 changes, leads to a strong affinity towards IL-10Rβ2. This combination phosphorylates JAK1 (Janus kinase), TYK2 (Tyrosine Kinase 2) and STAT3 (Signal transducer and activator of transcription 3), therefore impact downstream signaling. IL-22BP inhibits effects of IL-22 via a higher affinity than IL-22RA1 (Weidenbusch, Rodler et al. 2015).

1.2.3 IL-22-IL-22RA1 axis in the kidney

Initial studies on the expression of IL-22R identified low expression in the kidney (Aggarwal, Xie et al. 2001, Kotenko, Izotova et al. 2001, Tachiiri, Imamura et al. 2003, Wolk, Kunz et al. 2004), whereIL-22R is mainly expressed on tubular epithelial cells (Kulkarni, Hartter et al. 2014). So far, little is known about the function of IL-22 in the kidney. According to a previous study, IL-22 will be induced in the kidney after

stimulation by DAMPs (Kulkarni, Hartter et al. 2014) and it also can be seen in a polymicrobial peritonitis model (Weber, Schlautkotter et al. 2007). IL-22 was believed to function against the regeneration of the kidney because of the fact that blockade of IL-22 by IL-22BP-Fc can relieve renal damage and increase bacterial clearance from the kidney in polymicrobial peritonitis (Dumoutier, Van Roost et al. 2000, Weber, Schlautkotter et al. 2007). Moreover, one study found a correlation between polymorphisms in the IL-22R and nephropathy development (Suh, Cho et al. 2013). One study focused on IL-22BP demonstrated that urinary IL-22BP levels are correlated with active renal disease and IL-22BP is highly expressed in the renal tissue of patients with active renal disease, (Yang, Gao et al. 2014).

1.2.4 Signaling pathways of IL-22

The assembly of the IL-22-IL-22R1-IL-10R2 complex activates Janus tyrosine kinases (Jak1 and Tyk2) associated with the receptor complex, which results in the phosphorylation of these receptors as well as signal transducer and activator of transcription (STAT) proteins (Dudakov, Hanash et al. 2015). Phosphorylation of STAT3 is believed to be the primary mediator of IL-22 signaling. However, STAT1 and STAT5 phosphorylation have also been found to play a role (Lejeune, Dumoutier et al. 2002, Wolk, Kunz et al. 2004). In addition, IL-22 activates the mitogen-activated protein kinase (MAPK) pathway including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 (Lejeune, Dumoutier et al. 2002,

Brand, Dambacher et al. 2007, Lim and Savan 2014)as well as phosphatidylinositide 3-Kinase-Akt-mammalian target of rapamycin (PI3K-Akt-mTOR) pathway (Mitra, Raychaudhuri et al. 2012, Sabat, Ouyang et al. 2014). . STAT3 phosphorylation is an important downstream pathway in terms of regulating the effect of IL-22 in epithelial cells, and research on chemical-induced colitis indicated IL-22 -dependent phosphorylation of STAT3 in epithelial cells; meanwhile, conditional deletion of STAT3 *in vivo* in intestinal epithelial cells led to a similar phenotype seen in IL-22 deficient mice after chemical-induced colitis. This demonstrates an essential role for STAT3 in IL-22 mediated signaling (Pickert, Neufert et al. 2009).

1.2.5 IL-22 in inflammatory diseases

IL-22 is expressed in a wide range of organs such as skin, liver, breast, thymus, kidney, lung, pancreas, gastrointestinal tract, heart, eye, synovial tissues and adipose tissue, and its receptor is expressed on the stromal and epithelial cells of those organs (Witte, Witte et al. 2010, Sonnenberg, Fouser et al. 2011, Cordero-Coma, Calleja et al. 2013, Sabat, Ouyang et al. 2014, Dudakov, Hanash et al. 2015). As mentioned above, IL-22 is produced at sites of inflammation, therefore it may be involved in regulating a physiological response to repair tissue damage, but it may also lead to pathological inflammation (Dudakov, Hanash et al. 2015). There are several experimental models showing that IL-22 may play a protective role by regenerating epithelial tissues including pancreatitis, thymic injury, colitis and

hepatitis (Witte, Witte et al. 2010, Sonnenberg, Fouser et al. 2011, Dudakov, Hanash et al. 2012). However, IL-22 has been reported to induce proinflammatory molecule expression, such as IL-1, IL-6, IL-8, IL-11, G-CSF, GM-CSF and LPS binding protein (Andoh, Zhang et al. 2005, Sonnenberg, Fouser et al. 2011, Mortha, Chudnovskiy et al. 2014). Tissue damage disrupts tissue homeostasis and induces responses that intend to eliminate the injurious trigger and try to reinstall tissue homeostasis such as inflammation, atrophy and fibrosis (Hagemann, Haegele et al. 2013, Suarez-Alvarez, Liapis et al. 2016). Notch signalling is known to mediate development as well as homeostasis in various organs including kidney, heart, lung and intestine, and plays a main role in immune homeostasis as well (Radtke, Fasnacht et al. 2010, Barak, Surendran et al. 2012, Xu, Moghal et al. 2012, Radtke, MacDonald et al. 2013, Tsai, VanDussen et al. 2014, Luxan, D'Amato et al. 2016). Several studies found a correlation between Notch and IL-22, that Notch signalling induces IL-22 production by aryl hydrocarbon receptor ligands and in vivo the production of IL-22 is mediated by Notch signalling (Alam, Maekawa et al. 2010, Lee, Cella et al. 2012). In conclusion, IL-22 is involved in tissue homeostasis and plays a role in inflammation, atrophy and fibrosis via Notch signalling.

1.2.6 IL-22 in autoimmune diseases

Studies have shown that some inflammatory cytokines are involved in the pathogenesis of autoimmune disease (Pan, Li et al. 2013). IL-17 was believed to play

a pathogenic role in autoimmunity (Pan, Ye et al. 2008). Several research groups indicated that increased IL-17 levels are correlated with many chronic autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, psoriasis and Sjögren's syndrome (Garrett-Sinha, John et al. 2008, van den Berg and Miossec 2009, Mieliauskaite, Dumalakiene et al. 2012, Pollinger 2012). Similar to IL-17, IL-22 is highly expressed by Th17 cells as well, studies have revealed that IL-22 is expressed differentially in several autoimmune diseases (Cheng, Guo et al. 2009, Pan, Zhao et al. 2009, da Rocha, Duarte et al. 2012). Mouse models showed that IL-22 knockout or inhibition might ameliorate some autoimmune diseases (Ke, Sun et al. 2011, Semerano, Assier et al. 2012).

1.2.7 IL-22 in cancer

On the one hand, IL-22 plays a protective role in inflammation, on the other hand, as a double-edged sword, IL-22 can execute an inflammatory function in autoimmune diseases, and moreover, promote tumor development by inducing inflammation, proliferation, cell migration, angiogenesis and oxidative signaling via STAT3 signaling (Lim and Savan 2014). Aberrance of IL-22 signaling was shown to be important in various cancers. IL-22 was shown to be involved in cancer signaling in several sites, such as lung, pancreas, skin, colon, breast, stomach, thyroid, skin and cervix (Dudakov, Hanash et al. 2015).

Studies on colon cancer and hepatocellular cancer indicated that excessive IL-22 in the microenvironment of colon cancer, ulcerative colitis and hepatocellular carcinoma leads to tumor growth, apoptosis inhibition as well as metastasis promotion depend on STAT3 activation (Jiang, Tan et al. 2011, Jiang, Wang et al. 2013). *In vitro*, IL-22 stimulation enhances the migration and invasion of gastric cancer cells by regulating IL-22RA1/AKT/MMP-9 (matrix metalloproteinase 9) signaling axis (Ji, Yang et al. 2014). IL-22R is expressed on glioblastoma cells established *in vivo* and *in vitro*, and IL-22 induced glioblastoma cell survival in STAT3, ERK1/2 and PI3K/Akt pathways. In gastric cancer, IL-22 is important in establishing the tumor microenvironment and increased intratumoral IL-22 correlates with tumor progression and predicts poor survival in patients (Zhuang, Peng et al. 2012). Another study demonstrated that IL-22 levels are positively associated with MAP3K8 (mitogen-activated protein kinase kinase kinase 8) and Pin1 expression in breast cancer. IL-22-induced MAP3K8 signaling pathway may promote cancer-associated inflammation in the tumor microenvironment (Kim, Kim et al. 2014). In addition, IL-22 is frequently expressed in lung cancer, and increased IL-22RA1 expression and signaling in chemotherapy refractory cell lines are indicative of a pro-tumorigenic function of IL-22 and may contribute to a more aggressive phenotype. Overexpression of IL-22RA1 is an independent indicator of poor overall survival and supports the contribution of the IL-22-IL-22RA1 pathway in lung cancer progression (Kobold, Volk et al. 2013, Guillon, Gueugnon et al. 2016). Moreover, overall survival of patients with pancreatic ductal adenocarcinoma was significantly shortershorter

in patients with high expression of IL-22 and IL-22R than in those with low expression (Wen, Liao et al. 2014). One study on IL-22 and the RCC cell line A498 showed that IL-22 dose-dependently suppressed the growth of RCC A498 cells by regulation of STAT1 pathway (Zhang, Shang et al. 2011). Despite the known correlation between IL-22 and different types of cancers from previous studies, to our knowledge there is no published research on the relationship between the expression of IL-22/IL-22R1 and survival in ccRCC patients.

1.3 Hypotheses

Based on the above, we therefore hypothesized that:

1. Expression of IL-22-IL-22RA1 axis proteins is correlated with ccRCC patients' overall survival and disease-free survival. To this end, clinical and genomic data of ccRCC provided by TCGA were analyzed using the cBioPortal resource.
2. Expression of IL-22-IL-22RA1 axis proteins is correlated with clinicopathological characteristics of ccRCC patients. To verify this hypothesis, we quantified the expression of IL-22 and IL-22RA1 in human ccRCC tissues and correlated the expression with clinicopathological characteristics (age, sex, Fuhrman grade, primary T stage, present of necrosis, venous thrombus and relapse) of ccRCC patients.
3. ccRCC patients with lower expression level of IL-22, IL-22RA1, IL-22BP, Ki-67, Caspase-3 have a longer overall survival time and disease-free survival time. We

assessed the expression of IL-22, IL-22RA1, and IL-22BP, Ki-67 and Caspase-3 in human ccRCC tissues and correlated the expression with the outcomes of ccRCC patients in our patients' cohort.

4. IL-22RA1 and IL-22 are independent predictive factors for prognosis of ccRCC patients. To this purpose, a multivariate cox proportional hazards model was used to analyze the impact of prognostic factors on overall survival and disease-free survival.

2. Materials and Methods

2.1 Materials

100% ethanol	CLN, Germany
30% hydrogen peroxide	Merck KGaA, Germany
37℃ water bath	HAAKE, Germany
70% ethanol	CLN, Germany
96% ethanol	CLN, Germany
ABC reagent	Vector Laboratories, CA, USA
antigen unmasking solution	Vector Laboratories, CA, USA
autoclave	Melag, Germany
avidin blocking solution	Vector Laboratories, CA, USA
biotin blocking solution	Vector Laboratories, CA, USA
biotinylated anti-goat antibody	BA-9500, Vector Laboratories, CA, USA
biotinylated anti-mouse IgG1 antibody	406604, BioLegend, CA, USA
biotinylated anti-rabbit antibody	BA1000, Vector Laboratories, CA, USA
cleaved caspase-3 antibody	9661S, Cell Signaling, MA, USA
cover slips	VWR, Pennsylvania, USA
DAB	Sigma-Aldrich, Mo. USA
distilled water	made in our lab

drying oven	Memmert, Germany
goat serum	Thermo Scientific, USA
GraphPad Prism	GraphPad Prism 6.0
IBM SPSS	IBM SPSS 22
IL-22 antibody	sc-14436, Santa Cruz Biotech, Texas, USA
IL-22BP antibody	NBP1-85455, Novus Biologicals, CO, USA
IL-22RA1 antibody	ab5984, Abcam, UK
Image J	National Institutes of Health
Ki-67 antibody	BD 550609, BD Biosciences, CA, USA
methanol	Sigma-Aldrich, Mo. USA
methyl green	Sigma-Aldrich, Mo. USA
microscope	Leica Microsystems, Germany
microscope slides	Thermo Scientific, USA
microtome	Thermo Scientific Microm HM355S, USA
Microwave	Severin, Germany
mounting medium	Vector Laboratories, CA, USA
NiCl	Sigma-Aldrich, Mo. USA
PBS	PAN-Biotech, Germany
proteinase K	Qiagen, Germany
Tris-Hcl	made in our lab
Windows system	Microsoft Windows 7
xylene	Fisher Scientific, UK

2.2 The cancer genome atlas data analysis

The cancer genome atlas (TCGA) is a cooperation program by the National Cancer Institute (NCI) and National Human Genome Research Institute (NHGRI), it has synthetic multi-dimensional maps of the pivotal genomic changes as well as molecular and clinical data in 33 types of cancer so far (http://cancergenome.nih.gov/). The principal objectives of TCGA are vigorous quality control as well as to generate, , merge, analyze, and clarify molecular characteristics at the DNA, RNA, protein and epigenetic levels for hundreds of cancers from various tumor types and subtypes (Cancer Genome Atlas Research, Weinstein et al. 2013). Researchers can collect, select, and analyze human tumor tissues of the genomic database established by TCGA and this national network serves as a model for projects in the future. Tumors profiled by TCGA range from solid to hematological, from moderate to highly malignant. DNA, RNA and protein are extracted and analyzed for each tumor case. TCGA collects and analyzes high-quality tumor tissues and makes these following data usable on the TCGA Data Portal:

1) Clinical information about participants in the program
2) Metadata about the samples
3) Histopathology slides images from sample portions
4) Molecular information derived from the samples (e.g. mRNA/miRNA expression, protein expression, copy number, etc.) (NCI Wiki

https://wiki.nci.nih.gov/display/TCGA/About+TCGA+Data)

Pan-cancer is a coordinated initiative, its aim being the assemlyof coherent, consistent data sets from TCGA and separate disease projects, then analyzing and interpreting these data to build a well-coordinated joint data set sweeping into multiple tumor types (Cancer Genome Atlas Research, Weinstein et al. 2013). The Pan-Cancer analysis program was established as a collaboration among members of the TCGA network, however, many other interested institutions were involved soon after starting.

The findings from TCGA have provided a large amount of advanced knowledge on the cancers and therefore have given rise to the development of clinical diagnostic and prognostic biomarkers even redefinitions of preceding classifications of cancers (Colaprico, Silva et al. 2016). However, it is not easy for cancer researchers to translate the genomic data from this kind of large-scale cancer genomics projects into new biologic insights and clinical applications.

cBioPortal (http://www.cbioportal.org/) is an open-access resource for Cancer Genomics which provides visualization, analysis and download options of multidimensional cancer genomics data sets and makes the raw data generated by large-scale cancer genomics projects such as TCGA and ICGC (the International Cancer Genome Consortium) more easily and directly available (Cerami, Gao et al. 2012). cBioPortal enables researchers to explore genetic alterations and gene

expression interactively across different tissues and to link these underlying data to clinical outcomes (Gao, Aksoy et al. 2013).

In a single-cancer query, researchers can select cancer need to study, select genomic profiles and enter gene name. As shown in Figure 3, kidney renal clear cell carcinoma (TCGA, provisional) which includes 538 samples was selected for the present study.

