FUMARATE HYDRATASE DEFICIENT RENAL CELL CARCINOMA: A RETROSPECTIVE STUDY PERFORMED AT NHLS UNIVERSITAS ACADEMIC LABORATORIES, 2001 TO 2017

by

Michelle du Preez

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Department of Anatomical Pathology,
Faculty of Health Sciences,
The University of the Free State,
Bloemfontein

Promotor: Prof Jacqueline Goedhals,
Department of Anatomical Pathology,
Faculty of Health Sciences,
University of the Free State,
Bloemfontein

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DECLARATION

I, Michelle du Preez, declare that the coursework Master's Degree mini-dissertation that I herewith submit in a publishable manuscript format for the Master's Degree qualification in Anatomical Pathology at the University of the Free State is my independent work and that I have not previously submitted it for a qualification at another institution of higher education.

Michelle du Preez

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Date

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ABSTRACT

Abstract

Background: Fumarate hydratase deficient renal cell carcinoma (FH deficient RCC) has, in recent years, been described as part of a morphologic spectrum of renal tumours associated with hereditary leiomyomatosis and renal cell carcinoma (HLCRCC) syndrome and is currently recognized as a distinct entity in the 2013 International Society of Urological Pathology (ISUP) Vancouver classification of renal tumours and is included in the 2016 World Health Organization (WHO) classification. FH deficient RCC is an aggressive neoplasm which often presents at a high pathologic stage, with local and distant metastases at the time of diagnosis. FH deficient RCC has a wide variety of morphological patterns, however, the presence of pattern multiplicity, sarcomatoid/rhabdoid morphology and the presence of characteristic nuclear features, i.e. nuclei with prominent viral inclusion-like macronucleoli and perinuclear halos should prompt genetic testing of the tumour. Characteristic immunohistochemical staining patterns, i.e. loss of cytoplasmic expression of fumarate hydratase (FH) and aberrant nuclear expression of S-(2-succino)-cyteine (2SC) have a 100% positive predictive value for identifying FH deficient RCC, however, only FH is available for commercial use. Retention of cytoplasmic staining for FH does not exclude FH deficient RCC since the gene may still be functional in missense variants of the FH gene. To our knowledge, there is presently no published research regarding FH deficient RCC and its association with HLRCC syndrome in Africa. The impact of this disorder in South African patients, is therefore unknown.

Aim: The aim of this study was to determine the number and profile of patients with FH deficient RCC seen by the Department of Anatomical Pathology, University of the Free State over a 17-year period, from 1 January 2001 to 31 December 2017.

Methods: A retrospective, cross-sectional study was performed. All cases of primary renal cell carcinoma and RCC subtypes diagnosed between 1 January 2001 and 31 December 2017 were included. An immunohistochemical stain for FH was performed on all the cases. All the cases with the typical phenotype for FH deficient RCC were submitted for molecular analysis and genotyping.

Results: 172 patients were included in the study. Ninety (52.33%) were male and 82 (47.67%) were female. The mean age at presentation was 54.2 years and most patients presented in the 5th to 6th decades of life. All cases showed retained cytoplasmic staining with FH. One (0.58%) case of FH deficient RCC with the characteristic phenotype and a missense mutation in Exon 7 of the FH gene was identified. The patient was a 53-year-old black female and no information regarding the presence of uterine leiomyomas or family history of RCC was available.

Conclusion: All cases of RCC displaying the typical phenotype of FH deficient RCC (whether immunolabeling for FH is retained or lost) should be submitted for molecular testing in order to identify patients with HLRCC syndrome or confirm the diagnosis of sporadic FH deficient RCC to minimize morbidity and mortality in such patients and their families.

Keywords: Fumarate hydratase deficient renal cell carcinoma, hereditary leiomyomatosis and renal cell carcinoma syndrome, renal cell carcinoma, fumarate hydratase, South Africa

LIST OF ABBREVIATIONS

RCC:	Renal cell carcinoma
ccRCC:	Clear cell renal cell carcinoma
HLRCC:	Hereditary leiomyomatosis and renal cell carcinoma syndrome
PTEN:	Phosphatase and tensin homologue deleted on chromosome ten
BAP-1:	BRCA1 associated protein 1
FH:	Fumarate hydratase
HIF:	Hypoxia inducible factor
GLUT-1:	Glucose transporter isoform 1
NRF-2:	Nuclear factor erythroid 2-related factor 2
AMPK:	5’ adenosine monophosphate-activated protein kinase
ISUP:	International Society of Urological Pathology
WHO:	World Health Organization
2SC:	S-(2-succino-)-cysteine
PD-1:	Programmed cell death protein 1
PD-L1:	Programmed death ligand 1
DNA:	Deoxyribonucleic acid
WT:	Wild type
B:	Benign
US:	Uncertain significance
LP:	Likely pathogenic
F:	Fail
CA:	Competitive amplification
VUS:	Variant of unknown significance

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CHAPTER 1

LITERATURE REVIEW

1. Background

Renal cell carcinoma (RCC) includes a group of neoplasms which differentiate toward renal tubular epithelial cells. It is one of the ten most prevalent cancers around the world. Clear cell renal cell carcinoma (ccRCC) is the most prevalent among the different subtypes and is responsible for the bulk of malignancy-related deaths(1).

Cigarette use, obesity and high blood pressure have been found to contribute to the development of RCC. Genetic variants have, however, also been connected to the development of renal cancers, with around 3% of patients known to have a family history(2).

Hereditary RCC is often associated with an autosomal dominant mode of inheritance (penetrance, however, often varies). If the variant has, however, arisen de novo or if it is not penetrant in the parent carrier, there might be no family history of RCC. In the absence of a family history, inherited RCC should be considered when both kidneys are affected and/or multicentric tumours, which are of early onset, are present. Molecular genetic analysis should then be considered to confirm or exclude the diagnosis(2).

The major hereditary RCC syndromes are Birt-Hogg-Dube syndrome, Von Hippel-Lindau syndrome, hereditary type I papillary renal cell cancer, hereditary leiomyomatosis and renal cell cancer (HLRCC), succinate dehydrogenase subunit deficient RCC, chromosome 3 translocations, Cowden syndrome and familial BAP1 tumour syndrome(2).

Fumarate hydratase deficient renal cell carcinoma (FH deficient RCC) has, in recent years, been considered as part of an architectural variety of renal cancers associated with hereditary leiomyomatosis and renal cell carcinoma syndrome(3).

