



**Clinicopathological Features of the Renal Cell
Carcinoma Subtypes Diagnosed According to the
2016 WHO Renal Tumor Classification**

PhD Thesis

Levente Kuthi, MD

Thesis supervisor: Béla Iványi, MD, Dsc

Department of Pathology, Faculty of Medicine,

University of Szeged,

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INTRODUCTION

GENERAL FEATURES AND CLASSIFICATION OF RENAL CELL CARCINOMA

The renal cell carcinoma (RCC) derives from the epithelium of the kidney tubules, and it is the sixth most frequent cancer form in males and tenth in females worldwide. The tumors are mainly sporadic, and their risk factors are smoking, obesity, end-stage kidney disease along with occupational hazards like trichloroethylene exposure. RCC affects the elderly; namely, more than 70% of the cases are diagnosed in patients over 60. RCC is approximately twice frequent in males, and this difference might be caused by distinct lifestyle and occupational factors. Of all RCC cases, 2-4% are familial and belong to different kinds of cancer syndromes. RCC is not a single disease, but a heterogeneous group of malignant tumors, and the different subtypes have their unique clinical features and morphological patterns. The 1981 WHO classification of the renal tumors discussed the histological appearance, but it did not identify any distinct subtypes. Pioneered by Gyula Kovács, the Heidelberg classification took into account the genetic alterations as well and now is regarded as the forerunner of the modern renal tumor classifications. Later, the 2004 WHO classification relied on the approach and added new entities to the previous subsets. Nine years later, the Vancouver classification was published by the International Society of Urological Pathology, and it served the basis of the current 2016 WHO classification and defined 14 RCC subtypes. As regards the rare subsets, namely Xp11.2 translocation RCC, clear cell papillary RCC, mucinous tubular and spindle cell carcinoma, acquired cystic kidney disease-related carcinoma, their clinicopathological and diagnostic features have been studied in the past several years. A new nucleolar grading system was created as well, to replace the much-criticized Fuhrman grading system. The

Fuhrman grade was hardly reproducible; furthermore, its prognostic value was debated as well. However, a major limitation of the proposed ISUP grading system is that it has prognostic value solely in clear cell RCC and papillary RCC tumors. The ISUP investigated other prognostic factors like the presence of microscopic tumor necrosis, rhabdoid change, sarcomatoid transformation, and microvascular invasion. Sarcomatoid transformation is a long known negative prognostic factor with a median survival of 4 to 9 months. The rhabdoid change is another form of dedifferentiation seen in RCC, and it is associated with a dismal clinical course. In RCC, the presence of tumor necrosis is commonly noticed; however, its exact mechanism is still unknown. The presence of tumor necrosis inversely correlates with the patient outcome, but this seems to be subtype-specific because the studies reported so far did not find any link between the prognosis and the presence of necrosis in case of papillary RCC and chromophobe RCC. In clear cell RCC, this link between necrosis and an unfavorable clinical course was evident for a long time, and Delahunt et al. proposed a grading system that incorporated the presence of tumor necrosis.

GENETIC BACKGROUND OF RENAL CELL CARCINOMA

In the late 1980s, Kovács and his co-workers observed by cytogenetic studies that the short arm of chromosome 3 (3p) is frequently lost in **clear cell RCC**. Later, it turned out that the *VHL* gene located at the 3p25.3 band, is the critical tumor suppressor gene inactivated in clear cell RCC. The *VHL* gene encodes the VHL protein, which is the member of the VHL-elongin BC complex, and thereby it acts as a substrate of the E3 ubiquitin ligase. The later one degrades the hypoxia-inducible factor 1-alpha (HIF1 α) in normoxic circumstances. However, in case of the loss of the VHL protein, the HIF1 α remains intact and is relocated to the nucleus where HIF1 α is associated with the aryl hydrocarbon receptor nuclear translocator protein. This heterodimeric protein