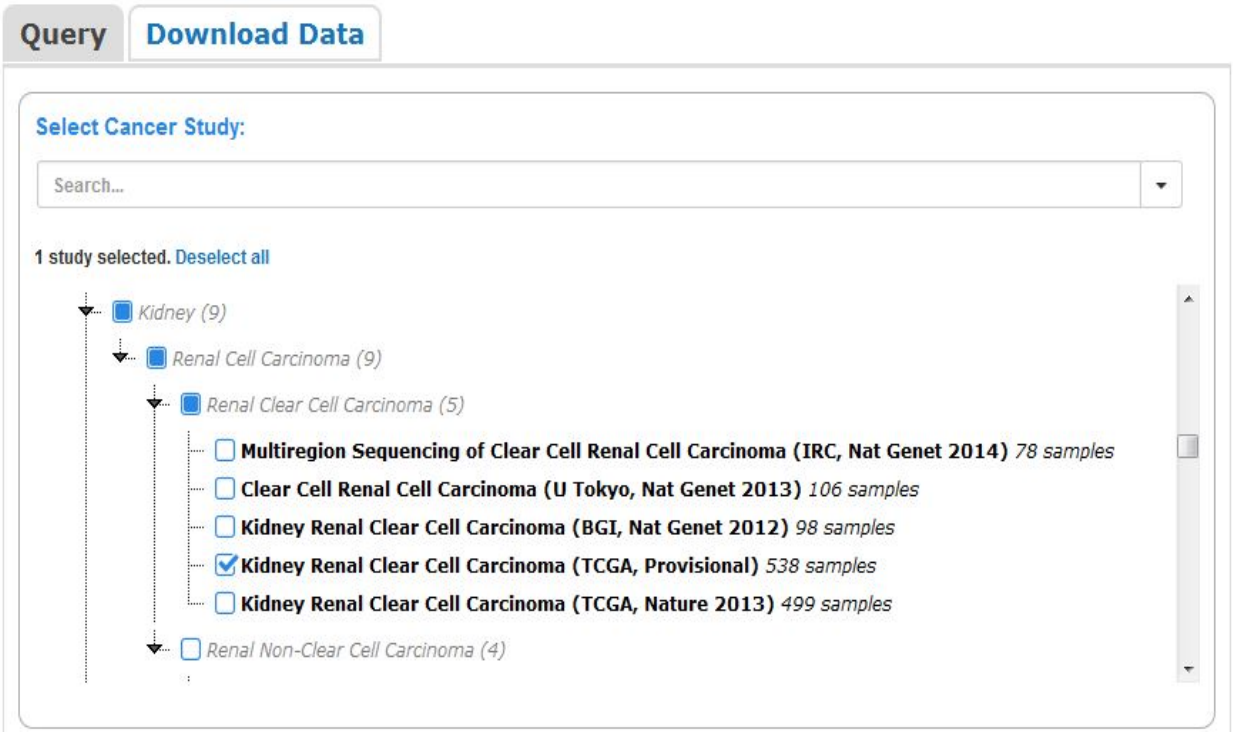

Figure 3 Select genomic profiles.

The next step was to select genomic profiles. We selected mRNA Expression z-Scores (RNA Seq V2 RSEM) which meant compared to the expression distribution of each gene tumors that were diploid for this gene, and then entered a z-score threshold. A z-score is also known as a standard score, is a measure of how many standard

deviations below or above the population mean a raw score is. z = (expression in tumor sample – mean expression in reference sample) / standard deviation of expression in reference sample. (Figure 4)

Figure 4 Select genomic profiles.

After that, “All Complete Tumors” was selected from the list. Then gene symbol was typed in the blank before submitting, as shown in Figure 5.

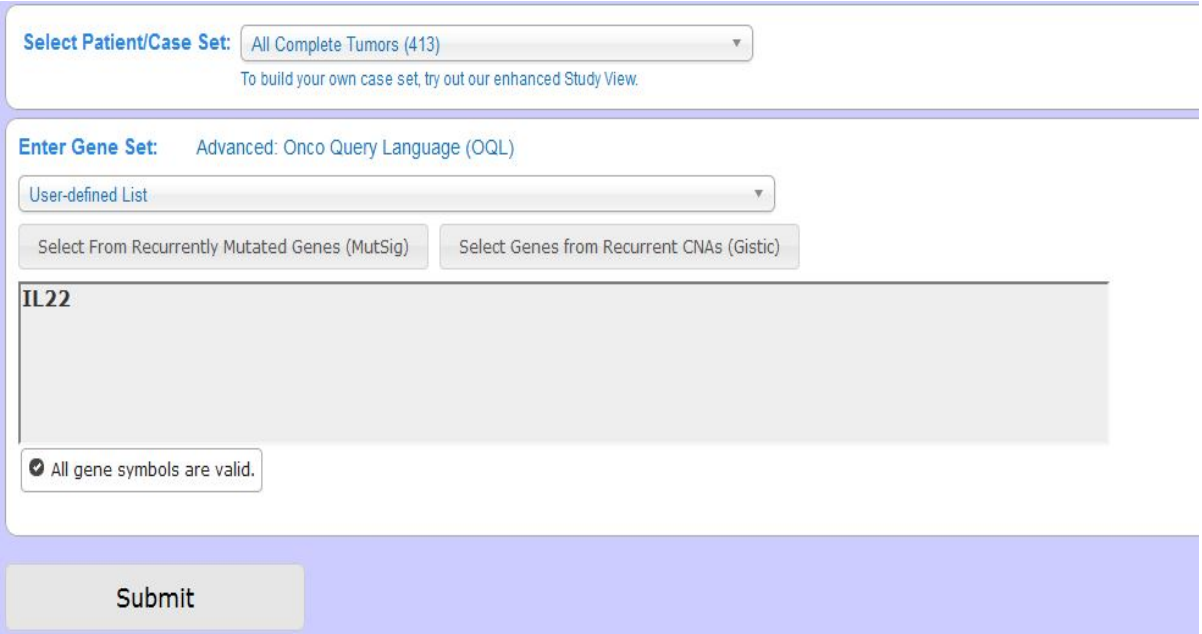

Figure 5 Select patient or case set, enter gene symbol.

Comprehensive results were come out after submitting. As shown in Figure 6, 35 (8%) of 413 cases exist IL-22 mRNA upregulation. Meanwhile, several different types of data were also shown here, such as mutual exclusivity, plots, mutations and so on.

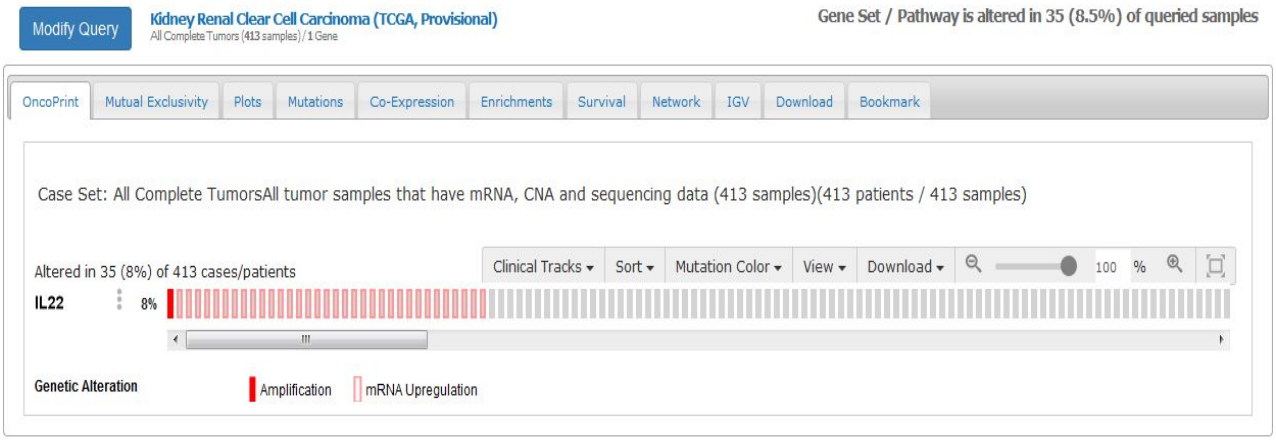

Figure 6 Comprehensive results were shown after submitting.

2.3 Patients and samples

60 cases tumor samples from non-metastatic ccRCC patients from Department of Urology, Hospital of the Ludwig-Maximilians-University Munich were included in the study. All patients were treated with radical or partial nephrectomy between December 1991 and July 2004. Diagnosis of ccRCC was confirmed by hematoxylin and eosin (H&E) staining by the Department of Pathology of the Hospital of the Ludwig-Maximilians-University Munich. Written, informed consent was obtained from all patients before surgery, and the study was approved by the Ethics Committee of the Hospital of the Ludwig-Maximilians-University Munich. Complete follow-up history was available for at least 5 years. Clinical and pathological parameters were entered into a database, included age, sex, Fuhrman grade, primary T stage, present of necrosis, venous thrombus and relapse. Cases in this study were re-assigned according to the 2010 AJCC TNM classification (Edge and Compton 2010)(Table 1). All the stages were classified into two groups, high stage (T3 and T4) and low stage (T1 and T2). All the tumor samples were embedded in paraffin after surgery, together with adjacent non-tumor tissue from the same patient. µ4µm-thick sections from tumor blocks were cut by microtome and allowed to air-dry overnight for the immunohistochemistry staining. Patients' clinical data are shown in Table 3.

Table 1 2010 AJCC TNM classification (Primary tumors stage)

Primary tumors (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Tumor ≤7 cm in greatest dimension, limited to the kidney
T1a	Tumor ≤4 cm in greatest dimension, limited to the kidney
T1b	Tumor >4 cm but ≤7 cm in greatest dimension, limited to the kidney
T2	Tumor >7 cm in greatest dimension, limited to the kidney
T2a	Tumor >7 cm but ≤10 cm in greatest dimension, limited to the kidney
T2b	Tumor >10 cm, limited to the kidney
T3	Tumor extends into major veins or perinephric tissues but not into the ipsilateral adrenal gland and not beyond the Gerota fascia
T3a	Tumor grossly extends into the renal vein or its segmental (muscle-containing) branches, or tumor invades perirenal and/or renal sinus fat but not beyond the Gerota fascia
T3b	Tumor grossly extends into the vena cava below the diaphragm
T3c	Tumor grossly extends into the vena cava above the diaphragm or invades the wall of the vena cava
T4	Tumor invades beyond the Gerota fascia (including contiguous extension into the ipsilateral adrenal gland)

2.4 Immunohistochemistry

Immunohistochemistry staining for IL-22, IL-22 receptor (IL-22RA1), IL-22 binding protein (IL-22BP), ki-67 and caspase-3 was performed as follows. Appropriate positive and negative controls were utilized for each run of immunohistochemistry

staining.

First, tissue slides were deparaffinizedn. For this, sections were incubated in two washes of xylene for 5 minutes each at room temperature. Then slides were incubated in three washes of 100% ethanol, two washes of 96% ethanol and one wash of 70% ethanol for 2 minutes each at room temperature, followed by washing sections twice in phosphate buffered saline (PBS) for 7 minutes each at room temperature.

The second step was endogenous peroxidase blocking. Sections were incubated in 3% hydrogen peroxide (20ml 30% hydrogen peroxide in 180ml methanol) for 20 minutes in dark at room temperature, followed by washing sections in PBS for 7 minutes at room temperature.

The third step was antigen unmasking.Slides were boiled (Microwave) in 10 mM sodium citrate buffer (pH 6.0): 300ml distilled water + 3ml antigen unmasking solution and then cooled on bench top for 30 minutes. Then slides were autoclaved with sodium citrate buffer for 20 minutes followed by 40 minutes cooling down. Sections were washed in PBS for 7 minutes at room temperature. For IL-22 and IL-22RA1 staining, proteinase K solution (1:400) was used for antigen unmasking instead of sodium citrate buffer. Added µ200µl proteinase K solution (1:400) to each section, incubated for 10 minutes at room temperature, and then washed in PBS for

7 minutes at room temperature.

The next step was antigen detection. Each section was first blocked with several drops of avidin blocking solution for 15 minutes at room temperature, and then washed briefly in PBS. Sections were then blocked with several drops of biotin blocking solution for 15 minutes at room temperature, followed by washing sections in PBS for 7 minutes. Before antibody incubation, tissue sections were incubated with 10% normal goat serum for 10 minutes at room temperature to block non-specific binding from the goat second antibody.

Primary and second antibody incubation was performed at this step. Antibody dilutionsare shown in table 2. µ200 µl of the diluted primary antibody solution were added to each section and incubated for 1 hour at room temperature or overnight at 4℃ and sections were washed twice in PBS for 7 minutes after incubation. 200µl biotinylated diluted second antibody solution were then added to each section and incubated for 30 minutes at room temperature followed by washing twice in PBS for 7 minutes.

The next step was ABC reagent incubation. ABC solution preparation: 15µl reagent A and 15µl reagent B to 1 ml PBS. 200µl ABC solution was added to each section and then incubated for 30 minutes at room temperature and then sections were washed twice in PBS for 5 minutes followed by washing in Tris-Hcl buffer (pH 7.6-7.8) twice for 5 minutes.

The last step was staining. Staining solution (37℃) preparation: 200 ml Tris-Hcl + 4ml DAB (3,3'-diaminobenzidine) solution + 1ml NiCl (Nickel chloride) + 500µl 3% H_2O_2 (hydrogen peroxide). At first sections were incubated in staining solution for 2 to 10 minutes at 37℃. The slides were then counterstained with methyl green for 2 minutes at room temperature. Then slides were incubated in three washes of 100% ethanol, two washes of 96% ethanol and one wash of 70% ethanol for 10 seconds each at room temperature, followed by incubating sections twice in xylene for 10 seconds each at room temperature, and then mounted coverslips with mounting medium. Images were captured with a LEICA AMIL instrument.

Table 2 Primary and second antibodies

Primary antibody	dilution	incubate time	second antibody
IL-22	1:100	1 hour	anti-goat antibody
IL-22RA1	1:200	1 hour	anti-rabbit antibody
IL-22BP	1:100	overnight	anti-rabbit antibody
Ki-67	1:50	1 hour	anti-mouse antibody
Caspase-3	1:50	overnight	anti-rabbit antibody

2.5 Staining Evaluation

2.5.1 IL-22 and IL-22BP staining evaluation

5 representative areas (X 100) from the entire slides were selected under the microscope. Positive signals were counted in all the 5 areas. The observer was

blinded to the outcome or characteristics of the patient when evaluating the staining.

2.5.2 IL-22RA1 staining evaluation

5 representative areas (X 100) from the tumor tissue as well as one area from adjacent non-tumor tissue were selected under microscope. Pictures of these areas were analyzed via Image J software as following: Opened the picture of adjacent non-tumor tissue in Image J, then selected 'Image' from the main menu of Image J → Color → Split Channels, the picture was split into three different channels—blue, green and red. Kept the green one, and selected 'Image' from the main menu of Image J → Adjust → Threshold, then adjusted the parameters until all the background was removed and positive signals were shown in the picture. Recorded the parameters and clicked 'Apply', then selected 'Analyze' from the main menu → Measure, then the staining value was calculated automatically. Same processing for pictures of tumor tissues was performed under the same parameters, in this way, the staining values could be compared between adjacent non-tumor tissue and tumor tissues from the same case, as well as normalised the tumor staining values by adjacent non-tumor staining values. The observer was blinded to the outcome or characteristics of the patient when evaluating the staining.

2.5.3 Ki-67 and Caspase-3 staining evaluation

A semiquantitative scoring method was used to evaluate ki-67 and caspase-3 staining. Both the staining intensity and the proportion of cells stained were assessed (Zhu, Cai et al. 2012). The total immunostaining scores were calculated as the intensity score (0= no staining, 1=weak staining, 2=moderate staining, 3=strong staining) × the proportion of positively stained cells (0 to 100%) The observer was blinded to the outcome or characteristics of the patient when evaluating the staining.

2.6 Statistical Analysis

Correlations between immunohistochemical variables and clinicopathological characteristics were analyzed by Fisher exact and chi-square tests. According to the NCI definition, overall survival (OS) refers to the length of time from either the date of diagnosis or the start of treatment for a disease, such as cancer, that patients diagnosed with the disease are still alive. Disease-free survival (DFS) was considered as the length of time after primary treatment for cancer ends that the patient survives without any signs or symptoms of that cancer. Depending on this, death and relapse were selected as two different end points during follow-up period to perform the Kaplan-Meier analyses, with log-rank tests assessing the differences between the groups. Multivariate Cox proportional hazards models were used to

analyze the impact of prognostic factors on overall survival and disease-free survival. All statistical tests were performed assuming significance at a level of $p<0.05$. Analyzes were performed using GraphPad Prism, version 5.04 and IBM SPSS Statistics, version22.