HLRCC syndrome, which also goes by the name "Reed syndrome", is an uncommon disorder which has only been found in approximately 200 families worldwide(4,5). The ensemble of syndromic findings in HLRCC was first mentioned and documented in an article by Reed *et al.* in 1973(6). Literature describing a familial susceptibility to uterine leiomyomas and renal cell carcinoma in two families from Finland, was published in 2001(4,7).

HLRCC clinically manifests as:

1. Multiple cutaneous leiomyomas (associated with the arrector pili muscle), which present as hard, papular or nodular, erythematous lesions on the skin, often involving the trunk and limbs,
2. The early onset, usually in patients not older than 40 years of age, of multiple and, in many cases, symptomatic uterine leiomyomas which may lead to early surgical interventions, and
3. Onset of aggressive renal cell carcinoma, often in patients younger than 40 years of age, in up to 20% to 30% of these patients, which tends to metastasize early(7).

HLRCC syndrome is an autosomal dominant disorder, characterized by germline heterozygous 5' missense variants and deletions in the fumarate hydratase genetic code, located on chromosome 1q43.2-q43(3,9-12). Characteristically, there is somatic inactivation of the corresponding allele, which suggests that the fumarate hydratase gene is a tumour suppressor gene(8).

Kiuru *et al*. reported that homozygous or compound heterozygous fumarate hydratase variants lead to a recessive disease, fumarate hydratase deficiency, which is characterized by neurological disability and retardation of growth and development(9). These individuals generally only survive a few months and no tumours have been reported in them or in their first degree relatives(9).

The fumarate hydratase gene encodes the enzyme fumarate hydratase, or fumarase, a vital component of the Krebs cycle (tricarboxylic acid cycle), which actuates the change of fumarate to a different substance named malate(3,11,15). When fumarase activity is suboptimal, the collection of fumarate in the cell serves as an oncometabolite and epigenetic modifier, both in the mitochondria and cytoplasm(16,17).

Several mechanisms of tumorigenesis may contribute to the development of the renal tumours in HLRCC syndrome, including:

1. A metabolic shift from oxidative phosphorylation to aerobic glycolysis,
2. Impeding hypoxia inducible factor (HIF) prolyl hydroxylase enzymes which leads to HIF upregulation and upregulation in HIF target genes such as GLUT1,
3. Aberrant succination with accumulation in the cell of S-(2-succino)-cysteine, and
4. Altered activity of transcription factors, including "pseudohypoxic" upregulation of HIF1a and NRF2 as well as AMPK (5,16-18).

These changes stimulate renal oncogenesis by way of the Warburg effect, may cause epithelial to mesenchymal transition(6) and ultimately lead to increased cell proliferation and resistance to programmed cell death/apoptosis(3,5,18).

Malignant cells change their metabolism to aid growth, endurance, proliferation and lengthy preservation. The mutual characteristic of this change in metabolism is promotion of glucose uptake by the tumour cells and fermentation of glucose to lactate. This occurrence is seen even when there are functioning mitochondria and is known as the Warburg effect(10).

HLRCC syndrome has incomplete and variable penetrance – approximately 80% of carriers will develop utero-cutaneous leiomyomas, while only 5-20% of carriers will develop a high-grade renal cell carcinoma(11,12).

Another study also suggested highly penetrant cutaneous (>90% of males and females) and uterine (>70% of females) leiomyomatosis and less penetrant (10-30% of males and females) FH deficient RCC(13).

Thus, most of the women suffering from HLRCC will develop bothersome uterine leiomyomas, which require surgical intervention at a young age – generally before renal cell carcinoma manifests(14). If selection bias is negated, the real penetrance of renal cancer is closer to 10-15%(15).

HLRCC syndrome associated renal cell carcinoma, i.e. FH deficient RCC, is presently accepted as a distinct disease(16) in the International Society of Urological Pathology (ISUP) Vancouver Classification of renal tumours, 2013 and is also part of the 2016 World Health Organization classification(4,7,26).

Renal tumours associated with HLRCC syndrome are usually aggressive and tend to present at a high pathologic stage(17,18), with local and distant metastases at the time of diagnosis – even in the event of a small primary carcinoma(19). Many patients die of disease within five years of initial diagnosis(12,30).

Due to the broad spectrum of morphological patterns, it may be problematic to recognise FH deficient RCC, and many of the tumours are diagnosed as type II papillary-, collecting duct-, unclassified renal cell carcinoma or renal medullary carcinoma. Recognition of FH deficient RCC is of paramount importance because of its aggressive behaviour and its association with a familial cancer syndrome(19).

Detection of these patients in a timely fashion, with implementation of feasible screening guidelines and preventative measures could potentially decrease the morbidity and mortality associated with the renal tumours in this syndrome(12,17).

2. Clinical presentation

Renal cell carcinomas associated with HLRCC are usually unilateral, single lesions – in contrast to other hereditary renal tumours, which are often bilateral and multifocal(3,7) – and present at a younger age(16).

The renal cell carcinomas associated with HLRCC are highly aggressive and usually have a higher rate of distant metastases at presentation than those patients with other hereditary renal cancer syndromes, such as Von Hippel-Lindau disease(8,31). FH deficient RCC also presents at higher pathological stages and between 57% and 75% of patients present with pT3 and pT4 tumours(3,19).

Many patients have both local metastases and metastases to extrarenal sites at the time of diagnosis and a high number die of disease less than five years after diagnosis(20,21). Metastases to the brain, lungs, adrenals, liver, bone, ovaries, peritoneum, tonsils and lymph nodes have been observed(4,19).

Recent studies have shown that the median age at presentation varies between 34 years and 51.7 years, with another study reporting a median age of 44 to 46 years(3,8,20,29-30). The youngest patient with confirmed HLRCC syndrome and FH deficient RCC was 11 years old at the time of diagnosis(10,11). Some studies show a slight male predominance and the highest reported male to female ratio is 3.1:1(3,19,22,23).

Studies report that the most common symptoms and signs at presentation include back, abdominal and flank pain, anaemia, as well as haematuria(4,13).

3. Pathology

a. Macroscopic pathology

A review of the literature with regards to macroscopic pathology of FH deficient RCC shows a median dimension which ranges from 6.5 cm to 11.6 cm(11,19), with the

smallest tumour in the literature measuring 2 cm in size and the largest measuring 30 cm(3,12).