complex formed acts as a transcription factor resulting in angiogenesis, increased cell survival, elevated mitotic activity and enhanced immune evasion. In terms of genetic background, RCCs with papillary morphology are different. **Type 1 papillary RCC** harbors activating mutation or amplification of the *c-MET* gene, besides the gains of chromosome 3, 7, and 17, along with loss of chromosome Y are observed roughly 80% of the cases. The distinction between type 1 papillary RCC and **mucinous tubular and spindle cell carcinoma** might be problematic because they share some histopathological features, but the trisomies of the chromosomes discussed above cannot be observed in the former one. The genetic changes in **type 2 papillary RCC** are much less consistent that observed in type 1 papillary RCC; namely, the tumor cells frequently have chromosomal losses and/or gains. For **chromophobe RCC**, multiple losses of chromosomes are present. **Clear cell papillary carcinoma** has no specific genetic anomaly, and more importantly, it harbors no *VHL*-related changes despite the clear cell phenotype. In terms of **Xp11.2 translocation carcinoma**, the critical genetic alteration detected is a rearrangement of the microphthalmia-associated transcription factor genes, including the *TFE3* and *TFEB*. A homozygous loss of the fumarate hydratase gene is typical for **hereditary leiomyomatosis and renal cell carcinoma syndrome associated RCCs**. As regards **renal medullary carcinoma**, the bilateral loss of the *INII* gene is the hallmark genomic change. In terms of **acquired cystic kidney disease-related RCC**, comparative genomic arrays and FISH studies discovered multiple chromosomal losses and gains. **Collecting duct RCC** harbors no consistent genetic change. Among the emerging entities, we discuss here only the *ALK* rearrangement-associated and *TCEB1*-mutated RCC. Although the former is an infrequent entity, its specific genetic change is also a promising therapeutic target because *ALK* inhibitors are available for years. The *TCEB1* encodes the elongin C protein, which binds exactly the VHL protein to the

E3 ubiquitin ligase. The *TCEB1*-mutated RCC harbors the bilateral loss of the *TCEB1* gene, which is resulted by a loss of 8q and a nonsense mutation.

AIMS

In 2010-2015, besides nephrectomy specimens, tumor resections and core biopsy samples were introduced in Hungary, hence the pathological standpoint had to be reconsidered. The diagnosis of clear cell RCC, papillary RCC, and chromophobe RCC is straightforward in most of the nephrectomy cases, but tumors with overlapping features can pose diagnostic difficulties. Also, from uncertain renal lesions and inoperable tumors, a biopsy sample is frequently taken. The proper diagnosis of such small and often fragmented material requires in-depth knowledge of histologic and immunohistochemical features of renal tumors to achieve a diagnostic certitude. However, before 2013, the knowledge on immunohistochemical features of RCC subsets was limited on reviews and expert opinions, and no unified recommendations had been published earlier. Besides, the diagnostic experience with the rare entities was minimal at the time of the introduction of the Vancouver classification; therefore, the following aims were set:

To investigate the incidence, clinicopathological, and immunohistochemical characteristics of RCCs according to the 2016 WHO.

To test the influence of grade, stage, resection line positivity, and the presence of a rhabdoid/sarcomatoid morphology, giant cells, and microscopic tumor necrosis on patient survival to prompt better patient care.

To analyze the clinicopathological, immunohistochemical, and molecular features of two rare subsets, namely the clear cell papillary RCC and Xp11.2 translocation RCC.

MATERIALS AND METHODS

GENERAL ASPECTS

Here, nephrectomy specimens were enrolled, and the slides were reviewed, and the histological subtype was identified according to the 2016 WHO classification. For immunohistochemistry, tissue microarray blocks were created applying a 2 mm core diameter. After a literature review, the following antibodies were used carbonic anhydrase 9 (CA9), CK7, CD10, AMACR, MelanA, HMB45, TFE3, TFEB, and Cathepsin K. The slides were evaluated microscopically by estimating the proportion of immunopositive cells.

MOLECULAR PATHOLOGICAL ANALYSIS

Fluorescent in situ hybridization

FISH assays were carried out to detect either the loss of chromosome 3p and chromosome Y or gain of chromosome 7 and 17; furthermore, to identify a *TFE3* gene rearrangement.

***VHL* Gene Sequence and *VHL* Gene Promoter Region Hypermethylation**

Genomic DNA was extracted from tumor tissue, then the *VHL* exons were amplified via specific primer pairs. The reaction products were checked for size and purity by agarose gel electrophoresis and then utilized for DNA sequencing. In the case of pathological mutation, the tumor-free renal tissue was analyzed as well. The methylation status of the *VHL* gene promoter region was determined using the methylation-specific PCR method.