3. Results

3.1 Correlations between IL-22 and ccRCC patients' overall survival and disease-free survival from the cancer genome atlas

Initially the correlation between levels of IL-22 and ccRCC patients' survival from TCGA was analyzed. There were 35 cases (8%) with upregulation of IL-22 and other 378 cases (92%) without upregulation of IL-22, and the cases deceased were 18 and 127, respectively. Median survival was 62.81 and 90.8 months, respectively. Log-rank test showed p-value=0.0159, patients with IL-22 overexpression shorterlinked to shorter overall survival compared to cases without upregulation of IL-22. (Figure 7A) Similarly, in the disease-free survival Kaplan-Meier estimate, there were 28 cases (8%) with high level of IL-22 and 307 patients (92%) with low level of IL-22, the cases relapsed during the follow-up period were 14 and 94, respectively. Median disease-free survival was 32.52 and 123.72 months. Log-rank test showed p-value=0.0047, patients with upregulation of IL-22 had a shorter disease free survival compared to patients without this alteration in IL-22. (Figure 7B)

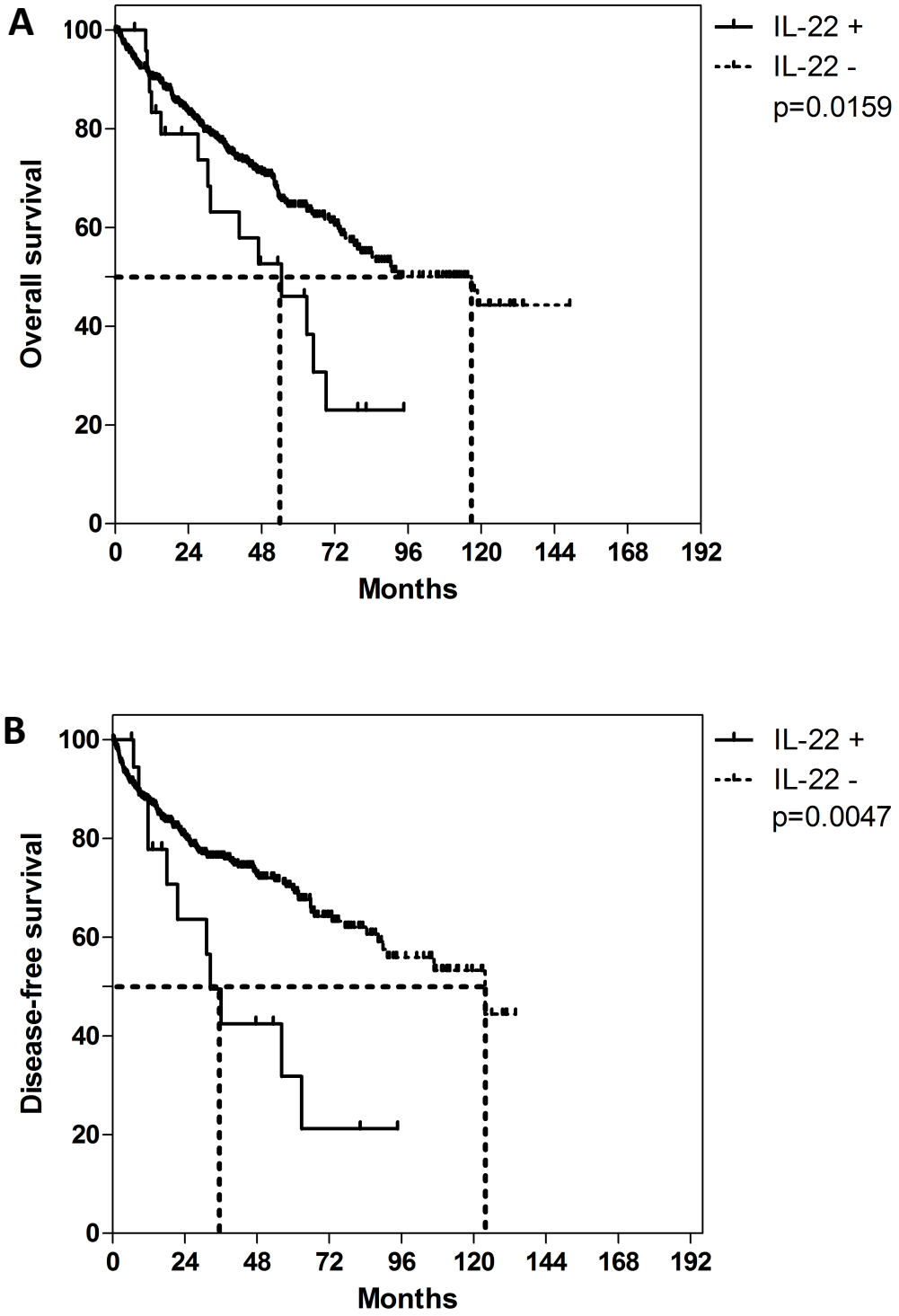

Figure 7 IL-22 Kaplan-Meier survival estimates. A Patients without IL-22 upregulation have a better overall survival. B Patients without IL-22 upregulation have a better disease-free survival.

3.2 Correlations between IL-22RA1 and ccRCC patients' overall survival and disease-free survival from the cancer genome atlas

Regarding IL-22RA1, the number of patients with and without IL-22RA1 was 33 (8%) and 380 (92%). 20 of the 33 cases died during the follow-up period, and 125 of the 380 cases died during this period. Median survival time was 45.27 months and 90.8 months, respectively. P-value=0.0013, which meant the overall survival was better in patients with low level of IL-22RA1 than patients with high level of IL-22RA1 (Figure 8A). Disease-free survival analysis is shown in Figure 8B. There were 23 cases (7%) with upregulation of IL-22RA1 and 312 cases (93%) without alteration, during the follow-up period, there were 12 cases and 96 cases relapsed, respectively. Meanwhile, the median disease-free time was 56.14 months and 123.72 months respectively. P-Value from the log-rank test was 0.0404, manifested a better disease-free survival in cases with low level of IL-22RA1, compared to patients with IL-22RA1 upregulation.

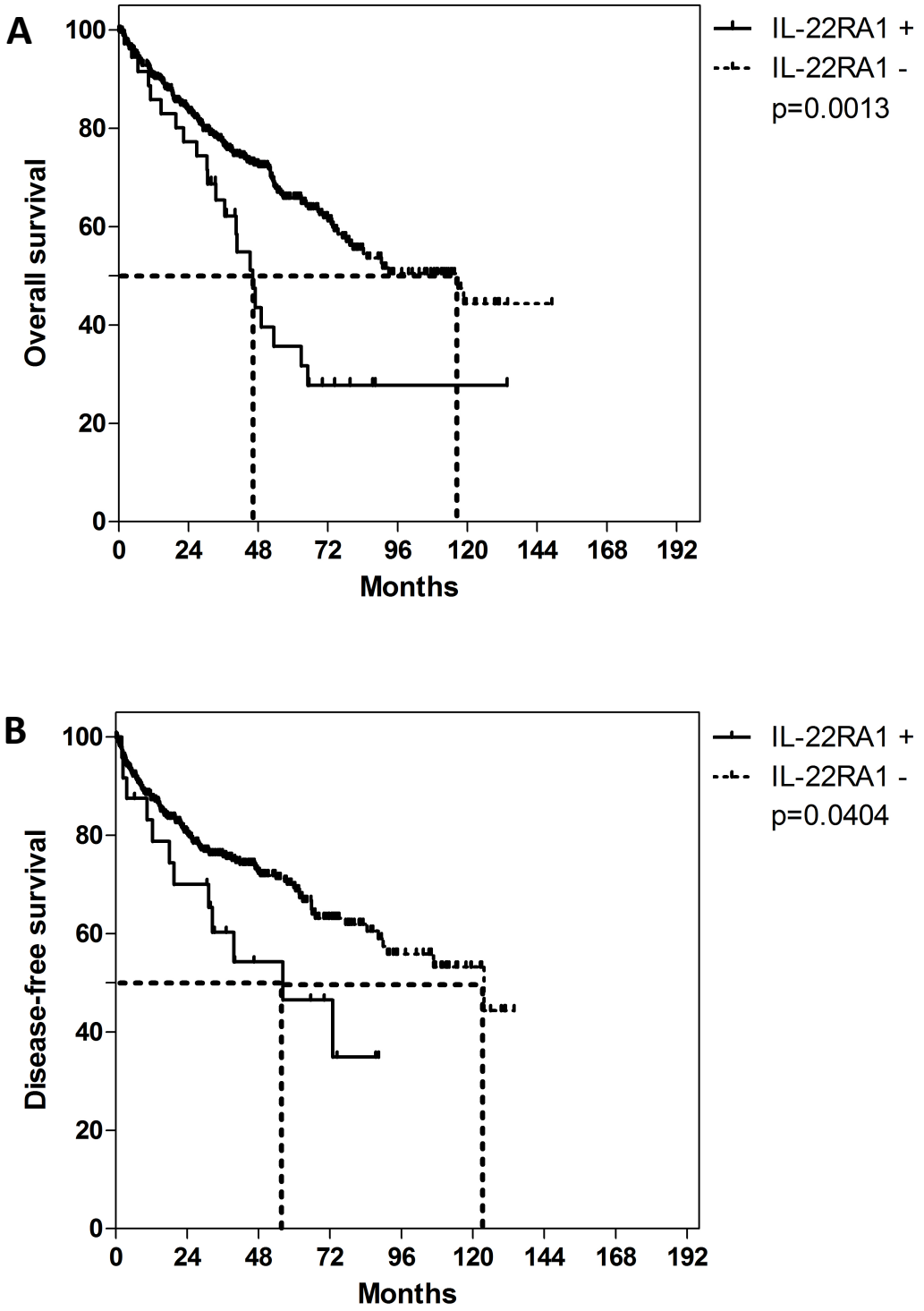

Figure 8 IL-22RA1 Kaplan-Meier survival estimates. A Patients without IL-22RA1 upregulation have a better overall survival. B Patients without IL-22RA1 upregulation have a better disease-free survival.

3.3 Clinical data of patient cohort of Munich

As listed in Table 4, among the 60 patients with ccRCC, 30 were female (50%), and 30 were male (50%), age ranged from 31 years to 77 years, the mean age was 59 years, among them, 27 (45%) patients aged less than or equal to 60 years, 33 (55%) patients aged more than 60 years. Fuhrman grades were estimated by the department of pathology using the Fuhrman grading system, among these 60 patients, 46 cases were in low Fuhrman grade (G1 or G2) (76.7%), and 14 cases were in high Fuhrman grade (G3 or G4) (23.3%). Pathological stages were re-assigned according to the 2010 American Joint Committee on Cancer (AJCC) TNM classification. The primary T stages were low (T1 or T2) in 46 patients (76.7%) and high (T3 or T4) in 14 patients (23.3%). The existence of tumor necrosis was judged by conventional hematoxylin and eosin staining by pathologists. Tumor necrosis was presented in 16 cases (26.7%) and absent in 44 cases (73.3%). The presence of venous thrombus was determined by imaging examination such as computerised tomography (CT) scan, Magnetic Resonance Imaging (MRI) and ultrasonic examination, as well as venous thrombus observation during or after surgery. Venous thrombus was presented in 8 patients (13.3%) and absent in 52 patients (86.7%).

During the follow-up period, 18 patients (30%) had disease recurrence and a total of 23 patients (38.3%) died due to all causes. The presence of relapse was assessed by

imaging examination and pathological examination during the follow-up period. Relapses included local kidney tumor recurrence in the original location, a regional recurrence that occurred in the lymph nodes as well as metastasis in other parts of the body or organs. In these 60 cases, 18 patients had disease recurrence, including local recurrence in 5 patients, lymph nodes recurrence in 2 cases, bone metastasis in 7 cases, brain metastasis in 3 patients, liver metastasis in 2 patients, lung metastasis in 8 cased and skin metastasis in one patient. As more than one recurrence site was found in some patients, the number of recurrence sites is shown in Table 3. The follow-up period ranged from 13 months to 182 months, and the median follow-up time was 78 months for the cohort.

Table 3 Patients' clinical data.

NO.	Age	Sex	Fuhrman grade	Primary T stage	Necrosis	Venous thrombus	Relapse	Vital status	Follow-up time (month)
1	50	0	1	T1A	0	0	0	0	106
2	37	0	1	T1B	3	0	0	0	175
3	63	1	1	T1B	1	0	0	0	143
4	66	1	1	T1B	0	0	0	0	48
5	74	1	1	T1B	0	0	0	0	75
6	65	1	1	T2B	0	0	0	0	178
7	49	1	1	T3A	1	0	0	0	179
8	68	0	2	T1A	0	0	0	0	22
9	73	0	2	T1A	0	0	0	0	54
10	34	1	2	T1A	0	0	0	0	129
11	47	1	2	T1A	1	0	0	0	182
12	55	1	2	T1A	0	0	0	0	105
13	58	1	2	T1A	0	0	0	0	132
14	62	1	2	T1A	0	0	0	0	145
15	65	1	2	T1A	0	0	0	0	172
16	66	1	2	T1A	1	0	0	0	182
17	31	0	2	T2A	1	0	0	0	124
18	34	0	2	T2A	0	0	0	0	13

19	49	0	2	T2A	3	0	0	0	142
20	49	0	2	T2A	0	0	0	0	26
21	51	0	2	T2A	0	0	0	0	100
22	55	0	2	T2A	0	0	0	0	106
23	64	0	2	T2A	0	0	0	0	86
24	65	0	2	T2A	0	0	0	0	148
25	68	0	2	T2A	0	0	0	0	98
26	62	1	2	T2A	0	0	0	0	26
27	75	1	2	T2A	0	0	0	0	122
28	52	0	2	T2B	0	0	0	0	78
29	67	0	2	T2B	0	0	0	0	70
30	72	0	2	T2B	0	0	0	0	89
31	36	1	2	T2B	0	0	0	0	77
32	73	1	2	T2B	0	0	0	0	42
33	71	0	1	T1A	0	0	0	1	80
34	41	1	1	T1A	0	0	0	1	143
35	77	1	1	T1B	0	0	0	1	54
36	69	1	1	T2A	0	0	0	1	88
37	45	0	2	T2A	0	0	0	1	52
38	66	0	2	T2A	0	0	0	1	42
39	75	0	2	T2A	0	0	0	1	75
40	76	0	2	T2A	0	0	0	1	51

41	57	1	3	T3B	3	1	0	1	74
42	60	1	3	T3B	3	2	0	1	36
43	53	1	2	T3B	0	2	1	1	54
44	58	1	2	T2A	0	0	2	0	111
45	63	1	3	T1A	0	0	2	0	96
46	50	0	3	T2A	0	0	2	0	58
47	65	0	2	T3B	1	1	2	0	13
48	76	1	2	T3B	0	1	2	0	24
49	38	0	2	T3A	2	0	2	1	39
50	57	0	3	T1B	0	0	2	1	60
51	62	0	3	T2A	0	0	2	1	31
52	58	1	3	T2A	1	0	2	1	151
53	62	0	3	T3B	0	1	2	1	52
54	69	0	3	T3B	1	1	2	1	38
55	61	1	3	T3B	3	1	2	1	124
56	66	1	2	T3A	0	0	3	1	37
57	72	1	3	T1B	0	0	3	1	36
58	59	1	3	T3A	0	0	3	1	57
59	44	0	4	T4	1	0	3	1	26
60	62	0	3	T4	3	0	3	1	80

Sex: 0=female; 1=male. Vital status: 0=alive; 1=dead

Table 4 Patients' cohort.

		NO (60)	%
Age	<=60	27	45
	>60	33	55
Sex			
	Male	30	50
	Female	30	50
Fuhrman grade			
	Low(G1/G2)	46	76.7
	High(G3/G4)	14	23.3
Primary T stage			
	Low(pT1/pT2)	46	76.7
	High(pT3/pT4)	14	23.3
Necrosis			
	Absent	44	73.3
	Present	16	26.7
Venous thrombus			
	Absent	52	86.7
	Present	8	13.3
Relapse			
	Absent	42	70
	Present	18	30
Vital status			
	Alive	37	61.7
	Dead	23	38.3

3.4 Immunohistochemical detection of IL-22, IL-22RA1 IL-22BP, Ki-67 and caspase-3

IL-22 staining was present predominantly in the cytoplasm. The staining pattern is shown in Figure 9. There is only a little expression in adjacent normal kidney tissue. High expression of IL-22 was identified as equal or more than 20 positive signals in all 5 areas randomly selected under microscope, low expression of IL-22 was identified as less than 20 positive signals or negative result, the number of cases expressing high and low IL-22 was 28 (46.7%) and 32 (53.3%), respectively.