FH deficient RCCs have a yellow, light-brown, brown, white or tan cut surface, and appear both solid and cystic (3,4,13,17,24,25). The tumours are generally unencapsulated but up to half can have a well-circumscribed border(3,13,24). Some tumours bulge from, but are confined to the kidney(13,20), while others infiltrate the perirenal fat, the renal sinus adipose tissue and the adrenal gland(25). These tumours can also have areas of haemorrhage and necrosis(4,24).

b. Microscopic pathology

Originally, FH deficient RCC was reported as a malignant neoplasm with a predominantly papillary architecture. The papillary structures are bordered by cells with ample, pink cytoplasm and nucleoli which resemble viral inclusions(19). However, lately it has been recognised that the tumour may display a much wider array of architectural patterns and the prominent nuclear features may only be present focally(19).

Due to these features, FH deficient renal cell carcinoma may be difficult to recognise and be misdiagnosed as type II papillary RCC, renal medullary carcinoma, collecting duct carcinoma, Xp11.2 translocation RCC, tubulocystic carcinoma, high-grade unclassified RCC, or high-grade clear cell RCC.(19,21,23).

Different patterns observed in FH deficient RCC in syndromic tumours include papillary, cribriform/sieve-like, solid, sarcomatoid, clear cell features, tubular, tubulopapillary, tubulocystic, cystic, nested with desmoplasia and low-grade oncocytic, morphologically resembling succinate dehydrogenase-deficient renal cell carcinoma(13,19,21,26). This pattern multiplicity is usually not seen in non-variant carriers(27).

A prominent, pink macro-nucleolus with a halo around the nucleus is characteristic of HLRCC syndrome associated RCC according to both the 2012 ISUP and 2016 WHO kidney tumour classification (27). However, studies have shown that, although the prominent pink nucleoli with perinuclear clearing were prevalent in most every FH deficient RCC, they were also found in approximately 58% of type II papillary renal cell carcinomas from patients with a wild-type allele(27). Other studies conducted showed that the cytomegalovirus inclusion-like nucleoli surrounded by a clear halo were present only focally in some tumours(4).

In a study by Muller *et al.*, the researchers urge pathologists to tie less importance to the prominent nucleoli and perinuclear halos, as although these findings appear to be sensitive markers for FH deficient renal cell carcinoma, they are not very specific(27).

The characteristic nuclear features, as well as other characteristics such as hypercellularity, pleomorphic nuclei and the appreciation of globular, pink intracytoplasmic inclusions can also be observed in the uterine leiomyomas associated with HLRCC. These findings have not, however, been seen with similar prevalence in the cutaneous leiomyomas associated with HLRCC(15).

Due to the remarkable overlapping features of many aggressive renal cell carcinomas, recent studies have highlighted the usefulness of immunohistochemistry for fumarate hydratase (FH) and S-(2-succino-)-cysteine (2SC) in diagnosing FH deficient RCC(11,15,22).

Many renal cell carcinomas have been identified with morphologic and immunohistochemical findings suggestive of HLRCC syndrome, however, since little data is available with regards to family history, other stigmata of the syndrome or a history of genetic testing, the term "FH deficient RCC" has been proposed for cases with the typical phenotype. The proposed term avoids diagnosis of a genetic syndrome without adequate work-up and can also be utilized for sporadic, non-syndrome associated cases of FH deficient RCC due to somatic variations(3,11,19). It is also

suggested that "FH deficient RCC" be used with a recommendation in the pathology report for genetic work-up, which is frequently positive in these cases(11).

4. Immunohistochemistry

Antibodies against 2SC and FH, in association with pattern multiplicity and a rhabdoid/sarcomatoid morphology, are the most useful tools to diagnose FH deficient renal cell carcinoma and prompt genetic testing rather than the so-called "characteristic" nuclear features(27).

FH deficient RCCs are regularly FH negative and 2SC positive. The loss of immunohistochemical staining for FH is directly linked to the loss of function of the fumarate hydratase gene.

An increase in the intracellular fumarate level will cause spontaneous interaction with the cysteine residues of a lot of proteins. This will result in succination and accumulation of 2SC in the nucleus(27). The abnormal succination can be demonstrated by polyclonal antibodies against 2SC(21). Bardella *et al.* concluded that positivity for 2SC in the tumour cells predicts variations in the FH gene in subjects referred for variant analysis. This positive staining was absent in a heterogenous group of non-HLRCC-related tumours evaluated in the study(21,28).

The antibody for FH is commercially available and it is often used to confirm fumarate hydratase malfunction in a renal cell carcinoma, prompting genetic testing(21,27). The antibody for 2SC is not available for commercial use and is currently only used in a research context(4).

FH deficient RCC is characterized by loss of FH by immunohistochemical staining. The loss of FH is 100% specific in identifying FH deficient RCCs, however, its sensitivity is only about 87.5%. Coarse, cytoplasmic expression of FH is considered positive expression. FH is also expressed in non-neoplastic cells such as vascular endothelial cells and inflammatory cells, as demonstrated in Figure 1.1(3,27). The FH stain is considered negative when there is

loss of coarse, cytoplasmic staining in the tumour cells, with adequate positive staining in the non-neoplastic tissues(3,27).

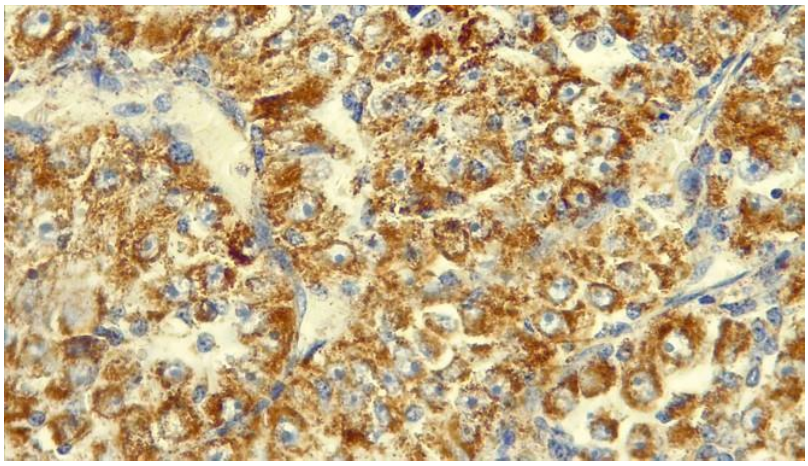

Figure 1.1 Immunohistochemistry against fumarate hydratase of case number 7 in our study. 40X magnification. Note the granular, cytoplasmic staining indicative of a positive stain.

An important pitfall in the interpretation of the FH stain, is that expression can be retained in cases with a missense variant, since the capability to make the fumarate hydratase protein is still there(3,12,22).

The 2SC stain is appreciated as positive when there is positive staining in both the cytoplasm and the nuclei of the tumour cells(3,21,22). Non-neoplastic cells show no staining. The 2SC stain has been demonstrated, by several authors, to be extremely sensitive. Sensitivity and specificity for 2SC were both shown to be 91.7%(27). The combined sensitivity and specificity for FH negative and 2SC positive tumors was 100%(3,27).