ANALYSIS OF THE CLINICOPATHOLOGICAL, IMMUNOHISTOCHEMICAL AND MOLECULAR FEATURES OF CLEAR CELL PAPILLARY RCC

It was a three-institutional retrospective study, in which the Department of Pathology, University of Szeged, the 1st Department of Pathology and Experimental Cancer Research Institute and the 2nd Department of Pathology, Semmelweis University participated. From these departments, 2326 RCC samples were reexamined for clear cell papillary RCC-like tumors. The inclusion criteria were as follows, low-grade nuclei, the presence of any degree of tubulopapillary growth pattern of tumor cells with clear cytoplasm, linear arrangement of nuclei from the basal membrane, along with the presence of a leiomyomatous stroma. The diagnosis of clear cell papillary RCC was made if the formerly mentioned morphology together with characteristic immunophenotype along with the lack of genetic alterations indicating clear cell RCC, and papillary RCC were detected.

ANALYSIS OF THE CLINICOPATHOLOGICAL, IMMUNOHISTOCHEMICAL AND MOLECULAR FEATURES OF XP11.2 RCC

Lastly, another retrospective study was completed which covered a large part of Hungary, because not just the departments mentioned above participated, but the Pathology Unit, Bács-Kiskun County Teaching Hospital, Pathology Unit, Hetényi Géza County Hospital, Surgical, and Molecular Tumor Pathology Centre, National Institute of Oncology and the Department of Pathology, University of Pécs were involved as well. In total, 2804 RCC samples were reevaluated for translocation RCC. The diagnostic criteria for Xp11.2 RCC were the typical morphological pattern or moderate-to-strong nuclear positivity with TFE3 immunohistochemistry or a positive *TFE3* break-apart FISH analysis.

RESULTS

HISTOLOGICAL SUBTYPES OF RCC AND PROGNOSTIC FACTORS ACCORDING TO THE 2016 WHO RENAL TUMOR CLASSIFICATION

Here, we reviewed 928 RCC cases. Based on the light microscopic appearance of the tumor and the results of immunostainings assessed, 83.5% of the samples were classified as clear cell RCC, 6.9% as papillary RCC, 4.5% as chromophobe RCC, 2.3% as RCC unclassified, 1.1% as Xp11.2 translocation RCC, 0.9% as clear cell papillary RCC, 0.4% as collecting duct carcinoma and 0.1% as mucinous tubular and spindle cell RCC. RCC occurred in 16 patients with end-stage kidney disease. The following morphotypes were encountered: clear cell on eleven occasions, papillary type 1 three times, and clear cell papillary on two occasions. Although the features of ACKD were observed in 9 end-stage kidneys with RCC, the histological evaluation did not lead to the suspicion of ACKD-associated RCC in any of these cases.

Correlation between the morphotype and CSS

Follow-up data sets were accessible for 804 patients. One hundred thirty-one patients with clear cell RCC, three patients with type 1 papillary RCC, seven patients with type 2 papillary RCC, seven patients with RCC unclassified, six patients with Xp11.2 translocation RCC, and three patients with collecting duct carcinoma had died from an RCC-related cause. The median follow-up of these patients was 29 months (range 1-254 months), whereas the median follow-up for all survivors was 68 months (range 2-313 months). The 5-year CSS was significantly different between patients with clear cell RCC and with chromophobe RCC ($p=0.021$) or with RCC unclassified ($p<0.001$) or with Xp11.2 translocation RCC ($p<0.001$), but not between patients with clear cell RCC and those with papillary RCC ($p=0.39$).