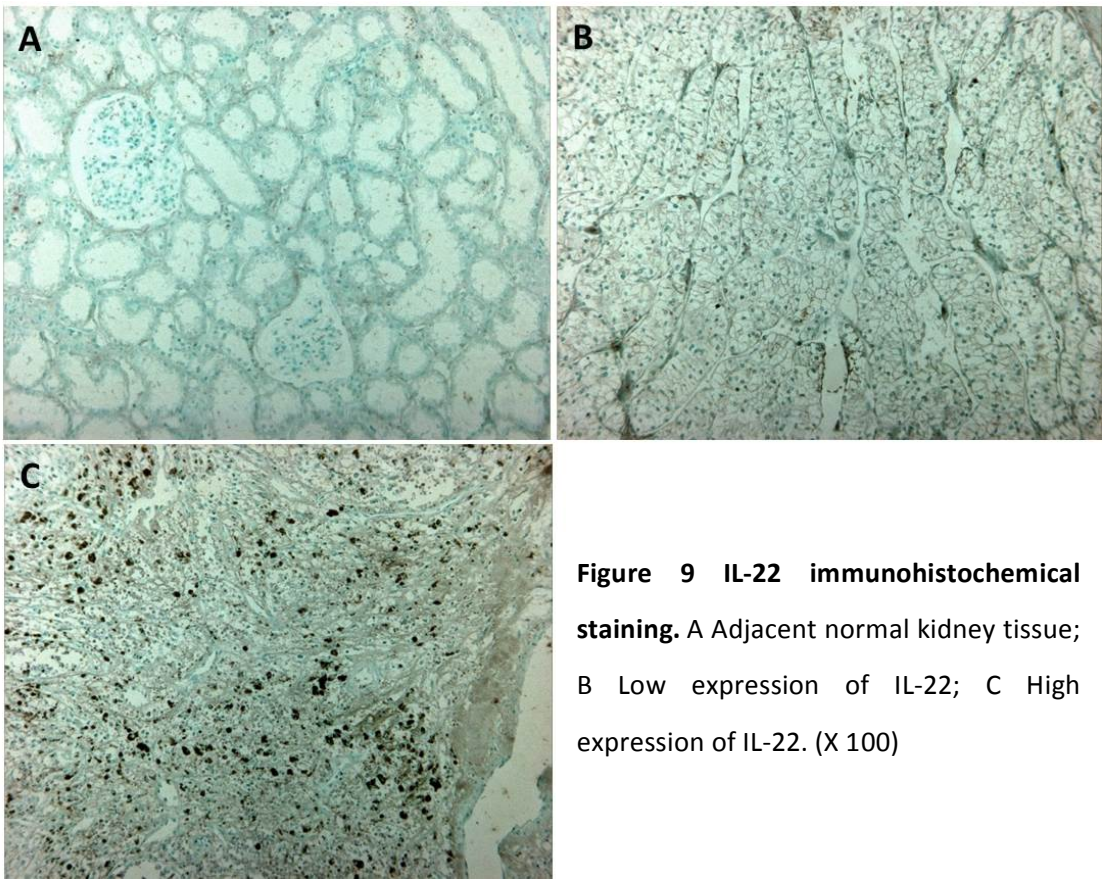

Figure 9 IL-22 immunohistochemical staining. A Adjacent normal kidney tissue; B Low expression of IL-22; C High expression of IL-22. (X 100)

Similarly, IL-22RA1 staining was present in the cytoplasm of tumor cells, as shown in Figure 10. There is a moderate expression of IL-22RA1 in adjacent normal kidney tissue. High expression of IL-22RA1 was defined as equal or more than 20 score measured by Image J software mentioned before; low expression of IL-22RA1 was defined as less than 20 score. The number of cases with high expression and low expression of IL-22RA1 was 34 (56.7%) and 26 (43.3%), respectively.

IL-22BP staining was only found in adjacent normal kidney tissue with moderate cytoplasmic staining and luminal membrane positivity in tubules. Positive staining for IL-22BP in tumor tissue slides was not detected, as shown in Figure 11.

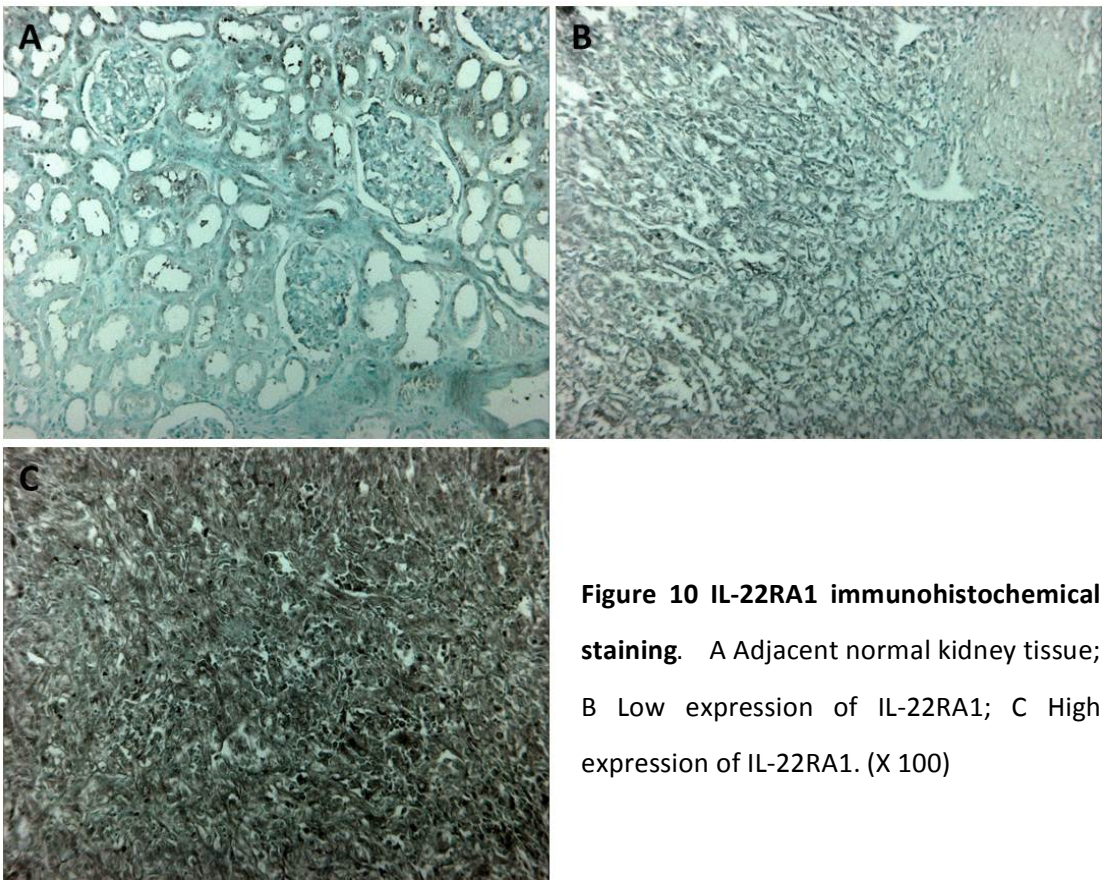

Figure 10 IL-22RA1 immunohistochemical staining. A Adjacent normal kidney tissue; B Low expression of IL-22RA1; C High expression of IL-22RA1. (X 100)

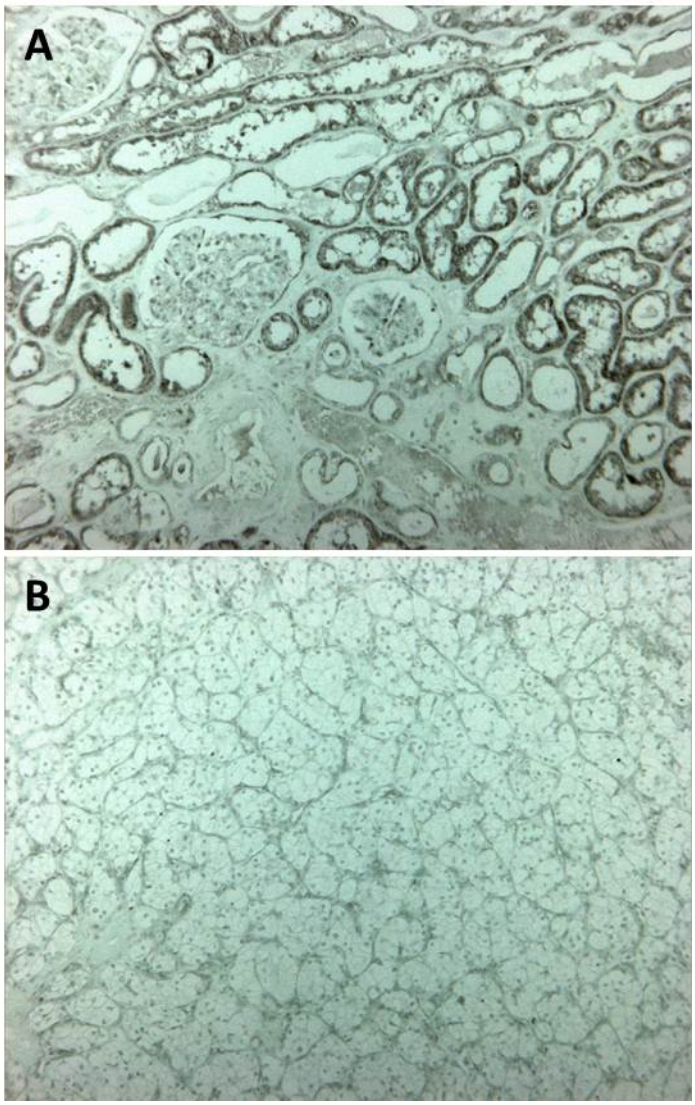

Figure 11 IL-22BP immunohistochemical staining. A Adjacent normal kidney tissue; B ccRCC tumor tissue. (X 100)

Ki-67 staining is shown in Figure 12. Very few positive cells can be seen in adjacent normal kidney tissue. Depending on the results, High expression of Ki-67 was identified as equal or more than 30 score (mean value), low expression of Ki-67 was identified as less than 30 score or negative result.

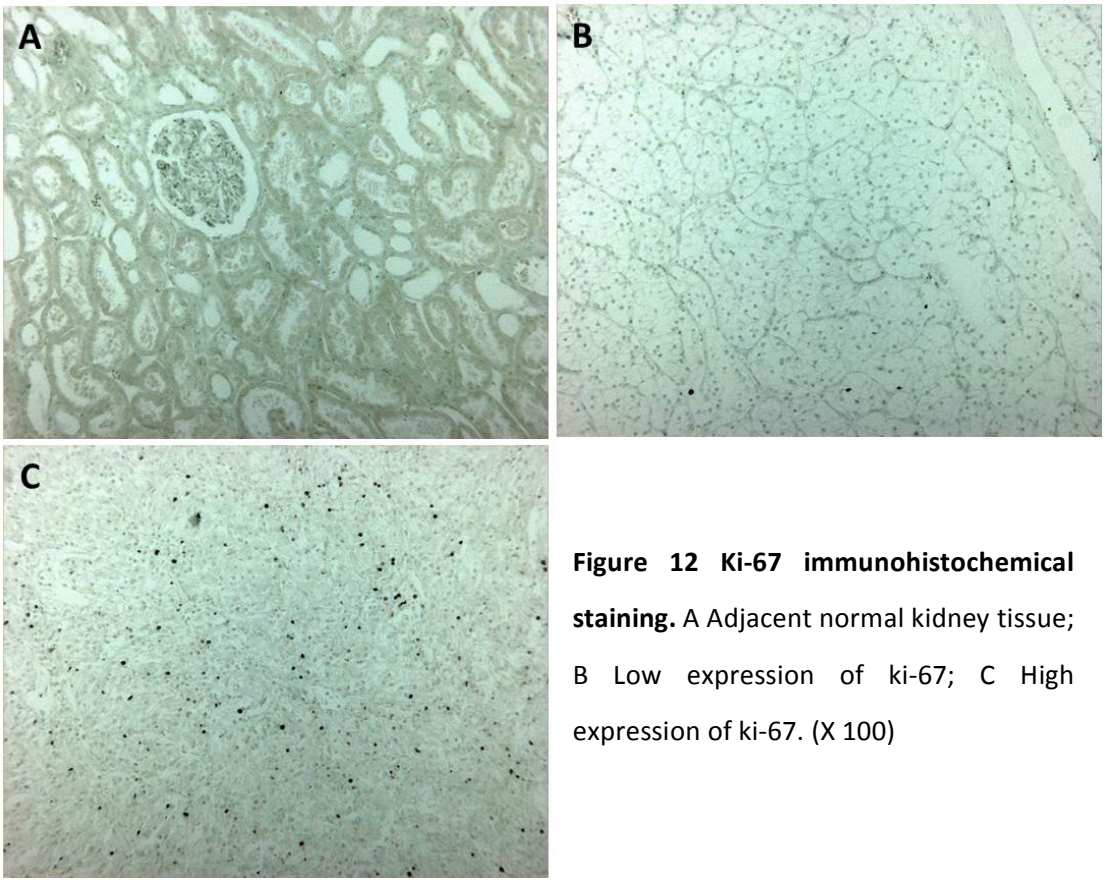

Figure 12 Ki-67 immunohistochemical staining. A Adjacent normal kidney tissue; B Low expression of ki-67; C High expression of ki-67. (X 100)

Caspase-3 staining is shown in Figure 13. Several cells were stained in adjacent normal kidney tissue. Depending on the staining evaluation results, High level of caspase-3 was also identified as equal or more than 30 score (mean value), low level of caspase-3 was identified as less than 30 score or negative result.

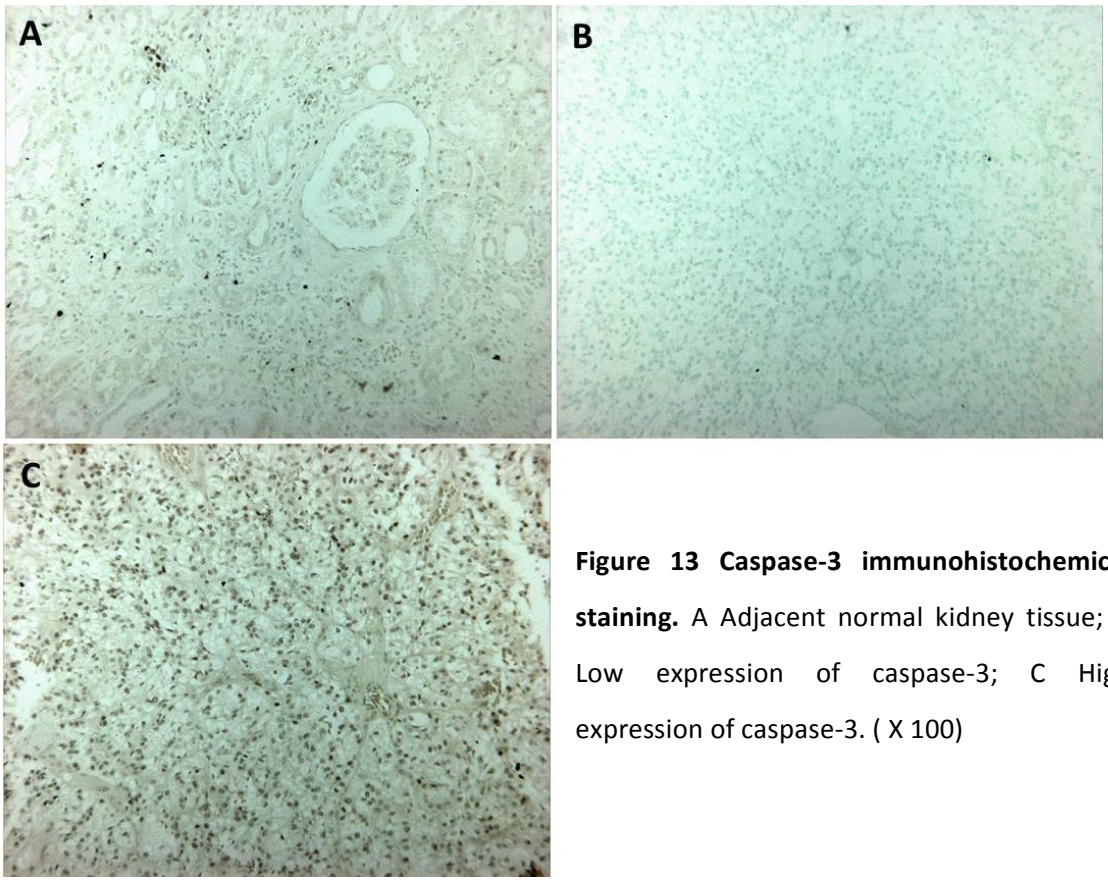

Figure 13 Caspase-3 immunohistochemical staining. A Adjacent normal kidney tissue; B Low expression of caspase-3; C High expression of caspase-3. (X 100)

3.5 Correlations between IL-22 expression and ccRCC patients' clinicopathologic characteristics

As listed in Table 5, in the group which patients aged equal or less than 60 years old, there were 15 cases and 12 cases with high IL-22 expression and low IL-22 expression, respectively. While in the group aged more than 60 years old, the cases expressing high level and low level of IL-22 were 13 and 20, respectively. There was no significant difference in IL-22 expression between two groups. (p=0.2988)

In terms of gender, in male patients, there were 14 cases and 16 cases expressing

high and low levels of IL-22. Meanwhile there were also 14 cases and 16 cases with high and low expression of IL-22, respectively in female patients. This meant there was no significance of IL-22 expression between genders. (p=1)

In the aspect of Fuhrman grade, the numbers of cases with high and low expression of IL-22 were 17 and 29 in low Fuhrman grade (G1 and G2) group. However, these two values were 11 and 3 in patients with high Fuhrman grade (G3 and G4). According to Fisher's exact test analysis, there was significance of IL-22 expression between low Fuhrman grade group and high Fuhrman grade group. (p=0.0126)

Similarly, there was also a significant difference in IL-22 expression in terms of primary T stage between low stage (pT1 and pT2) and high stage (pT3 and pT4). Same as Fuhrman grade, there were 17 and 29 cases expressing IL-22 in high levels and low levels in low primary T stage group, and 11 cases expressing high IL-22 in high T stage group, which only 3 patients had IL-22 low expression.