In the ideal setting, both antibodies (for FH and 2SC) would be used for confirmation of an FH deficient RCC, however, as mentioned, 2SC is not commercially available(3,27). In the case of a positive or equivocal FH stain and unavailable 2SC stain, one should rely on characteristic morphology to guide further investigation into the patient's personal and family history(3,7,27).

5. Molecular analysis

Individuals who suffer from HLRCC have one wild-type and one mutated allele of the FH gene. As Knudson's "two-hit" hypothesis has predicted, a tumour will develop when the wild-type allele is also knocked out by a somatic variant during the course of the life of a patient with HLRCC(9,12,18,29).

The heterozygous variant state results in HLRCC, whereas homozygous or compound heterozygous variants in FH result in fumarate deficiency syndrome, which is deadly within the first decade of life(12).

Tomlinson *et al.* have mapped the FH gene to chromosome 1q42.3-q43. HLRCC associated variants are thought to lead to absent or truncated protein, or substitutions or deletions of highly conserved amino acids. The FH gene encodes the enzyme FH and activity of FH is reduced in the lymphoblastoid cells from patients with HLRCC(29).

Screening programs using immunohistochemistry for either 2SC or FH should, as noted before, act as investigational and prompt further genetic work-up(18). Martinek *et al.* have found that variants in the FH gene are basically evenly distributed along the ten exons of the gene and most of them (81%) are missense or other types of point variants with only a few large whole gene deletions(18). Sanger sequencing of the whole coding region of the gene will thus detect more than 97% of all known variants(18).

The FH gene plays a role in the development of FH deficient RCC associated with HLRCC. It is, however, not clear whether the risk of renal cell carcinoma is associated with a certain subtype of FH germline variants, environmental exposure or with a

specific alterant gene because of the relative low penetrance of renal cell carcinoma in HLRCC syndrome(7,29).

At present, molecular genetic testing is the only diagnostic procedure which is able to accurately diagnose individuals with HLRCC(18).

6. Prognosis

The renal cell carcinomas associated with HLRCC syndrome are usually at an advanced stage at presentation, they are aggressive and generally have poor clinical outcomes. In a study conducted by Toro *et al.*, 9 out of 13 patients demised of metastatic renal cell carcinoma within five years of diagnosis(12).

In a study conducted by Hubert *et al.*, after a median follow-up time of 16 months in 26 patients, 19% were disease free, 31% were alive with disease, while 50% were dead from disease(19).

It is thus clear that FH deficient RCC often displays aggressive behavior and tends to present with either locally extensive and/or metastatic disease(19).

The diagnosis of FH deficient RCC should prompt urgent referral for appropriate surgical interventions and systemic therapy, as well as genetic counseling, testing and subsequent stringent follow-up for the patient and their family members(19).

It is difficult to develop monitoring guidelines for HLRCC, however, the possible effect from genetic anticipation, as demonstrated by Wong et al. underlined the need to start monitoring of these patients at an early age(30).

The goal of surveillance is minimization of morbidity and mortality via pre-symptomatic detection of renal tumours and adequate treatment which in turn alters the natural course of the disease(30).

Van Spaendonck-Zwartz *et al*. suggested that kidney surveillance in patients with HLRCC should include the use of non-invasive modalities such as renal ultrasound, executed by an expert radiologist, followed by MRI surveillance at a later stage. The ultrasound surveillance should start at the age of 10 years and performed every 6 months until an MRI scan (which is often distressing for young children) can be done from the age of 16 to 18 years. MRI surveillance should also take place biannually(24).

Further data is needed for future guidelines on monitoring and treatment of HLRCC(24).

7. Treatment

a. Surgical treatment

Approximately 30 years ago, the standardized treatment approach for localized renal cancer was nephrectomy with a lymph node dissection. Currently, however nephron sparing surgery is implemented for small renal cancers(7).

This approach is of great value for patients with hereditary renal carcinoma syndromes, which are associated with the occurrence of multifocal and bilateral tumours. Thus, synchronous and metachronous tumours may necessitate multiple surgeries and sparing of renal function is of paramount importance in said patients.

In Von Hippel-Lindau Syndrome, hereditary papillary renal cell carcinoma and Birt-Hogg-Dube syndrome, surgical intervention is advised when the size of the biggest tumour is larger than 3cm. The above rule of thumb applies because of the relatively indolent growth of tumours in these syndromes(7). HLRCC, however, does not follow this rule since metastases can occur even in the setting of a small primary tumour, which is usually unilateral and single(7).

Since FH deficient RCC is biologically more aggressive than other types of kidney cancer, active surveillance is not advocated(7). The recommended treatment is usually

partial nephrectomy with wide surgical resection planes and retroperitoneal lymphadenectomy(7). If there is doubt that a partial nephrectomy would be curative, a radical nephrectomy is advised. Radiofrequency ablation or cryotherapy is not recommended for cancers in patients with HLRCC(7).

b. Systemic treatment for metastatic carcinoma in HLRCC

Currently, there is no standard systemic treatment for metastatic FH deficient RCCs(7). A study of erlotinib and bevacizumab, targeting vascular endothelial growth factor and epidermal growth factor receptor, is under way for the treatment of advanced HLRCC associated renal cell carcinoma and sporadic papillary renal cell carcinoma. The rationale behind these treatments is the implied role of lessened FH activity in HIF stabilization and activation of HIF target receptors(7).

A study conducted by Alaghehbandan *et al.* found that PD-1/PD-L1 expression in FH deficient RCC is only present in a small number of cases(31). Targeted therapies may, however, be beneficial in the positive cases, if the expression is correlated by immunohistochemistry and molecular testing(31).

8. Rationale behind this study

To our knowledge, there is currently no published research regarding FH deficient RCC and its association with HLRCC syndrome in Africa. The impact of this disorder in South African patients is therefore unknown.

9. Aim and objectives

Aim

The aim of this study is to determine the number and demographics of patients with fumarate hydratase deficient renal cell carcinoma seen by the Department of Anatomical Pathology at the University of the Free State and in collaboration with the NHLS.

Objectives

1. To establish the number of cases of renal cell carcinomas seen with salient morphological features and immunohistochemical staining signatures suggesting HLRCC and their correlation with FH mutation analysis by DNA sequencing over a 16-year period from January 2001 to December 2017 by the Department of Anatomical Pathology at the University of the Free State in collaboration with the NHLS.
2. To evaluate the demographic and pathological features of the patients diagnosed with Fumarate Hydratase deficient renal cell carcinoma.