Grade and microscopic tumor necrosis in clear cell RCC

The Kaplan Meier estimation did not reveal any difference in biological behavior between grade 1 vs. grade 2 tumors ($p=0.550$), and grade 3 vs. 4 tumors ($p=0.226$). When the grade 1 and 2 tumors were lumped together into low-grade carcinomas, and grade 3 and 4 tumors into high-grade carcinomas, the survival analysis revealed a significant difference between the two groups ($p<0.0001$). When CSS according to the presence or absence of microscopic tumor necrosis was analyzed, the necrotic tumors exhibited a significantly poorer outcome than the non-necrotic tumors ($p<0.001$). When the presence or absence of tumor necrosis was tested in patients with low-grade tumors vs. high-grade tumors, necrosis was associated with a significantly poorer outcome only in high-grade tumors. In univariate Cox proportional hazard analysis, the ISUP grade, TNM stage, tumor necrosis, giant tumor cells, rhabdoid/sarcomatoid change, and positive surgical margins all proved to be negative predictors of CSS. In multivariate Cox proportional hazards analysis, however, only the ISUP grade, TNM stage, and positive surgical margin turned out to be independent prognostic factors.

Subtypes and grade in papillary RCC

When the 5-year CSS was calculated according to the ISUP grade, 100% was observed for grade 1, 94% for grade 2, 74% for grade 3, and 33% for grade 4 samples, respectively. The 5-year survival rate was significantly better for patients with grade 2 tumors than for those with grade 3 tumors ($p=0.011$). However, there was no significant difference in survival rates between cases with grade 1 vs. grade 2 ($p=0.696$), and grade 3 vs. grade 4 ($p=0.445$); and, therefore, samples with grades 1 and 2, and grades 3 and 4 were merged to low-grade and high-grade categories. The 5-year CSS rates according to the two-tiered grading system exhibited a significant difference. In a Cox

proportional hazard analysis, the ISUP grade and TNM stage, but not the morphotype, exerted a significant effect on the patient outcome.

CLINICOPATHOLOGICAL, IMMUNOHISTOCHEMICAL AND MOLECULAR FEATURES OF CLEAR CELL PAPILLARY RCC

In this retrospective study, using the inclusion criteria, we retrieved 31 samples. All tumors coexpressed CK7 and CA9, and the CD10 and the AMACR reactions were negative in 27 and 30 cases, respectively. The FISH assays for papillary RCC and deletion of chromosome 3p yielded negative results. The histomorphology, the results for *VHL* mutation and *VHL* methylation testing, and the immunophenotype confirmed 21 cases as clear cell papillary RCC and 10 cases as clear cell RCC.

General features of clear cell papillary RCCs

Here, the tumors were obtained from 12 females and 9 males. The mean age was 60 years. All the tumors were solitary, and the mean size was 23 mm.

Microscopic findings on clear cell papillary RCCs

Each tumor was circumscribed, and at least one thin fibrous, or fibromuscular capsule was present, which contained smooth muscle in 13 tumors. A minimal infiltration of renal sinus fat was observed in one case, but another invasive pattern was not seen at all. The dominant growth pattern was tubulo-acinar, with cyst formation in a continuum from microscopic to macroscopic cystic spaces in 12 samples. Also, a papillary architecture was observed in 14 tumors and was detected mainly focally. Substantial areas with compact cell nests and trabeculae were seen in 11 samples. The linear arrangement of nuclei, together with its orientation away from the basement membrane, was observed in 16 tumors. Stromal smooth muscle was found in 18 cases.

Immunohistochemical and molecular findings on clear cell papillary RCCs

All exhibited a strong and diffuse CK7 expression. Immunoreaction for CA9 resulted in diffuse staining in 17 tumor samples and focal staining in 4 tumor samples. The “cup-shaped” pattern was detected in 17 cases. CD10 was focally positive in two samples. The mutation status of the *VHL* gene was investigated in 11 samples, and no pathological mutation was found. The *VHL* gene methylation status was analyzed in 16 samples, and none of these harbored promoter region hypermethylation.

General features of clear cell RCCs with diffuse CK7-positivity

In this group, we analyzed 10 cases, and the mean age was 51 years, with 5 females and 5 males. The mean size of the tumors was 29 mm.

Microscopic findings on clear cell RCCs with diffuse CK7-positivity

The predominant growth pattern was a tubulo-acinar, followed by cystic, papillary, and solid. An apical linear nuclear arrangement was seen in 6 samples, and 2 cases contained a smooth muscle rich stroma. Infiltration of the renal vein, sinus, and perinephric fat was not seen.