As to necrosis, in patients whose tumor without necrosis, there were 18 patients expressing high levels of IL-22 and the other 26 cases expressing low levels of IL-22, while in patients with necrosis, these values were 10 and 6. There was no significance of IL-22 expression between these two groups. (p=0.1569)

When it comes to venous thrombus, there were 23 cases without venous thrombus

expressing high levels of IL-22, and this number was 29 in low levels of IL-22. However, in venous thrombus present patients, 5 of them expressing high levels of IL-22 and the other 3 expressing low levels of IL-22. Also here there was no significance between two groups. (p=0.4544)

Next, the correlation between expression of IL-22 and relapse of disease was analyzed. As shown in the table 5, among patients who did not suffer relapse, there were 15 and 27 cases expressing high levels of IL-22 and low levels of IL-22, respectively. While in patients that relapsed, these two numbers turned to 12 and 6. Interestingly, this difference was statistically significant between two groups (p=0.0463).

A similar result was shown in vital status, there were 37 patients still alive at the end of our follow-up period, among them 12 patients showed a high level IL-22 expression, the other 25 patients showed low level of IL-22 expression. In patients who were dead during the follow-up period, the numbers of cases expressing high levels and low levels of IL-22 were 16 and 7. Similar to result of relapse, there was also statistical significance between two different groups. (p=0.0077)

Table 5Correlations between IL-22 expression and clinicopathologic characteristics

	IL-22 High	IL-22 Low	p-value
Age			0.2988
<=60	15	12	
>60	13	20	
Sex			1
Male	14	16	
Female	14	16	
Fuhrman grade			0.0126
Low(G1/G2)	17	29	
High(G3/G4)	11	3	
Primary T stage			0.0126
Low(pT1/pT2)	17	29	
High(pT3/pT4)	11	3	
Necrosis			0.1569
Absent	18	26	
Present	10	6	
Venous thrombus			0.4544
Absent	23	29	
Present	5	3	
Relapse			0.0463
Absent	15	27	
Present	12	6	
Vital status			0.0077
Alive	12	25	
Dead	16	7	

3.6 Correlations between IL-22RA1 expression and ccRCC patients' clinicopathologic characteristics

As shown in Table 6, in the aspect of age, in the group which patients aged equal or less than 60 years old, there were 15 cases and 12 cases with high IL-22RA1 expression and low IL-22RA1 expression, respectively. However, in the other group which patients were more than 60 years old, the number of cases expressing high levels and low levels of IL-22RA1 were 19 and 14. There was no significant difference of IL-22RA1 expression between two groups. (p=1)

Regarding gender, there were 21 cases and 9 cases expressing a high and low level of IL-22RA1, respectively in male patients. Meanwhile there were also 13 cases and 17 cases with high and low expression of IL-22RA1 in female patients. This meant there was no significance of IL-22R1 expression between genders (p=0.0673).

In the aspect of Fuhrman grade, the numbers of cases with high and low expression of IL-22RA1 were 21 and 25 in low Fuhrman grade (G1 and G2) group. However, these two values were 13 and 1 in patients with high Fuhrman grade (G3 and G4). According to Fisher's exact test analysis, there was a significance of IL-22RA1 expression between low Fuhrman grade group and high Fuhrman grade group (p=0.0018).

However, there was no significant difference (p=0.0718) of IL-22RA1 expression in terms of primary T stage between low stage (pT1 and pT2) and high stage (pT3 and pT4). There were 23 and 23 cases expressing IL-22RA1 in high levels and low levels, respectively in low primary T stage group, and 11 cases expressed high IL-22RA1 in high T stage group, which only 3 patients had IL-22RA1 low expression.

As to necrosis, in patients whose tumor lacked necrosis, there were 22 patients expressing high levels of IL-22RA1 and low levels of IL-22RA1 each, while in necrosis present patients, there were 12 patients expressed a high level of IL-22RA1 and another 4 cases expressed a low level of IL-22RA1. Obviously, the difference of IL-22RA1 expression was not statistically significant between these two groups. (p=0.1399)

In terms of venous thrombus, there were 28 cases without venous thrombus expressing high levels of IL-22RA1, and this number was 24 in low levels of IL-22RA1. However, in venous thrombus present patients, 6 of them expressing high levels of IL-22RA1 and the other 2 expressing low levels of IL-22RA1. The difference of IL-22RA1 expression was not statistically significant between these two groups. (p=0.4463)

The correlation between expression of IL-22RA1 and relapse was analyzed. As shown in the table 5, in patients who did not have relapse, there were 18 and 24 cases

expressing high levels of IL-22RA1 and low levels of IL-22RA1, respectively. While in patients that relapsed, these two numbers became 16 and 2. Interestingly, the difference of IL-22RA1 expression was statistically significant between these two groups. (0.0014).

A similar result was seen in terms of vital status, there were 37 patients still alive at the end of follow-up period, among them 21 patients showed high levels of IL-22RA1 expression, the other 16 patients showed low levels of IL-22RA1 expression. In patients who were dead during the follow-up period, the numbers of cases expressing high levels and low levels of IL-22RA1 were 18 and 5, respectively. Similar to relapse, this difference was statistically significant between two groups. (p=0.015)

Table 6 Correlations between IL-22RA1 expression and clinicopathologic characteristics

	IL-22RA1 High	IL-22RA1 Low	p-value
Age			1
<=60	15	12	
>60	19	14	
Sex			0.0673
Male	21	9	
Female	13	17	
Fuhrman grade			0.0018
Low(G1/G2)	21	25	
High(G3/G4)	13	1	
Primary T stage			0.0718
Low(pT1/pT2)	23	23	
High(pT3/pT4)	11	3	

Necrosis				0.1399
	Absent	22	22	
	Present	12	4	
Venous thrombus				0.4463
	Absent	28	24	
	Present	6	2	
Relapse				0.0014
	Absent	18	24	
	Present	16	2	
Vital status				0.0150
	Alive	16	21	
	Dead	18	5	

3.7 Correlations between IL-22 and ccRCC patients' overall survival and disease-free survival

As mentioned above, patients were separated to two groups, IL-22 low group and IL-22 high group, depending on IL-22 expression. First, the overall survival was compared between these two groups. As shown in Figure 14A, the difference of overall survival was statistically significant between two groups (p=0.0031). High expression of IL-22 was associated with shorter overall survival compared with low expression of IL-22. Log-rank test showed Chi-square value was 8.719, hazard ratio was 3.616, 95% CI of ratio was 1.541 to 8.488. Next, the disease-free survival between two groups was compared. The difference was also significance (p=0.0422). High expression of IL-22 was associated with shorter disease-free survival compared

with low expression of IL-22. Log-rank test showed Chi-square value was 4.128, hazard ratio was 2.641, 95% CI of ratio was 1.035 to 6.741. (Figure 14B) Patients with high expression of IL-22 had a shorter overall survival and disease-free survival compared to patients with low expression of IL-22.

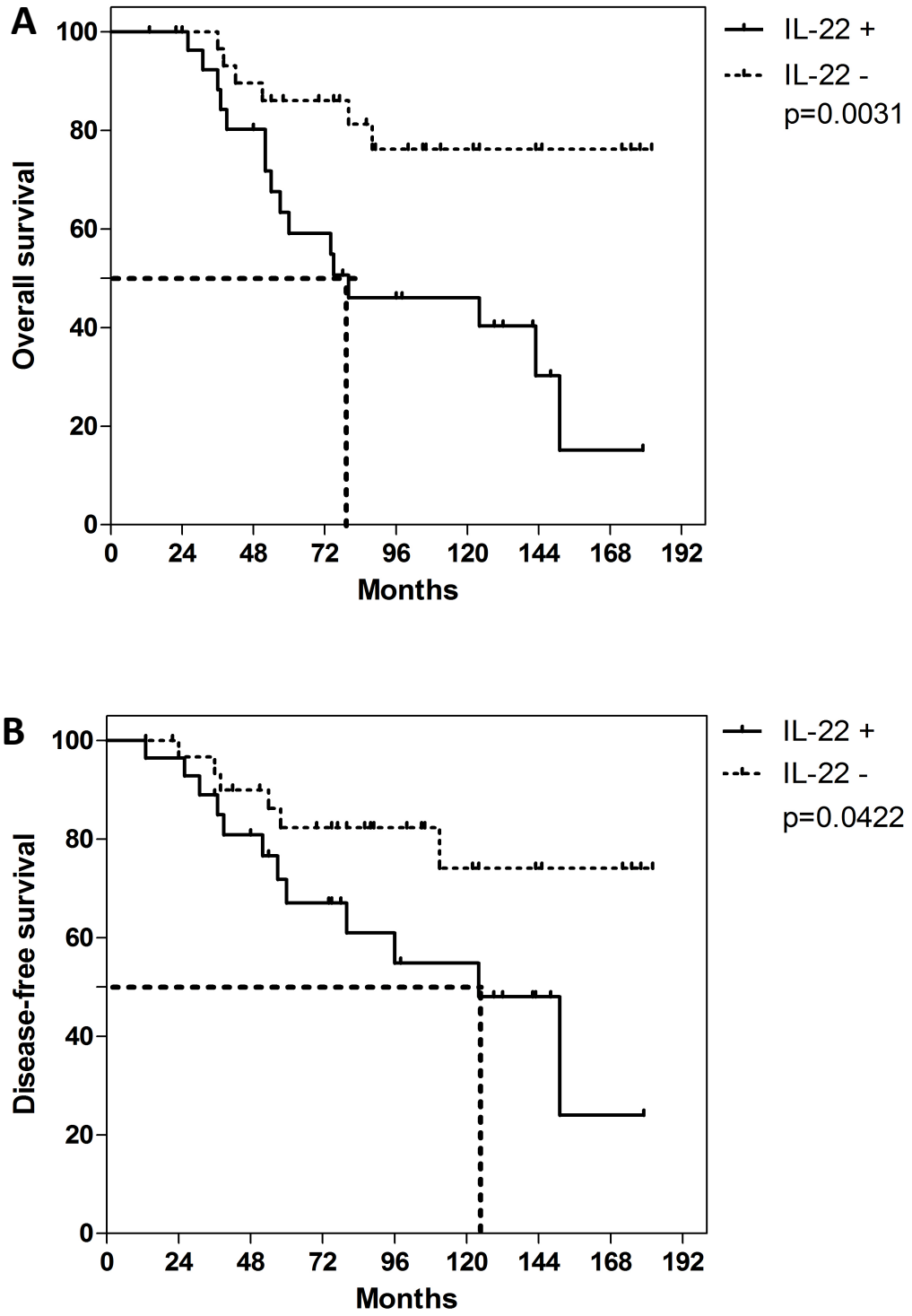

Figure 14 IL-22 Kaplan-Meier survival estimates. A Patients with low IL-22 expression have a better overall survival. B Patients with low IL-22 expression have a better disease-free survival.

3.8 Correlations between IL-22RA1 and ccRCC patients' overall survival and disease-free survival

In the same way, the overall survival and disease-free survival were compared between two groups which expressing low levels of IL-22RA1 and high levels of IL-22RA1. As shown in Figure 15A, the difference of overall survival was significant between these two groups of patients (p=0.0196). High expression of IL-22RA1 was associated with poor patient overall survival compared with low expression of IL-22RA1. Chi-square was analyzed by log-rank test, which was 5.451. Hazard ratio was 2.671, 95% CI of ratio was 1.171 to 6.095. Similarly, the difference of disease-free survival was also significant between high and low levels of IL-22RA1 expression patients (p=0.0036), high expression of IL-22RA1 was associated with poor patient disease-free survival compared with low expression of IL-22RA1. Log-rank test showed Chi-square was 8.470. Hazard ratio was 3.978 and 95% CI of ratio was 1.570 to 10.080 (Figure 15B). Patients with high expression level of IL-22RA1 had a shorter overall survival and disease-free survival compared to patients with low expression pattern of IL-22RA1

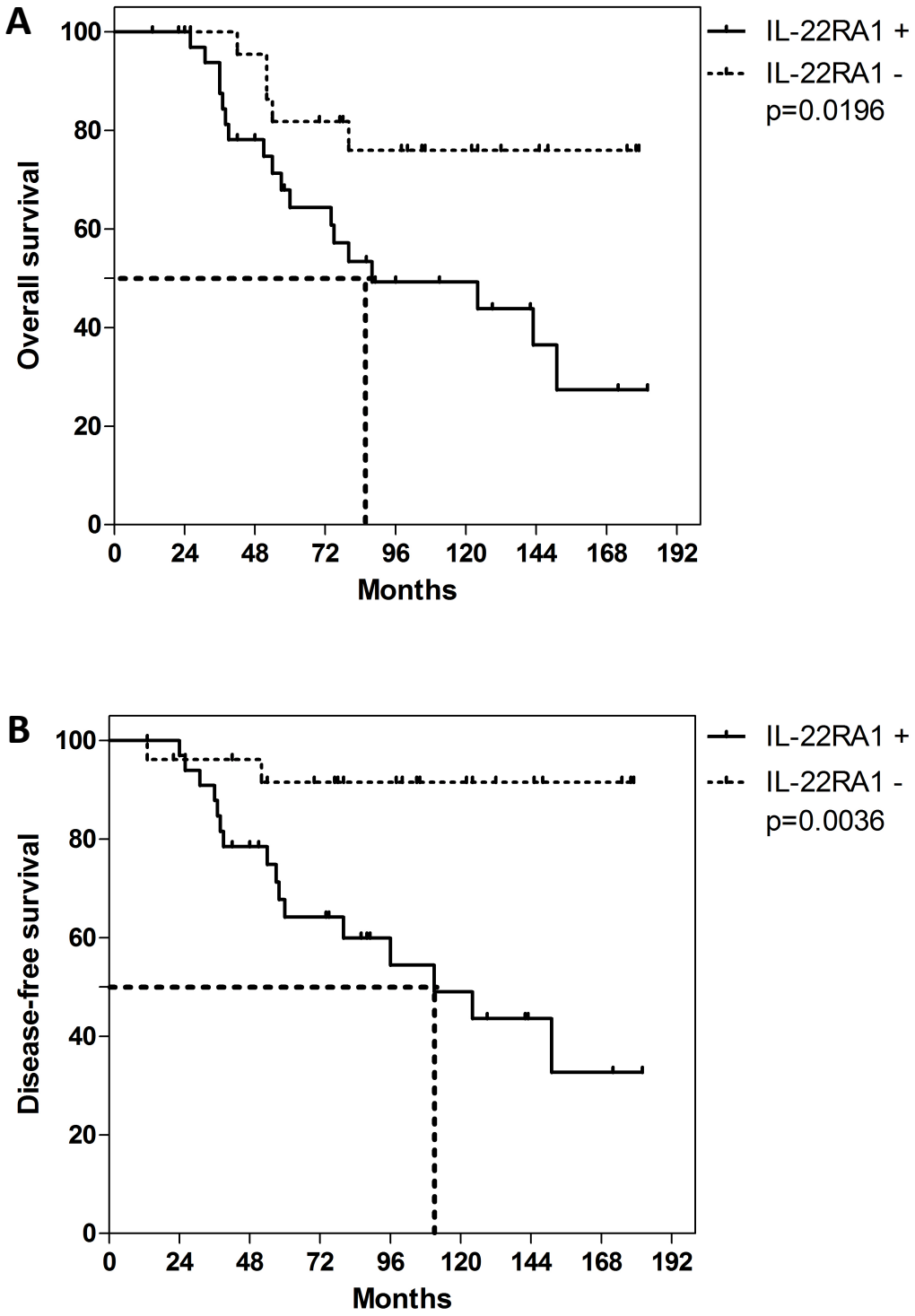

Figure 15 IL-22R1 Kaplan-Meier survival estimates. A Patients with low expression level of IL-22RA1 have a better overall survival. B Patients with low expression level of IL-22RA1 have a better disease-free survival.