Should cases of FH-deficient RCC be identified, the FH immunohistochemical stain will be added to the Department of Anatomical Pathology diagnostic platform to be used in future cases of renal cell carcinoma.

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Chapter 2

FUMARATE HYDRATASE DEFICIENT RENAL CELL CARCINOMA: A RETROSPECTIVE STUDY PERFORMED AT NHLS UNIVERSITAS ACADEMIC LABORATORIES, 2001 TO 2017

Du Preez M[1], Oosthuizen J[2], Goedhals J[1]

1. Department of Anatomical Pathology, Faculty of Health Sciences, University of the Free State and National Health Laboratory Service, Bloemfontein, South Africa
2. Division of Human Genetics, Faculty of Health Sciences, University of the Free State and National Health Laboratory Service, Bloemfontein, South Africa

Contact person: Dr M du Preez, mflooi429@gmail.com, +27514053050

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Abstract

Background: Fumarate hydratase deficient renal cell carcinoma (FH deficient RCC) is a distinct and aggressive neoplasm associated with hereditary leiomyomatosis and renal cell carcinoma (HLCRCC) syndrome. Pattern multiplicity, sarcomatoid/rhabdoid morphology, presence of characteristic nuclear features and loss of cytoplasmic staining for FH by immunohistochemistry should prompt genetic testing. Retention of FH expression does not exclude FH deficient RCC since the functionality of the gene is usually preserved in missense variants. To our knowledge, no published research regarding FH deficient RCC and its association with HLRCC syndrome in Africa, is available.

Aim: To determine the number and profile of patients with FH deficient RCC seen at our institution from 1 January 2001 to 31 December 2017.

Methods: All cases of primary RCC diagnosed within the time frame, were included in this retrospective, cross-sectional study. An immunohistochemical stain for FH was performed on all cases. Molecular analysis was performed on cases with suggestive morphology.

Results: Of 172 patients, 90 (52.33%) were male and 82 (47.67%) were female. Most patients presented in the 5^{th} to 6^{th} decades of life. All cases showed retained cytoplasmic staining with FH immunohistochemistry. One (0.58%) case of FH deficient RCC with a missense variant in Exon 7 of the FH gene was identified in a 53-year-old black female. No information regarding the presence of uterine leiomyomas or family history of RCC was available.

Conclusion: All cases of RCC displaying typical features of FH deficient RCC should be submitted for molecular testing to confirm the diagnosis of syndromic or sporadic FH deficient RCC.

Keywords: Fumarate hydratase deficient renal cell carcinoma, hereditary leiomyomatosis and renal cell carcinoma syndrome, renal cell carcinoma, fumarate hydratase, South Africa

Article

Introduction

Renal cell carcinoma (RCC), one of the ten most prevalent cancers in the world, is a diverse group of tumours which differentiate toward renal tubular epithelial cells(1). Smoking, obesity and hypertension have been found to play a role in the development of RCC and genetic variants have also been implicated in its pathogenesis, with about 3% of patients known to have a family history(2).

The major hereditary RCC syndromes are Birt-Hogg-Dube syndrome, Von Hippel-Lindau syndrome, hereditary type I papillary renal cell cancer, hereditary leiomyomatosis and renal cell cancer (HLRCC), succinate dehydrogenase subunit deficient RCC, chromosome 3 translocations, Cowden syndrome and familial BAP1 tumour syndrome(2).

Fumarate hydratase deficient renal cell carcinoma (FH deficient RCC) has, in recent years, been described as part of an architectural variation of renal tumours associated with hereditary leiomyomatosis and renal cell carcinoma syndrome, also referred to as Reed syndrome, an uncommon disorder which has only been found in approximately 200 families worldwide(3–5). HLRCC syndrome associated renal cell carcinoma is currently recognized as a distinct entity(6) in the International Society of Urological Pathology (ISUP) Vancouver Classification, 2013 of renal tumours and is also included in the 2016 World Health Organization classification(4,7,8).

HLRCC syndrome is clinically characterized by three main features: multiple cutaneous leiomyomas associated with the arrector pili muscle, early onset of multiple often symptomatic uterine leiomyomas and onset of aggressive kidney cancer before the age of 40 years(7). It is an autosomal dominant illness, defined by germline heterozygous 5’ missense variants and deletions in the fumarate hydratase genetic code, located on chromosome 1q43.2-q43(3,9-10). Characteristically, there is somatic inactivation of the corresponding allele, which suggests that the fumarate hydratase gene is a tumour suppressor gene(11).

The fumarate hydratase gene encodes the enzyme fumarate hydratase, or fumarase, a vital component of the Krebs cycle (tricarboxylic acid cycle), which actuates the change of fumarate to malate(3,12-13). When fumarase activity is suboptimal, the accumulation of fumarate in the cell serves as an oncometabolite and epigenetic modifier, both in the mitochondria and cytoplasm(6,14).

HLRCC syndrome has incomplete and variable penetrance and approximately 80% of carriers will develop utero-cutaneous leiomyomas, while only 5-20% of carriers will develop a high-grade renal cell carcinoma(10,12). If selection bias is negated, the real penetrance of kidney tumours is closer to 10-15%(13).

Originally, FH deficient RCC was reported as a malignant neoplasm with a predominantly papillary architecture. The papillary structures are bordered by cells with ample pink cytoplasm and nucleoli resembling viral inclusions(16). However, lately it has been recognised that the tumour may display a much wider array of architectural patterns and the prominent nuclear features may only be present focally(16).

Many renal cell carcinomas have been identified with morphologic and immunohistochemical findings suggestive of HLRCC syndrome, however, since little data is available with regards to family history, other stigmata of the syndrome or a history of genetic testing, the term "FH deficient RCC" has been proposed for cases with the typical phenotype. The proposed term avoids diagnosis of a genetic syndrome without adequate work-up and can also be utilized for sporadic, non-syndrome associated cases of FH deficient RCC due to somatic variants(3,12,16). It is also suggested that "FH deficient RCC" be used with a recommendation in the pathology report for genetic work-up, which is frequently positive in these cases(12).

Antibodies against 2SC and FH, in association with pattern multiplicity and a rhabdoid/sarcomatoid morphology, are the most useful tools to diagnose FH deficient renal cell carcinoma and prompt genetic testing rather than the so-called "characteristic" nuclear features(18).