Immunohistochemical and molecular on clear cell RCCs with diffuse CK7-positivity

There was coexpression of CK7 in a diffuse fashion and CA9 in a diffuse (8 cases) or focal fashion (2 cases). The cup-shaped distribution of CA9 was present in 6 cases. Diffuse CD10-positivity was observed in two cases. The *VHL* gene mutation status was analyzed in 9 samples, and in three cases, a pathogenic mutation was identified. Also, a *VHL* gene promoter hypermethylation was seen in 7 cases.

Follow-up data of both patient group

The median time was 52.5 months (with a range of 1 to 184 months) for clear cell papillary RCC patients and 31.6 months (with a range of 3 to 100 months) for clear cell RCC patients. Only three patients had no follow-up data, and two patients died in non-cancer-related causes. No tumor progression and recurrence was documented for the 26 surviving patients.

CLINICOPATHOLOGICAL, IMMUNOHISTOCHEMICAL AND MOLECULAR FEATURES OF Xp11.2 TRANSLOCATION RCC

Twenty-eight tumors proved to be Xp11.2 RCC among 2804 nephrectomies reviewed from the pathology departments listed above. The diagnosis was later confirmed by immunohistochemistry in each case and by FISH analysis except for three patients.

Clinical and follow-up data

Thirteen male and fifteen female patients were included in our cohort. The median age was 60 years (with range 8 to 72), and 3 tumors occurred in children. Follow-up information was accessible in 21 patients, and the mean follow-up time was 14 months (with a range 2 months to 321 months). Regional lymph node or distant metastasis developed in 13 patients (9 had been discovered before surgery; 6 distant and 3 regional lymph node metastases). Seven patients died from cancer-related causes, and one patient died from a non-cancer-related cause.

Morphological findings on Xp11.2 RCCs

All the tumors that were examined were unilateral and unifocal. The diameter of the tumors ranged from 15 mm to 160 mm, and the average was 78.5 mm. The invasion of the renal vein, sinus, and adipose capsule were observed in 7, 8, 6 cases, respectively. The predominant architectural appearance was a

solid pattern, followed by a papillary pattern, while both a solid and papillary pattern was seen in a small proportion of the cases. The presence of foamy cells, intracytoplasmic pigment, cholesterol clefts, psammoma bodies, and necrosis was observed in 7, 4, 1, 11, and 17 cases, respectively. Most of the tumors had high-grade features.

Immunohistochemical findings on Xp11.2 RCCs

Three cases displayed positivity with CA9, although two of these were necrotic tumors. All the cases investigated were negative with CK7, while CD10 was strongly positive in 17 cases. The diagnostic TFE3 reaction strongly labeled the nuclei in 26/28 cases, but Cathepsin K displayed positivity only in 6 tumors. MelanA was positive only in four cases, and HMB45 showed a weak-to-diffuse positivity in three patients.

FISH findings on Xp11.2 RCCs

The FISH reaction was performed in 25 cases because in three patient the quality of the tumor tissue was not sufficient for a proper analysis. In 21 tumors, typical split signals were seen, while in two patients mostly truncated signal pattern was observed. In one patient, the signals were separated, but they were unusually close to each other. Also, in another patient (a female), an entire break-point region was utterly absent. Hence, in this case, only one signal pair was detected in the nuclei of the tumor cells, while in the surrounding renal parenchyma, two unaffected signal pairs were present.

DISCUSSION

In our first study, the distribution and prognostic features of RCC subtypes in 928 Hungarian patients were analyzed. A relatively low incidence of papillary RCC was observed. ACKD-associated RCC did not occur in a cohort of 16 end-stage kidney disease with RCC. Immunohistochemistry

provides better subtyping of the cases, and in our hands, the panel of CA9, CK7, CD10, AMACR, and TFE3 is quite useful for the daily diagnostic service. For clear cell RCCs and papillary RCCs, low-grade and high-grade groups were assigned, and these groups were associated with different survival rates. Lastly, in clear cell RCC, microscopic tumor necrosis did not prove to be an independent predictor of outcome.