3.9 Correlations between Ki-67 and ccRCC patients' overall survival and disease-free survival

Moreover, the overall survival and disease-free survival between patients expressing low levels and high levels of Ki-67 cells within tumors was compared. As shown in Figure 16A, the difference of overall survival was significant between these two groups of patients (p=0.0461). High expression of Ki-67 was associated with shorter patient overall survival compared with low expression of Ki-67. Chi-square was analyzed by log-rank test, which was 3.976. Hazard ratio was 2.309, 95% CI of ratio was 1.014 to 5.258. Similarly, the difference of disease-free survival was also significant between patients with high and low levels of Ki-67 expression (p=0.0171). High expression of Ki-67 was associated with shorter patient disease-free survival compared with low expression of Ki-67. Log-rank test showed Chi-square value was 5.681, hazard ratio was 3.096 and 95% CI of ratio was 1.222 to 7.839. (Figure 16B) Patients with high expression level of Ki-67 had a shorter overall survival and disease-free survival compared to patients with low expression pattern of Ki-67.

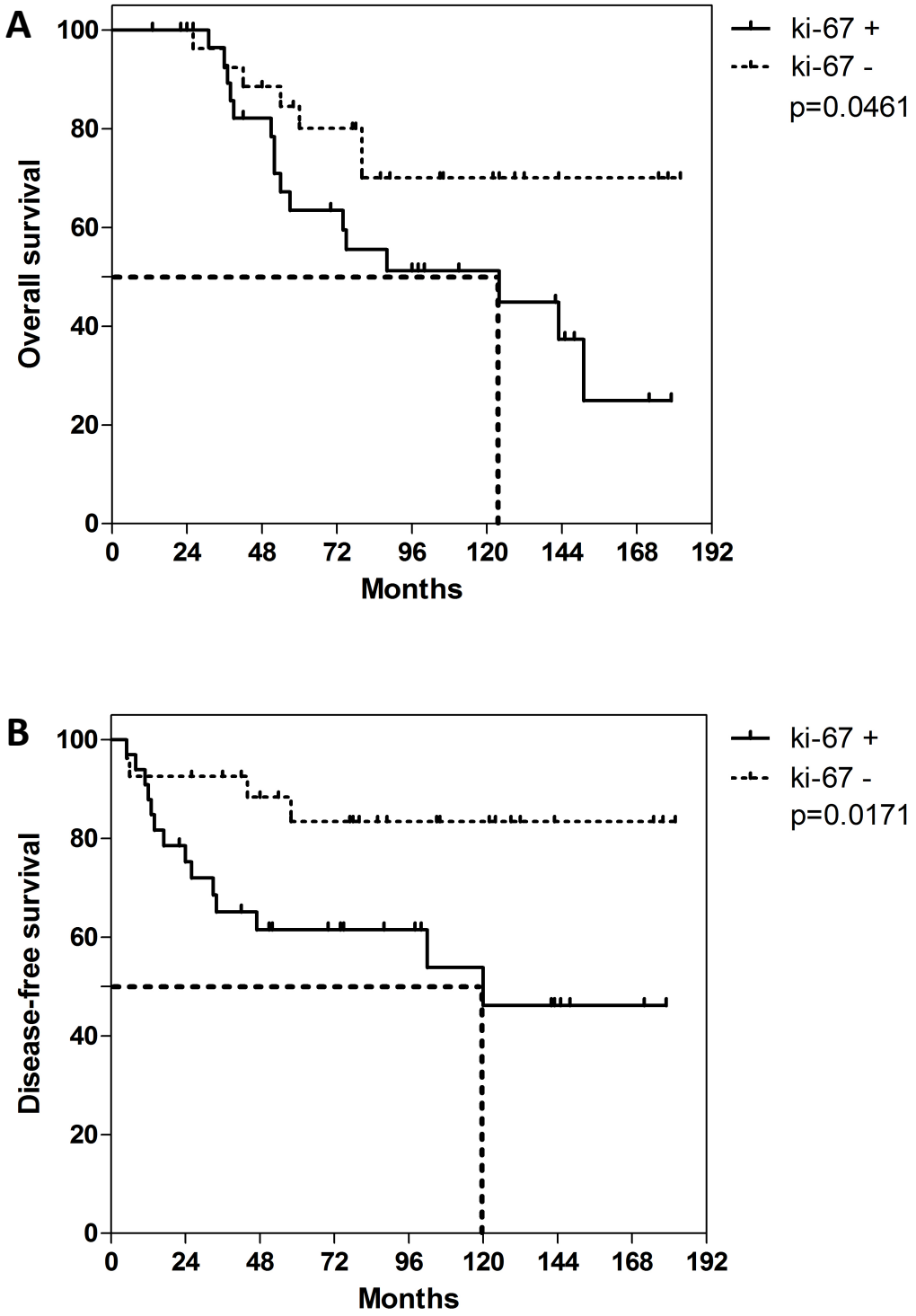

Figure 16 Ki-67 Kaplan-Meier survival estimates. A Patients with low expression level of ki-67 have a better overall survival. B Patients with low expression level of ki-67 have a better disease-free survival.

3.10 Correlations between Caspase-3 and ccRCC patients' overall survival and disease-free survival

In addition, a comparison between patients expressing low levels and high levels of caspase-3 was performed. As shown in Figure 17A, there was no significance (p=0.4953) in the difference of overall survival between these two groups of patients. However, there was significance between them in disease-free survival, p=0.0432 (Figure 17B). High expression of caspase-3 was associated with shorter disease-free survival of ccRCC patients. Chi-square value was 4.078, hazard ratio was 2.727, and 95% CI of ratio was 1.031 to 7.211. Patients with high expression level of caspase-3 had a shorter disease-free survival compared to patients with low expression pattern of caspase-3.

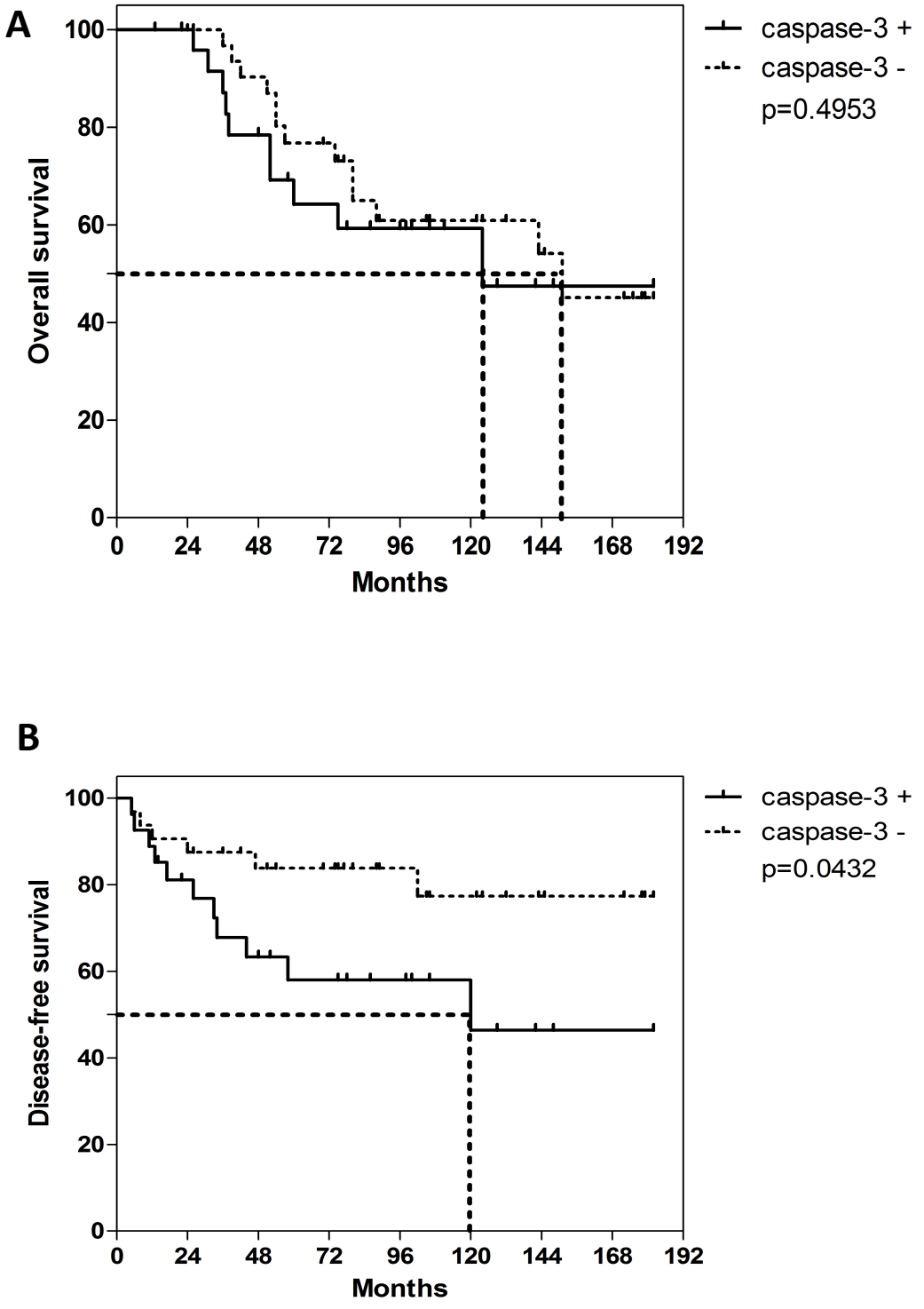

Figure 17 Casspase-3 Kaplan-Meier survival estimates. A Patients with high expression and low expression level of caspase-3 have no significant difference in overall survival. B Patients with low expression level of caspase-3 have a better disease-free survival.

3.11 Multivariate Cox proportional hazards regression models for ccRCC patients' overall survival and disease-free survival

As list in Table 7 and 8, the independent prognostic values of various parameters were assessed via the multivariate Cox proportional hazards model. Parameters included age (<=60 years vs. >60 years), sex (male vs. female), Fuhrman grade (G1/2 vs. G3/4), stage (pT1/2 vs. pT3/4), necrosis (absent vs. present), venous thrombus (absent vs. present), IL-22 expression (high vs. low), IL-22RA1 expression (high vs. low), Ki-67 expression (high vs. low) and caspase 3 expression (high vs. low). The results showed that tumor stage (HR, 6.046; 95% CI, 0.041 to 0.659; p=0.011), necrosis in tumor (HR, 6.78; 95% CI, 1.562 to 29.433; p=0.011) and IL-22RA1 expression (HR, 3.242; 95% CI, 0.096 to 0.991; p=0.048) were independent prognostic markers for mortality in patients with ccRCC after controlling for conventional clinicopathologic factors. Meanwhile, Fuhrman grade (HR, 5.780; 95% CI, 0.035 to 0.853; p=0.031), tumor stage (HR, 55.296; 95% CI, 0.001 to 0.258; p=0.003), venous thrombus (HR, 22.082; 95% CI, 1.584 to 36.722; p=0.021) and IL-22RA1 expression (HR, 75.355; 95% CI, 1.87 to 20.798; p=0.022) were independent prognostic marker for recurrence.

Table 7 Multivariable Cox proportional hazards regression model for overall survival

Parameter	Variable	HR	95% CI	p Value
Age	<=60 vs. >60	0.938	0.382 to 2.301	0.889
Sex	male vs. female	2.557	0.906 to 7.213	0.076
Fuhrman grade	G1/2 vs. G3/4	2.019	0.510 to 7.998	0.317
Stage	p1/2 vs. p3/4	6.046	1.516 to 24.102	0.011
Necrosis	absent vs. present	6.780	1.562 to 29.433	0.011
Venous thrombus	absent vs. present	2.143	0.488 to 9.419	0.313
IL-22	high vs. low	1.738	0.461 to 6.549	0.414
IL-22RA1	high vs. low	3.242	1.010 to 10.412	0.048
Ki-67	high vs. low	0.819	0.274 to 2.447	0.720
Caspase-3	high vs. low	0.565	0.173 to 1.843	0.344

Table 8 Multivariable Cox proportional hazards regression model for disease-free survival

Parameter	Variable	HR	95% CI	p Value
Age	<=60 vs. >60	0.629	0.141 to 2.798	0.543
Sex	male vs. female	1.950	0.235 to 16.192	0.536
Fuhrman grade	G1/2 vs. G3/4	5.780	1.172 to 28.518	0.031
Stage	p1/2 vs. p3/4	55.296	3.883 to 787.444	0.003
Necrosis	absent vs. present	0.273	0.026 to 2.868	0.279
Venous thrombus	absent vs. present	22.082	1.584 to 307.906	0.021
IL-22	high vs. low	5.698	0.884 to 36.772	0.067
IL22-RA1	high vs. low	75.355	1.870 to 3037.342	0.022
Ki-67	high vs. low	0.478	0.048 to 4.760	0.529
Caspase-3	high vs. low	4.773	0.155 to 147.125	0.372

4. Discussion

In this study, we hypothesized that: 1. The expression of IL-22-IL-22RA1 axis proteins is correlated with ccRCC patients' overall survival and disease-free survival in The Cancer Genome Atlas dataset. To this end, we analyzed the clinical and genomic data of ccRCC provided by TCGA using the cBioPortal resource. We found that patients with alterations in IL-22 or IL-22RA1 expression had a significantly shorter overall survival and disease-free survival compared to patients without alterations in IL-22 or IL-22RA1 expression; 2. The expression of IL-22-IL-22RA1 axis proteins is correlated with clinicopathological characteristics of ccRCC patients. To verify this hypothesis, we estimated the expression of IL-22 and IL-22RA1 in our own cohort of ccRCC cases and correlated the expression with clinicopathological characteristics (age, sex, Fuhrman grade, primary T stage, present of necrosis, venous thrombus and relapse) of ccRCC patients. The results showed that IL-22 expression was correlated with Fuhrman grade, primary T stage, relapse and vital status of ccRCC patients. And IL-22RA1 expression was associated with Fuhrman grade, relapse, and vital status of ccRCC patients. 3. ccRCC patients with lower expression level of IL-22, IL-22RA1, IL-22BP, Ki-67, Caspase-3 have a longer overall survival time and disease-free survival time. To verify this point, we estimated the expression of IL-22, IL-22R, and IL-22BP, Ki-67 and Caspase-3 in human ccRCC tissues and correlated the expression with the outcomes of ccRCC patients in our patients' cohort. Immunohistochemical staining

detected IL-22, IL-22RA1, Ki-67, and caspase-3 positive tumor cells, but no IL-22BP positive cell was detected. The result showed that ccRCC patients with high expression of IL-22 had a poor overall survival and disease-free survival compared to patients with low expression of IL-22. This was true also for IL-22RA1 and Ki-67. However, ccRCC patients with high expression of caspase-3 only showed a significantly shorter disease-free survival, but no change in the overall survival. 4. IL-22RA1 and IL-22 are independent predictive factors for prognosis of ccRCC patients. To this purpose, multivariate cox proportional hazards models were used to analyze the impact of prognostic factors on overall survival and disease-free survival. The result revealed that IL-22RA1 but not IL-22, is an independent predictive factor for prognosis of ccRCC patients.