FH deficient RCCs are regularly FH negative and 2SC positive. The loss of immunohistochemical staining for FH is directly linked to the loss of function of the fumarate hydratase gene. An increase in the intracellular fumarate level will cause spontaneous interaction with the cysteine residues of a lot of proteins. This will result in succination and accumulation of 2SC in the nucleus(18). The abnormal succination can be detected by a polyclonal antibody against 2SC(19).

In the ideal setting, antibodies for both FH and 2SC would be used for identification of an FH deficient RCC, however, 2SC is not currently commercially available(3,18). In the case of a positive or equivocal FH stain and unavailable 2SC stain, one should rely on characteristic morphology to guide further investigation into the patient's personal and family history(3,7,18). At present, molecular genetic testing is the only diagnostic procedure which is able to accurately diagnose individuals with HLRCC(15).

To our knowledge, there is presently no published research regarding FH deficient RCC and its association with HLRCC syndrome in Africa. The goal of our research was therefore to determine the number and profile of patients with FH deficient renal cell carcinoma seen by the Department of Anatomical Pathology at the University of the Free State (UFS) and the National Health Laboratory Service (NHLS).

Materials and methods

A retrospective cross-sectional study was performed. The study included cases of renal cell carcinoma identified by the Department of Anatomical Pathology at the UFS and NHLS over a 17-year time frame. The department provides histopathological services to all government health institutions in the Free State province and at times from the Northern Cape and North West provinces. A manual search of archived pathology request forms was performed to identify cases between 1 January 2001 and 31 March 2004 and a Systematised Nomenclature of Medicine (SNOMED) search of the NHLS electronic databases was utilised to identify cases diagnosed between 1 April 2004 and 31 December 2017. Men and women of all ages were included. Cases were excluded from the study if there was insufficient tissue available in archived wax blocks and if the tumour was identified as a metastasis or a benign renal tumour.

The slides and wax blocks of each case were sourced from the laboratory archives and the slides were reevaluated by two investigators and a representative wax block was chosen (MdP and JG).

The wax blocks were sectioned at 4 micrometres and stained using an anti-fumarate hydratase rabbit monoclonal antibody (clone EPR211104, Abcam Inc., Cambridge, MA). A Benchmark XT automated slide stainer was used to stain the slides. Immunohistochemistry was performed with the antibody against FH/Fumarase at a 2:1000 dilution, succeeded byGoat Anti-Rabbit IgG H&L (HRP) ready to use. Mayer's haematoxylin was used to counterstain the slides, followed by dehydration. The slides were then cover slipped.

The immunohsitochemical stains were evaluated by two investigators (MdP and JG). Any coarse cytoplasmic staining in the tumour cells was interpreted as positive. Complete absence of coarse cytoplasmic staining in the tumour cells with positive staining in the endothelial cells in the blood vessels was regarded as negative. Any cases that were negative for FH or had nuclear features suggestive of FH deficient RCC underwent molecular testing.

DNA extraction

DNA was extracted from formalin fixed paraffin embedded (FFPE) tumour blocks using a QIAamp DNA FFPE Tissue Kit with the addition of a QIAgen supplementary protocol for the deparaffinization step according to the manufacturer's instructions (QIAamp DNA FFPE Tissue Kit, cat. no. 56404; Deparaffinization Solution, cat. no. 19093).
Selected tumour blocks were cut into 6 µm sections of which two sections per block were placed immediately in a 1.5ml microcentrifuge tube containing 160 µl deparaffinization solution whereafter the QIAgen deparaffinization supplementary protocol was followed to step 12 and continued from step 14 of the QIAamp DNA FFPE

Tissue handbook. Extracted DNA quality and quantity were verified on a Nanodrop-1000 spectrophotometer.

PCR Amplification

Primer sequences for targeted amplification were obtained from literature (Kiuru *et al.*, 2002) and synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). Targeted amplification was performed on a LightCycler 480 II using the LightScanner Master Mix (Idaho Technology Inc., Salt Lake City, UT) according to the manufacturer's instructions. Each 10 µl reaction contained 30 ng genomic DNA, 0.3 µM of each primer, 0.4 mM $MgCl_2$ and 4 µl LightScanner® Master Mix according to the manufacturer's instructions. Annealing temperature was set to 60 degrees Celsius.

Sanger sequencing

Illustra™ ExoStar™ 1-Step Enzymatic PCR and Sequence reaction clean-up was performed prior to Sanger sequencing according to the manufacturer's instruction (Life Sciences, GE Healthcare, UK). Sequencing of the FH gene targets were performed using previously described primers (Kiuru et al., 2002) and BigDye® Terminator v3.1 cycle sequencing kit (Life Technologies Corporation, Austin, TX) whereafter purification was performed with ethanol/EDTA according to the manufacturer's instructions. Sequencing products were separated on an ABI 3530XL automated sequencer (Life Technologies, Foster City, CA).

Variant Classification

Sanger sequences quality and base calling were performed using Chromas v2.6.6 (Technelysium Pty Ltd, Queensland, Australia). Variant nomenclature was annotated according to the Human Genome Variation Society (HGVS) recommendations (https://varnomen.hgvs.org/) using the FH gene reference transcript NM_000143.4 and human genome reference hg19 (GRCh37). The clinical significance of the variants was determined using the American college of Medical Genetics guidelines (ACMG, Richards et al., 2015) and the Varsome genomics search engine (ttps://doi.org/10.1093/bioinformatics/bty897).

In addition, the patients' age, sex, race, type of renal cell carcinoma, results of diverse immunohistochemistry and clinical manifestation, including the presence of leiomyomas were noted. This information was obtained from the pathology reports.

Results

Over the seventeen year period, a total of 172 cases of renal cell carcinoma conformed to the inclusion criteria of this study. An average of 10.12 cases were diagnosed per year, with the number of cases per year ranging from 5 to 16. The number of cases per year is summarized in Table 1.

Table 1. Number of renal cell carcinoma cases diagnosed per year.

Year	Number of cases
2001	13
2002	8
2003	5
2004	16
2005	11
2006	15
2007	16
2008	5
2009	11
2010	12
2011	11
2012	7
2013	8
2014	9
2015	7
2016	12
2017	6
Total:	**172**

The median age of the patients was 56 years, with a mean age of 54.2 years and a range of 7 to 81 years. Most patients were diagnosed in the 5th and 6th decades of life. Fifty-one patients were identified in the age group 51 – 60 years, while 41 patients were diagnosed in the age group 61 – 70 years. The ages of the patients are summarized in Table 2.

Table 2. Ages of the patients diagnosed with renal cell carcinoma.