In the subsequent paper of ours, the immunophenotype and the genetic profile of 31 RCCs composed of clear cells, low-grade nuclei, and a tubulopapillary architecture were investigated. Twenty-one cases were classified as clear cell papillary RCC, and 10 as clear cell RCC with diffuse CK7-positivity, and the following conclusions were drawn. First, clear cell papillary RCCs rarely exhibit a predominant papillary architecture, hence their name is misleading, and in our eyes, the tubulo-papillary term is more favored. Second, a linear nuclear arrangement away from the basement membrane and cup-like CA9-positivity is not an essential feature. Third, the evidence for their malignant potential is still lacking. Fourth, RCCs with clear cell papillary RCC morphology, diffuse CK7-positivity, and with an altered *VHL* status do exist; and they can be differentiated from clear cell papillary RCCs only by carrying out molecular tests for the *VHL* status. Last but not least, the biological behavior of both clear cell papillary RCCs and clear cell RCCs with diffuse CK7-positivity seems to be indolent with a favorable clinical outcome.

Lastly, we studied 28 Xp11.2 RCC cases by descriptive light-microscopy, a panel of immunohistochemistry, and FISH tests. We had two tumors with a reasonably unusual morphology, one with an anaplastic carcinoma appearance and another with rhabdoid morphology. We observed a unique FISH pattern with the complete loss of the labeled break-point region. The mean follow-up period was more than 4 years, and it turned out that the prognosis of Xp11.2 RCC in adults is rather poor.

To sum up, we gathered diagnostic experience in both common and rare RCC subsets. This knowledge makes us able to deal with in-house and consultation cases with certainty regardless of the specimen type. By analyzing the validated survival data, we identified prognostic groups according to the histological subsets, and we believe this will facilitate a better patient follow-up and treatment. We studied the clear cell papillary and Xp11.2 RCCs firstly in detail in Hungary. We estimated their incidence rate and provided clinicopathological, immunohistochemical as well as genetic data. Regarding the latter two, in our eyes, immunohistochemistry, along with the genetic tests, have an essential role to discriminate against the overlapping and doubtful entities. Based on our experience, we created a summary table on the RCC subsets.

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| | CCRCC | PRCC T1 | PRCC T2 | ChRCC | CCPRCC | Xp11.2 RCC | CDC | MTSCC |
|-----------------|---|---------------------------------------|------------------------|----------------------|-------------------------|----------------------------------|---|--------------------------|
| Typical cells | Clear | Basophilic | Eosinophilic | Eosinophilic | Clear | Clear, eosinophilic | Eosinophilic | Basophilic |
| Typical pattern | Solid | Papillary, rarely solid | Papillary | Solid | Papillary, tubular | Papillary | Tubular, solid | Tubular |
| Other features | Rich capillary network | Foam cells, low-grade | Foam cells, high-grade | Perinuclear halos | Smooth muscle stroma | Psammoma bodies | Desmoplasia, inflammation | Mucin |
| CA9 | ++ | - | - | - | + (cup-shaped) | +/- | +/- | - |
| CK7 | +/- | ++ | +/- | ++ | ++ | - | ++ | ++ |
| CD10 | ++ | + | +/- | - | - | ++ | - | + |
| AMACR | +/- | ++ | + | - | +/- | + | + | ++ |
| Other IHC | Vim + | Vim + | Vim + | CD117 +, Vim - | ∅ | TFE3 +, melanocytic markers + | p63 -, CK20- GATA3 -, <i>U.europeus</i> + | Vim + |
| Genetics | -3p, <i>VHL</i> mutation methylation | <i>c-MET</i> mutation, +7, +17, -Y | ∅ specific | Poly- and monosomies | ∅ <i>VHL</i> alteration | <i>TFE3</i> translocation | ∅ specific | ∅; but no +7, +17, -Y |

If the histological appearance, immunohistochemistry, and genetic test are inconclusive, but the tumor is positive for PAX8, furthermore, urothelial carcinoma along with metastasis was excluded, the case can be treated as an unclassified RCC. [CCRCC = clear cell carcinoma, PRCC T1 = papillary carcinoma type 1, PRCC T2 = papillary carcinoma type 2, ChRCC = chromophobe carcinoma, CCPRCC = clear cell papillary carcinoma, Xp11.2 RCC = Xp11.2 carcinoma, CDC = collecting duct carcinoma, MTSCC = mucinous tubular and spindle cell carcinoma, Vim = vimentin]