The clinical behavior of ccRCC can be estimated by a combination of multiple prognostic factors. Accurate outcome prediction for patients with ccRCC is important for treatment stratification. Multiple clinical and pathological variables are crucial in predicting outcome in patients with ccRCC. Among them, the pathological stage is recognized as one of the most important variables (Javidan, Stricker et al. 1999, Gettman, Blute et al. 2001). However, some other features such as tumor nuclear grade and histological subtype also play an important role in the outcome. Tumor stage, nuclear grading and the presence of tumor necrosis are considered as the major prognostic indicators (Frank, Blute et al. 2002, Delahunt, McKenney et al. 2013)

Moreover, the tumor stage, size, grade, and necrosis (SSIGN) score was reported first in 2002 for clinicians to assess the effects of tumor characteristics on outcome so that to be used to improve patient management, stratify ccRCC patients for clinical trials to estimate the efficacy of adjuvant therapies as well as develop appropriate postoperative surveillance programs (Frank, Blute et al. 2002).

The Fuhrman grade has gained widespread adoption in clinical practice after Fuhrman's report in 1982 (Fuhrman, Lasky et al. 1982), which is based on tumor nuclear size, shape, and prominence of nucleoli. Following studies made Fuhrman grade more convincible by testing against survival for large series of tumors divided based on RCC subtype (Delahunt 2009). In studies focus on ccRCC, significant differences in survival were noted between each grade or combined grade 1 and 2, grade 3 and 4 (Ficarra, Righetti et al. 2001, Kim, Cho et al. 2004, Ficarra, Martignoni et al. 2005).

In spite of the effort of concerning studies on Fuhrman grade, there are controversies on its reliability. Some researchers demonstrated that Fuhrman grade should not be regarded as a reliable prognostic factor. For instance, one study reported that tumor grade did not provide additional survival data once tumor stage was assigned (Patard, Leray et al. 2005). On the contrary, several other studies noted a significant prognostic role of Fuhrman grade. One group reported that Fuhrman grade significantly associated with cancer stage, distant metastasis and patient

survival (Ficarra, Martignoni et al. 2005). Moreover, Ke-Hung Tsui, et al. reported a correlation between advanced tumor stage and high-grade lesion, which indicated that high tumor grade might make tumor more aggressive, thus leading to high stage, distant metastasis as well as diminished survival rates (Tsui, Shvarts et al. 2000).

In our study, the correlation between IL-22 expression and ccRCC patients' clinicopathological characteristics showed that Fuhrman grade was associated with IL-22 expression; a similar result was obtained by correlating Fuhrman grade with IL-22RA1 expression. In the multivariate Cox proportional hazards regression model, among all the variables, Fuhrman grade (G1/2 vs. G3/4) was not an independent predictor for overall survival of ccRCC patients. However it was an independent prognostic factor for disease-free survival of ccRCC patients. Above all, Fuhrman grade still can be a useful predictor of the outcome of ccRCC patients, especially in respect to recurrence prediction after surgery. The mechanism of how IL-22 and IL-22RA1 expression impact Fuhrman grade or the other way around is unknown and needs further studies in the future.

Tumor stage is regarded as one of the most powerful predictors of outcome for RCC patients (Selli, Hinshaw et al. 1983, Delahunt, Bethwaite et al. 1994). Over the past several decades, in order to make it even more accurate in patient prognostication, several evidence-based amendments have been added to the TNM staging system of RCC. Primary tumor size and regional spread have been regarded as significant

prognostic factors of RCC and there is a large body of evidence to indicate that tumor size can predict tumor infiltration into the kidney sinus, therefore is a dependent prognostic feature (Delahunt 2009).

Numerous studies on the prognostic significance of tumor size have been reported. From the results of present study, there was a significant correlation between primary T stage and IL-22 expression. Cases expressed a higher level of IL-22 tend to have a higher stage. However, this correlation was not observed in IL-22RA1, although the p-value was only a little higher than 0.05. Interestingly but not surprisingly, multivariate Cox proportional hazards regression analysis implied that primary tumor stage is an independent prognostic factor for both overall survival and disease-free survival in ccRCC patients. ccRCC patients with lower primary tumor stage may have a better outcome. This finding was consistent to previous investigations by other groups.

Coagulative tumor necrosis was first recognized as a prognostic marker of advanced RCC in the 1970s (Amtrup, Hansen et al. 1974, Mancilla-Jimenez, Stanley et al. 1976). Coagulative necrosis is the most common form of necrosis. Typical necrosis has homogeneous clusters, sheets of dead as well as degraded tumor cells that coalesce into an amorphous coagulum (Sengupta, Lohse et al. 2005). One large cohort research with 3009 patients was performed to characterize tumor necrosis as a prognostic feature of RCC, and indicated that histologic coagulative tumor necrosis is

an independent predictor of prognosis for ccRCC and chromophobe RCC and that the presence of necrosis indicated an increased hazard for death from RCC (Sengupta, Lohse et al. 2005). However, there are some other studies with different conclusions. One study by Foria et al. shows that extensive necrosis in RCC may imply better short-term prognosis, but this predictive value was abolished after adjusting for tumor pTNM stage (Foria, Surendra et al. 2005). Additionally, Leibovitch et al. performed a consecutive retrospective study of 173 RCC patients after radical nephrectomy, showing that extensive necrosis was not related to kidney tumor biology, but might rather reflect the relation between tumor size and vascularity (Leibovitch, Lev et al. 2001).

In our current study, as one of the clinicopathological features to be assessed, we first correlated the expression of both IL-22 and IL-22RA1 with intratumoral necrosis and did not find a significant correlation between necrosis and the expression of IL-22 or IL-22RA1. Moreover, the multivariate Cox proportional hazards regression analysis for disease-free survival implied that necrosis is not an independent predictor of disease-free survival, whereas it was an independent predictor of overall survival for ccRCC patients. The cause of extensive necrosis in RCC is not well understood. Normally it is not very common for RCC tumors to show extensive necrosis, and only 1.6% of all the RCC cases diagnosed with extensive necrosis during 8 years in a regional hospital, and it is a kind of tumor that rarely show spontaneous regression (Hamid and Poller 1998). The reasons for this phenomenon include

auto-infarction which results from renal vein thrombosis, the existence of renal artery atherosclerosis, tumor growth exceeding the vascular supply, or mechanisms of the autoimmune anti-tumor response (Childs, Chernoff et al. 2000). One previous report noted that extensive tumor necrosis might be result of renal tumor spontaneous regression (Hamid and Poller 1998). Since some studies had too small number of cases to perform valuable statistical analysis to estimate the correlation between tumor necrosis and prognosis, more studies should focus on the pattern of necrosis with RCC prognosis as well as the mechanism of necrosis or regression.

As to venous thrombus, there are 5% to 15% of RCC patients with venous thrombus extension, and 20% to 50% of these patients present with metastasis, and the prognosis for this subset is poor (Blute, Leibovich et al. 2004, Terakawa, Miyake et al. 2007, Wagner, Patard et al. 2009, Miyake, Terakawa et al. 2012). One study focus on venous thrombus of RCC demonstrated that inferior vena cava tumor thrombus volume (IVC-TV) may represent a clinical-associated variable for assessment of perioperative complications, disease progression, as well as overall survival, and has a greater predictive value in RCC than the tumor size and metastases (Zargar-Shoshtari, Sharma et al. 2015). Nevertheless, another study indicated that venous tumor thrombus consistency was not predictive of overall survival in RCC patients. They evaluated the prognostic value of venous tumor thrombus consistency in 147 RCC patients with or without venous tumor thrombus, result showed that venous tumor thrombus consistency was not predictive of survival, and

did not enhance the performance of a multivariable model which included series informative predictors (Antonelli, Sodano et al. 2015).

In our present study, the result of the correlation between IL-22 expression and clinicopathological variables showed no significant association between IL-22 expression and present of venous thrombus. The same was true for correlation with IL-22RA1 expression and with other clinicopathological characteristics. Moreover, the multivariate Cox proportional hazards regression model for overall survival indicated that venous thrombus consistency is not an independent predictor for overall survival of ccRCC patients. Interestingly, the multivariate Cox proportional hazards regression model for disease-free survival revealed that venous thrombus consistency is an independent predictor of disease-free survival of ccRCC patients. Considering all the conclusions drawn from previous studies along with our findings, further research, especially including large case cohorts with long-term follow-up periods are still required to decide whether venous thrombus is an independent predictor of survival of RCC patients.

In spite of all the conclusions above, ccRCC is an extremely complicated disease. The disease-free survival and overall survival of ccRCC patients are still not satisfactory. In the past decade, intensive molecular research has provided substantial insight into the biological behavior of RCC and is beginning to shape clinical practice (Shariat and Xylinas 2012). The increasing number of patients raises the need for new

molecular markers to evaluate the risk of death as well as recurrence. Thus, identification and incorporation of these molecular markers is considered to play an important role in the future.

There have been varieties of serum and tissue molecular biomarkers that are suggested as potential prognostic markers. Rabjerg et al. used TaqMan assays to identify superior prognostic makers in a long-term follow-up study and they showed a strong correlation between high expression levels of SPP1 and CSNK2A1 and a poor prognosis in ccRCC patients, while DEFB1 expression was on the contrary associated with a better DFS (Rabjerg, Bjerregaard et al. 2016). Several molecular biomarkers have been identified to guide therapy. The most promising results so far are VEGF, VEGF receptors, CAIX (Carbonic anhydrase IX), HIF-1α and VHL expression of the tumor (Czarnecka, Kukwa et al. 2014).

Among various molecular biomarkers that have been identified so far, Ki-67 is regarded as a significant prognostic marker in several neoplasms, including RCC (Gerdes 1990). Higher Ki-67 expression predicted shorter survival of RCC (Rioux-Leclercq, Turlin et al. 2000, Visapaa, Bui et al. 2003). Gayed et al. validated Ki-67 as a convictive independent molecular marker to predict outcomes in ccRCC patients (Gayed, Youssef et al. 2014). Another study by Tollefson et al. reported that both Ki-67 and coagulative tumor necrosis are independent predictors of poor outcome for ccRCC patients, however, Ki-67 and coagulative tumor necrosis should

not be regarded as surrogates for each other, and both of them should be taken into account when generating prognosis for ccRCC patients (Tollefson, Thompson et al. 2007). In the present study, we measured the expression of Ki-67 in 60 ccRCC patients and assess the correlation between Ki-67 expression and outcome of ccRCC patients. First, from the results of Kaplan-Meier analysis, there are significant differences between high level and low level of Ki-67 expression in both overall survival and disease-free survival, which revealed that ccRCC patients with high expression of Ki-67 tend to have a poor prognosis. Nevertheless, the results from multivariate Cox proportional hazards regression analysis for overall survival and disease-free survival demonstrated that Ki-67 is not an independent predict factor for the outcome of ccRCC patients. This result is somehow inconsistent to some of other studies, even though it is not very explicit in terms of this inconformity, the relatively small number of cases in our study may be one the reasons. Moreover, there may be a correlation between Ki-67 and the IL-22-IL-22R axis, so that the multivariate Cox proportional hazards regression analysis shows different results compared to studies not assessing the IL-22-IL-22R axis. Further studies should be designed to identify whether there is an association between expression of IL-22-IL-22 axis and Ki-67.

Apoptosis plays a crucial role in various physiological processes including maintenance of tissue homeostasis (Hengartner 2000). Disorders in physiological pathways of apoptosis contribute to different diseases such as autoimmunity,

neurodegeneration and cancer (Reed 1999). Activation of caspase-3 can initiate a specific death-inducing signal involved in the normal apoptotic process and down regulated expression of caspase-3 is correlated with enhanced malignant potential and decreased survival of cancer patients (Frisch and Francis 1994, Glinsky and Glinsky 1996, Kurabayashi, Furihata et al. 2001, Hsia, Chen et al. 2003, Bellini, Cury et al. 2010, Jiang, Gong et al. 2010). We performed immunohistochemical staining for caspase-3 in our tumor tissues and then analyzed the association between the expression of caspase-3 and outcome of ccRCC patients. Kaplan-Meier analysis reveals that there is no significant difference between high level and low level expression of caspase-3 for overall survival. However, there is significant difference between different levels of caspase-3 expression for disease-free survival, indicating that high levels of caspase-3 expression in tumor tissue are associated with a shorter disease-free, but not overall survival. In addition, the multivariate Cox proportional hazards regression analysis for overall survival and disease-free survival revealed that caspase-3 is not an independent predict factor for the outcome of ccRCC patients.

IL-22BP is encoded by an IL-22RA1 independent gene (Dumoutier, Lejeune et al. 2001). IL-22BP is found to be expressed on dendritic cells and the expression level decreases when dendritic cells undergo maturation (Chang, Hanawa et al. 2006, Huber, Gagliani et al. 2012, Martin, Beriou et al. 2014). Several studies reported IL-22BP expression in lymphatic organs, the gastrointestinal system, the lungs, the

skin, the placenta as well as the breast (Dumoutier, Lejeune et al. 2001, Gruenberg, Schoenemeyer et al. 2001, Kotenko, Izotova et al. 2001, Weiss, Wolk et al. 2004). One study reported that deficiency of IL-22BP leads to accelerated and increased tumorigenesis in a colitis-associated colon cancer mouse model (Huber, Gagliani et al. 2012). However, from the literature no study was found on the correlation between IL-22BP expression and kidney cancer. Interestingly, in the present study IL-22BP positive staining was only detected in adjacent normal kidney tissue, but no staining in tumor tissues, which may imply that IL-22BP has a therapeutic potential by blocking the function of IL-22-IL-22R1 system in IL-22-induced cancer or other diseases. Further studies should be performed to clarify the mechanism of this phenomenon.

In this study, one objective was to identify new prognostic and predictive biomarkers in ccRCC. From others' previous studies, IL-22-IL-22R axis is associated with several types of cancer. The IL-22-IL-22R system seems an important novel target in cancer research. One study performed by Xu et al. revealed a correlation between Notch1 signaling and ccRCC, that Notch1 activation increased ccRCC cell proliferation, enhanced anchorage independent growth regulated by PI3K/Akt pathway (Xu, Zhu et al. 2012). From others' previous studies, it is known that IL-22 does not regulate the function of immune cells directly (Wolk, Kunz et al. 2004). Instead, IL-22 targets cells at barriers of the body, including skin, cells of the pancreas, digestive and respiratory systems, liver, joints as well as kidney (Sabat, Ouyang et al. 2014). Considered that

IL-22 signaling pathway may be involved in Notch1 signaling, we assumed that IL-22 also plays a role in ccRCC.

Among all the studies focus on IL-22 and cancers, IL-22 is most unambiguously pro-tumoral in tumors of the gastrointestinal tract (Lim and Savan 2014). Patients suffering from gastric cancer have higher circulating frequencies of IL-17 and IL-22 producing T cells compared to healthy subjects, and these T cells correlated inversely with the survival time of patients, while correlating directly with the stage of cancer (Liu, Peng et al. 2012). Renal cell carcinoma is originating from the lining epithelium of proximal convoluted tubules (Capitanio and Montorsi 2016). In pancreatic cancer, expression of IL-22 and IL-22R was increased in pancreatic ductal adenocarcinoma tissue and both predict poor patient survival (Wen, Liao et al. 2014). One study on hepatocellular carcinoma indicated that high levels of IL-22 in the serum are associated with a poor outcome in both HBV (Hepatitis B Virus) and HCV (Hepatitis C Virus)-induced hepatocellular carcinoma (Waidmann, Kronenberger et al. 2014). In addition, aberrant expression of IL-22R1 was found in lymphoma patients. Compared to normal lymph nodes, primary central nervous system lymphomas showed higher IL-22R1 expression (Sung, Kim et al. 2011).