Age group	Number of patients
0 – 10 years	1
11 – 20 years	3
21 – 30 years	8
31 – 40 years	12
41 – 50 years	35
51 – 60 years	51
61 – 70 years	41
71 – 80 years	19
>80 years	2
Total:	**172**

A slight male predominance was seen with 90 (52.33%) male patients and 82 (47.67%) females with a male to female ratio of 1.1:1. One hundred and five (61.04%) of the patients were black, while 58 (33.72%) patients were white, and 8 (4.65%) patients were coloured. Only 1 (0.58%) patient was of Indian ethnicity. The race of the patients is summarized in Table 3.

Table 3. Race of the patients diagnosed with renal cell carcinoma.

Race	Number of patients
Black	105
White	58
Coloured	8

Indian	1
Total:	**172**

Eighty-four (48.84%) of the tumours were situated on the right, while 76 (44.19%) were situated on the left. The laterality of the tumours was not stated in 12 (12.79%) patients. One hundred and thirty-three (77.33%) cases were nephrectomy specimens, 13 (7.56%) were needle biopsy specimens, 11 (6.4%) were enucleation or resection specimens and 14 (8.14%) were biopsy specimens not otherwise specified. No information on the type of procedure performed was available in 1 (0.58%) case.

No information with regards to the presence of cutaneous or uterine leiomyomas, nor any information regarding a family history of RCC was noted in any of the pathology reports.

Ten (5.81%) cases with morphological features suggestive of FH deficient renal cell carcinoma, i.e. tumours consisting of large cells with abundant eosinophilic cytoplasm with at least focal viral inclusion-like macronucleoli and a perinuclear halo, were identified. Of the 172 cases that were stained with FH, all 172 (100%) of the cases showed granular cytoplasmic staining.

The age, sex, race, type of renal cell carcinoma that was originally diagnosed, results of other immunohistochemical stains and clinical presentation of the 10 cases that were selected for molecular analysis are summarized in Table 4.

Table 4. Summary of cases with morphological features suggestive of FH deficient renal cell carcinoma.

Case	Age	Sex	Race	Original diagnosis	Immunohistochemistry	Clinical presentation
1.	74yr	Female	White	ccRCC	None	Right renal tumour
2.	72yr	Female	Black	RCC	None	Right renal tumour
3.	38yr	Female	Black	RCC	None	Left renal tumour

4.	56yr	Male	Coloured	RCC	None	Right renal tumour
5.	42yr	Female	Black	Sarcomatoid RCC	None	Renal tumour (side not stated)
6.	52yr	Male	Coloured	RCC	None	Right renal tumour
7.	53yr	Female	Black	RCC, Fuhrman nuclear grade 3	None	Right renal tumour
8.	47yr	Female	Black	Sarcomatoid RCC	Cytokeratins -, CD10 +, Desmin +	Renal tumour (side not stated)
9.	58yr	Male	White	ccRCC	None	Right renal tumour
10.	58yr	Male	White	Sarcomatoid RCC	None	Left renal tumour

In all 10 of the samples, the Exon 1 primers of the FH gene could not be successfully optimized. The fragment sequenced showed the presence of off-target amplification which was localized to chromosome 1q21.3. The blasted sequence was predicted to originate from the glucosylceramidase beta pseudogene 1 (GBAP1). This could be ascribed to faulty primer design or the PCR regimen was not successfully optimized for this fragment.

Amplification of Exon 3 showed the presence of non-specific binding of the primer pair in 9 of the samples. The amplification regimen resulted in on target amplification for sample 1. Samples 2-4, and 6-10 presented with a heterozygous sequence profile. Interpretation of the sequence and blast analyses predicted amplification of FH Exon 3 and off target chromosome 6p23 amplification. Sample 5 only amplified the off target region on chromosome 6p23. The competitive amplification can be ascribed to either subtelomeric deletions on chromosome 1 where the FH gene is located which resulted in the loss of the template. Alternatively, the more feasible reason is single nucleotide polymorphisms (SNPs) in the binding site of the primer. The mismatches could result in irregular primer hybridization, as the primer successfully amplified on target for one individual. These results are of unknown significance and should be investigated in a future study.

Sequencing of Exon 6 in samples 5 and 6 failed due to highly fragmented DNA. The quality of the DNA extracted from these tumour blocks was low and Exon 6 was the largest amplicon at 381 base pairs. Hence, PCR efficiency was low and not enough DNA sample was left to concentrate, clean-up and repeat the PCR reaction and sequencing.

A limited number of variants were detected that might be due to the small size of the FH gene. Across the 10 samples, three synonymous, two intronic variants and two missense variants were detected. The seven variants were classified using the ACMG guidelines and resulted in two benign classifications, three likely benign classifications, one variant of unknown significance and one likely pathogenic classification. The location and classification of variants detected in the FH gene, is summarized in Table 5.

Table 5. Location and classification of variants detected in the FH gene.

Sample Number	FH Location	Variant detected	Ref SNP ID	ACMG Classification
3	Exon 9	c.1302C>T p.(Cys434=)	rs2070080	Benign
6	Exon 2	c.207C>T, p.(Gly69=)	rs370392829	Benign
	Exon 8	c.1197C>T p.(Ser399=)	No rs	Likely Benign
7	Exon 7	c.1087C>T p.(Pro363Ser)	No rs	Likely Pathogenic
	Intron 8	c.1391-3C>T	No rs	Likely Benign
9	Exon 4	c.382G>A p.(Ala128Thr)	rs1553341620	VUS
	Intron 4	c.555+18G>A	No rs	Likely Benign

Reason for classification

FH(NM_000143.4): c.1087C>T, p.(Pro363Ser)

The variant is located in Exon 7 of the FH gene. The variant has not been reported in the exome aggregate or GnomAD population control databases and is therefore an

extremely rare variant at the time of analysis. Varsome in silico predictions result in 12 out of 12 assays predicting a damaging or deleterious effect. PhyloP report the position not to be highly conserved across primates, but highly conserved across vertebrates. According to UniProt, the amino acid residue is located in the Lyase (carboxylase) domain where residue 366 is important for the substrate malate binding with residues 365 and 378 important in the catalytic activity. The 363 amino acid is located two residues away from a conserved active site. Sorting Intolerant From Tolerant (SIFT) predict the substitution from Proline to Serine to be damaging due to the change in physiochemical properties, hydrophobic to hydrophilic, that might influence the electron transport chain at residue 365 during catalytic activity. To our knowledge, no other missense variants at residue 363 was found in the literature. Based on all the above information, this variant is classified as likely pathogenic.