In terms of the kidney, no publication was found in PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) in respect of the correlation between IL-22 or IL-22R expression and RCC. Only one study focus on the effects of IL-22 on human

RCC cell line A498 *in vivo* and *in vitro*, showing that IL-22 suppressed A498 cells *in vitro* and inhibited A498 cell-bearing mouse xenografts (Zhang, Shang et al. 2011). This result seems to be inconsistent to our study. The result from the other study implied that IL-22 might play an anti-RCC role in A498 cell line partially mediated by STAT1 signaling pathway regulation as well as G2/M cell cycle arrest. However, our study showed that IL-22 might promote the progress of ccRCC. One explanation for these different results is that different study objects were used. The biological behavior of tumor cells is not always consistent between cell lines and real tumors from patients. Additionally, IL-22 may have different effects not only in cell lines and real tumors, but also in different phases of tumor progression. What's more, IL-22 may play different roles in different cell lines. For instance, because IL-22 plays its role by IL-22/IL-22RA1 signaling pathway, the cell lines with more expression of IL-22RA1 will have more IL-22 effect. Thus, different cell lines should be used in further studies covering this subject.

From the TCGA results, we gained knowledge on correlations between alterations in IL-22/IL-22RA1 expression and the outcome of ccRCC patients. Patients with high expression of either IL-22 or IL-22RA1 presented with shorter outcomes than patients with low expression of IL-22 or IL-22RA1. Based on the varieties of studies mentioned above, we measured the expression of IL-22 and IL-22RA1 in ccRCC and to estimate their relationship with clinicopathological characteristics as well as prognosis in ccRCC. Moreover, the study of TCGA data is based on gene expression,

and what we did next is to assess the effect of IL-22 and IL-22RA1 on protein expression levels from Munich ccRCC patients' cohort.

From the results of immunohistochemical staining, IL-22 expression was detected in the kidney cancer tissues. However, it is not easy to figure out where exactly this cytokine is located. As mentioned above, IL-22 belongs to a cytokine family that is involved in tissue homeostasis. IL-22 was originally found to be expressed in T cells of both human and mouse (Dumoutier, Louahed et al. 2000, Wolk, Kunz et al. 2002). In humans, the main producers of IL-22 are innate lymphoid cells (ILCs), TH17 cells, and TH22 cells, particularly at mucosal surfaces (Duhen, Geiger et al. 2009, Trifari, Kaplan et al. 2009, Sonnenberg, Fouser et al. 2011). IL-22 is produced by CD3+ (CD4-CD8-) oligoclonal T cells that are detected in epithelia of the organs mentioned above (Bonneville, O'Brien et al. 2010). In the current study, the expression of IL-22 in ccRCC is significantly correlated with Fuhrman grade, primary T stage, relapse and vital status, which indicated that IL-22 may impact the outcome of ccRCC. Kaplan-Meier analysis demonstrated that high expression of IL-22 is associated with poor prognosis in ccRCC. However, multivariate analysis showed that high expression of IL-22 is not an independent prognostic factor of either overall survival or disease-free survival. This result is not consistent with some other studies. For instance, Zhang Wen et al. demonstrated that IL-22 is an independent prognostic factor of overall survival in pancreatic ductal adenocarcinoma (Wen, Liao et al. 2014). Jiang et al. found IL-22 to be overexpressed in human hepatocellular carcinoma

microenvironment, which might lead to tumor growth, inhibition of apoptosis, and promotion of metastasis due to STAT3 activation (Jiang, Tan et al. 2011). In contrast, Kobold et al. did not observe a correlation between IL-22 expression by immunohistochemistry and prognosis in lung cancer, which suggested IL-22 not to be a predictor for lung cancer patients (Kobold, Volk et al. 2013). These findings indicate that the predictive role of IL-22 might differ between different organs.

When it comes to the therapeutic role of the IL-22/IL-22R axis in cancer patients, no investigation has been performed so far One study established hepatocyte-specific IL-22Ra1 knockout ($Il22Ra1^{Hep-/-}$) and Stat3 knockout ($Stat3^{Hep-/-}$) mouse models to study the therapeutic role of IL-22 in experimental intra-abdominal Klebsiella pneumonia infection in mice. They infected the mice with Klebsiella pneumonia that resulted in liver injury and necrosis, and the result indicated that IL-22 overexpression or therapeutic administration of rIL-22 (recombinant IL-22) reduced bacterial burden in both the liver and spleen, therefore IL-22 may be a useful adjunct in treating hepatic as well as intra-abdominal infections (Zheng, Horne et al. 2016). Moreover, in lung cancer cells IL-22 acts as an autocrine factor to make cancer cell survival and resistance to chemotherapy. Meanwhile, this therapeutic effect was found *in vivo* in a xenograft model by IL-22 RNAi (RNA interference) plasmids (Zhang, Chen et al. 2008). There are many other studies demonstrating that IL-22 expression was positively correlated with tumor growth, metastasis and tumor stages (Jiang, Tan et al. 2011).

Immunohistochemical staining for ccRCC tissues showed that IL-22RA1 was expressed at the cell membrane and in the cytoplasm. As we know, the functional IL-22RA1 is restricted to nonhematopoietic cells of the pancreas, liver, skin, intestine, lung and kidney (Wolk, Kunz et al. 2004). It is believed that immune cells are not the target cell of IL-22, monocytes, B cells, T cells, NK cells, monocyte-derived macrophages and dendritic cells do not express IL-22RA1 (Wolk, Kunz et al. 2002, Wolk, Kunz et al. 2004, Wolk, Witte et al. 2008). Both IL-22RA1 and IL-10R2 chains of the IL-22 receptor are expressed constitutively in many organs, and epithelial cell lines derived from these organs respond to IL-22 in vitro (Kotenko 2002). The expression of IL-22RA1 determines the sensitivity of the cells towards IL-22 (Sabat, Ouyang et al. 2014). IL-22 binds to its receptor chains in a temporal mechanism and IL-22 correlated with the extracellular domain of IL-22RA1 (Li, Tomkinson et al. 2004). IL-22 primarily acts on epithelial cells to perform barrier function (Aujla, Chan et al. 2008). Ligation of IL-22RA1 induces migration and proliferation of epithelia in these organs, so that the barrier integrity is enhanced by molecular variation such as phosphorylation of STAT3, activation of MAP kinase, and activation of proliferative genes like c-Myc and cyclin D1 (Xie, Aggarwal et al. 2000, Lejeune, Dumoutier et al. 2002). One study by Joseph Franz, et al. noted that IL-22RA1 is shuttled for degradation by a functionally non-described human protein named FBXW12 (F-BOX and WD Repeat Domain Containing 12). Increasing FBXW12 may suppress IL-22RA1 expression and IL-22 dependent cellular responses, in addition, FBXW12 upregulates IL-22RA1 expression level and signaling (Franz, Jerome et al. 2015).

In the recent study, IL-22RA1 seems to be a promising prognosis marker for ccRCC patients. Patients with a high level IL-22RA1 expression tend to have shorter overall and disease-free survivals, and, more interestingly, IL-22RA1 can be regarded as an independent predictor of both overall survival and disease-free survival from the result of multivariate Cox proportional hazards regression analysis. This conclusion is consistent with the results from TCGA, which also suggests a potential role of IL-22RA1 in ccRCC patients. Nevertheless, in the current study we only detected protein levels of IL-22 and IL-22RA1 expression in ccRCC tissues, hence mRNA levels of IL-22 and IL-22RA1 should be assessed as well, and further studies on the IL-22-IL-22R pathway and their mechanism in cancer are still necessary before clinical application of our findings.

Several potential therapeutic options may exist to target the molecules in IL-22-IL-22RA1 system. For instance, TNF -specific antibody or p40-specific antibody can block cytokines like TNF and IL-23, so that inhibit the activation and survival of T helper cells, as reported that adalimumab and ustekinumab can be used in psoriasis (Sabat, Ouyang et al. 2014). Antagonists can block T helper cell-specific transcription factors that promote IL-22 production such as Ahr. Additionally, IL-22 can be inhibited by antibodies or IL-22BP. Furthermore, antibodies targeting IL-22RA1 (IL-22RA1-blocking-antibody) can neutralize the function of IL-22RA1 by inhibiting the binding of IL-22 to IL-22RA1. Moreover, inhibitors that target downstream

molecules such as STAT3, JAK1 and TYK2 may also block the IL-22-IL-22RA1 pathway. The use of IL-22RA1 blocking antibodies seems to be one of the most appropriate therapeutic strategies for various reasons. The most convincing reason is this inhibition is highly specific in the IL-22-IL-22RA1 system, so that very limited unwanted effects will be produced. In addition, IL-22RA1 is not expressed in immune cells. Instead, it is only expressed by epithelial cells from several organs. This phenomenon makes it impossible to produce dominating systemic immune activation or inhibition (Sabat, Ouyang et al. 2014). The possible pathway of IL-22-IL-22RA1 in renal cell carcinoma is shown in figure 18.

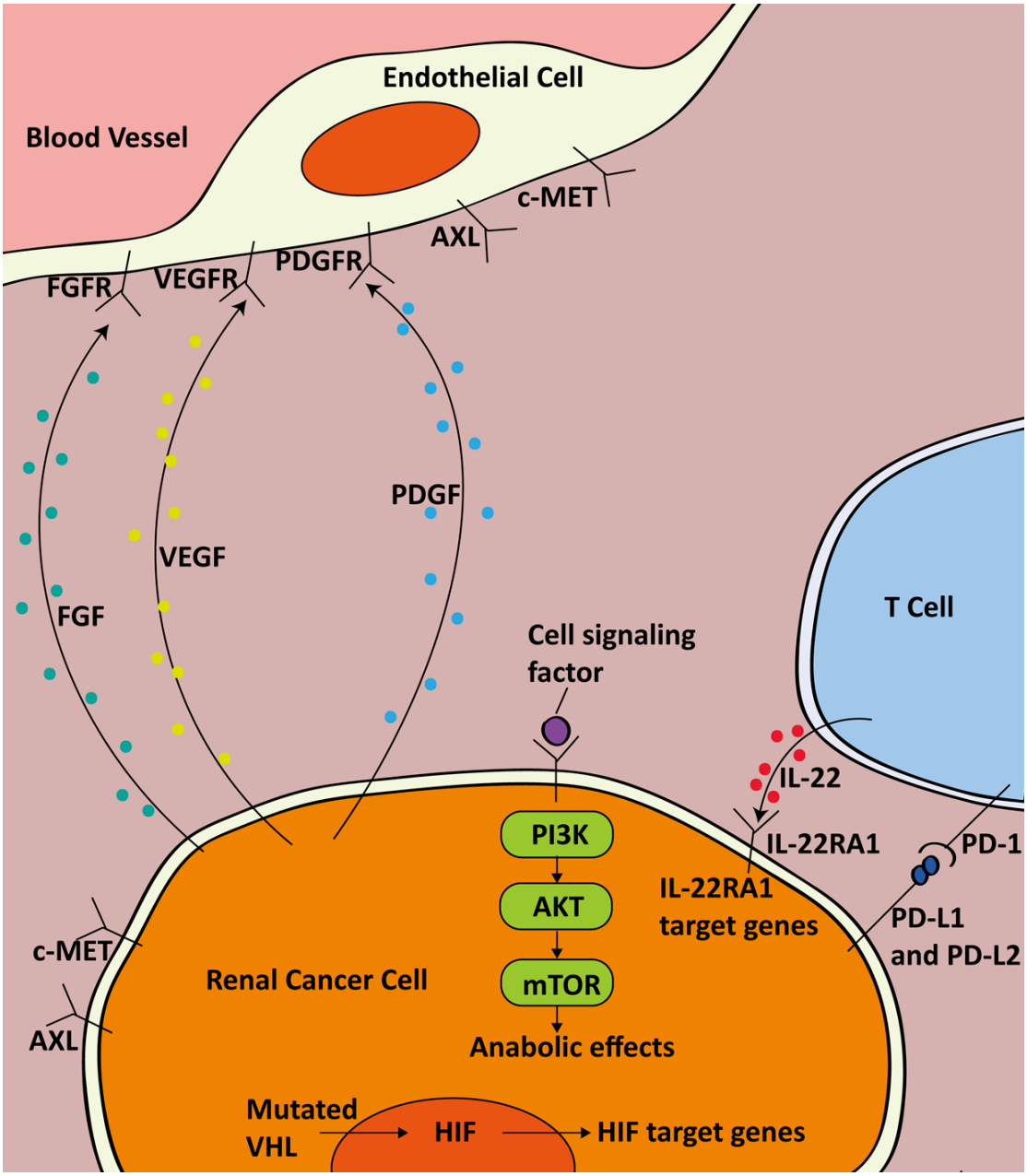

Figure 18 IL-22-IL-22RA1 pathway in renal cell carcinoma. IL-22 released from T cell binds to IL-22RA1 on renal cancer cell, leading to the activation of downstream IL-22RA1 target genes.

However, there are limitations to the conclusions drawn from the present study:

1. With regard to the TCGA cohort analysis, no multivariate analysis is provided to evaluate the independent prognostic significance of IL-22/IL-22R expression.

Further no data regarding the duration of follow-up or the number of events in this cohort was available.

2. With regard to the Munich cohort, the number of the cases might be too limited (n=60) to provide definitive results.
3. The number of events within this cohort is small (18 recurrence events and 23 deaths). The low event rate hampers the ability to control for multiple variables (Age, Sex, Grade, Stage, Necrosis, Venous Thrombosis, IL-22, and IL-22R) and might lead to a bias in the statistical results.

Obviously, evolving data suggest that inhibiting the activity of IL-22-IL-22R1 system may be a therapy for many diseases. Even though there is still a long way to go before using these approaches to patients, however, we should keep in mind that it is a promising potential therapeutic option for IL-22-IL-22R1 system-associated diseases. IL-22RA1 status may act as a prognostic factor potential target for therapy to overcome drug resistance.

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6. List of Abbreviations

ADAM17	a disintegrin and metalloprotease domain 17
AHR	aryl hydrocarbon receptor
AJCC	American Joint Committee on Cancer
Akt	protein kinase B
ARNT	aryl hydrocarbon receptor nuclear translocator
BMI	body-mass index
BSG	basigin
ccRCC	clear cell renal cell carcinoma
CI	confidence interval
CT	computerised tomography
CYP1A1	cytochrome P450 family 1 subfamily A member 1
CYP24A1	cytochrome P450 family 24 subfamily A member 1
DAB	3,3'-diaminobenzidine
DFS	disease-free survival
DLK	Delta-Like
DLL	Distal-less
DNA	deoxyribonucleic acid
ERK	extracellular signal-regulated kinases
FDA	Food and Drug Administration
G-CSF	granulocyte colony-stimulating factor
GISTIC	Genomic Identification of Significant Targets in Cancer
H&E	hematoxylin and eosin
H_2O_2	hydrogen peroxide
HES	hairy and enhancer of split
HEY	Hairy/enhancer-of-split related with YRPW motif protein
HIF	hypoxia-inducible factor
HR	hazard ratio
ICGC	the International Cancer Genome Consortium
IFN	Interferon

IL	Interleukin
IL-22BP	IL-22 binding protein
IL-22R	IL-22 receptor
ILCs	innate lymphoid cells
JAG	Jagged
JAK1	Janus kinase
JNK	c-Jun N-terminal kinase
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
MAP3K8	mitogen-activated protein kinase kinase kinase 8
MAPK	mitogen-activated protein kinase
miRNA	microRNA
ml	milliliter
MMP9	matrix metallopeptidase 9
MRI	Magnetic Resonance Imaging
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NCI	National Cancer Institute
NHGRI	National Human Genome Research Institute
NiCl	Nickel chloride
NK	natural killer
OS	overall survival
PBS	phosphate buffered saline
PD-1	programmed death 1
PD-L1	programmed death ligand 1
PI3K	phosphoinositide 3-kinase
PSEN1	presenilin 1
pT	primary tumor stage
PTEN	Phosphatase and tensin homolog
RBP-J	Recombination Signal Binding Protein For Immunoglobulin Kappa J Region
RCC	renal cell carcinoma
rIL-22	recombinant IL-22

RNA	ribonucleic acid
RNAi	RNA interference
SCNAs	somatic copy-number alterations
SSIGN	stage, size, grade, and necrosis
STAT3	Signal transducer and activator of transcription 3
TACE	TNF-α ADAM metalloprotease converting enzyme
TCGA	The Cancer Genome Atlas
TNF	tumor necrosis factor
TYK2	Tyrosine Kinase 2
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau
XRE	xenobiotic response elements
µl	microliter
µm	micrometre

7. List of figures

8. List of tables

9. Acknowledgments

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