FH(NM_000143.4): c.382G>A p.(Ala128Thr)

The variant is located in Exon 4 of the FH gene. The variant has not been reported in the exome aggregate of GnomAD population databases consisting of ~254000 alleles, indicating that this variant is extremely rare. In ClinVar, this variant has been reported in HLRCC syndrome as a variant of unknown significance. Varsome in silico predictions result in 9 out of 12 assays predicting a damaging or deleterious effect. PhyloP predicts the region not to be conserved across primates or vertebrates, yet SIFT predicts the variant as damaging due to a physiochemical change from a very small hydrophobic to small hydrophilic amino acid. According to UniProt, residue 128 is located in the Lyase domain and form part of a helical structure with the closest residues of importance being amino acids 145 – 147, which are important in fumarate substrate binding. No other missense variants has been reported as pathogenic at this position. With the lack of functional evidence, this variant is classified as a variant of unknown significance.

Case with likely pathogenic mutation:

Case number 7, which was selected for molecular analysis, was a 53-year-old black female who presented with a tumour in the right kidney. A right-sided nephrectomy

was performed. The tumour measured 90 x 80 x 50mm and was eccentrically located in the kidney. The tumour also involved the renal pelvis. Microscopically, the tumour showed large nuclei with prominent nucleoli with extension of the tumour into the renal pelvis and perirenal fat. A final diagnosis of renal cell carcinoma, Fuhrman nuclear grade 3, was made. No immunohistochemical stains were performed at the time of the diagnosis. No further history regarding the presence of uterine leiomyomas or a family history of renal cell carcinoma, was present in the pathology report.

Microscopic review of the tumour showed the presence of a renal tumour with a fibrous pseudocapsule and solid as well as focal papillary architecture. The papillae were lined by large cells with abundant eosinophilic cytoplasm and the nuclei were hyperchromatic with large, viral inclusion-like nucleoli and perinuclear halos.

An immunohistochemical stain against fumarate hydratase was performed and showed coarse cytoplasmic staining in the tumour cells. The expression of fumarate hydratase was thus retained. The microscopic appearance of the tumour is shown in Figure 2.1.

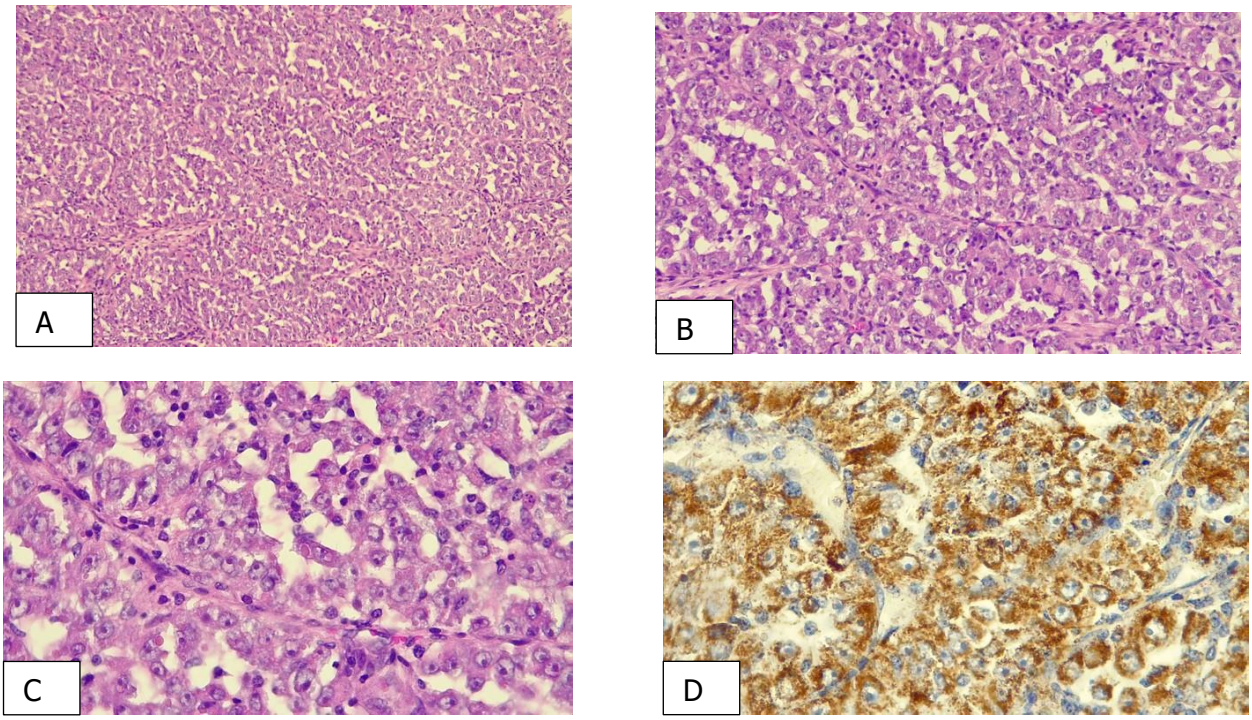

Figure 2.1: From the top left to right:

A: 10x magnification of case number 7, showing solid and papillary architecture. B: 40x magnification of case number 7, showing high-grade nuclear features. C: 100x magnification of case number 7, showing the viral inclusion-like nucleoli one expects to see in FH deficient renal cell carcinoma. D: Immunohistochemical stain against FH, showing retained granular, cytoplasmic staining.

Case with variant of unknown significance

Case number 9, which was selected for molecular analysis, was a 58-year-old white male patient who presented with a right sided flank mass. Imaging studies raised the possibility of a possible renal cell carcinoma and a radical right sided nephrectomy was performed. The tumour measured 92mm in maximum diameter and was multinodular in appearance. The tumour infiltrated through the renal capsule into the perirenal adipose tissue and also involved the renal sinus. The tumour had not invaded the renal vasculature. Microscopically, the tumour consisted of a clear cell renal cell carcinoma, with a Fuhrman nuclear grade of 4. No immunohistochemical stains were performed at the time of the diagnosis. No further history with regards to a family history of renal cell carcinoma, cutaneous leiomyomas or female relatives with uterine leiomyomas was available in the report.

Microscopic review of the tumour showed the presence of a tumour with a multinodular growth pattern surrounded by a fibrous pseudocapsule. The tumour had both solid and papillary growth patterns and the cells were large with abundant pale, eosinophilic and granular cytoplasm with prominent cell membranes. The nuclei were enlarged and hyperchromatic with prominent viral inclusion-like nucleoli and perinuclear halos.

An immunohistochemical stain against fumarate hydratase was performed. The stain showed retained coarse, cytoplasmic staining in the tumour cells. The microscopic appearance of the tumour is shown in Figure 2.2.

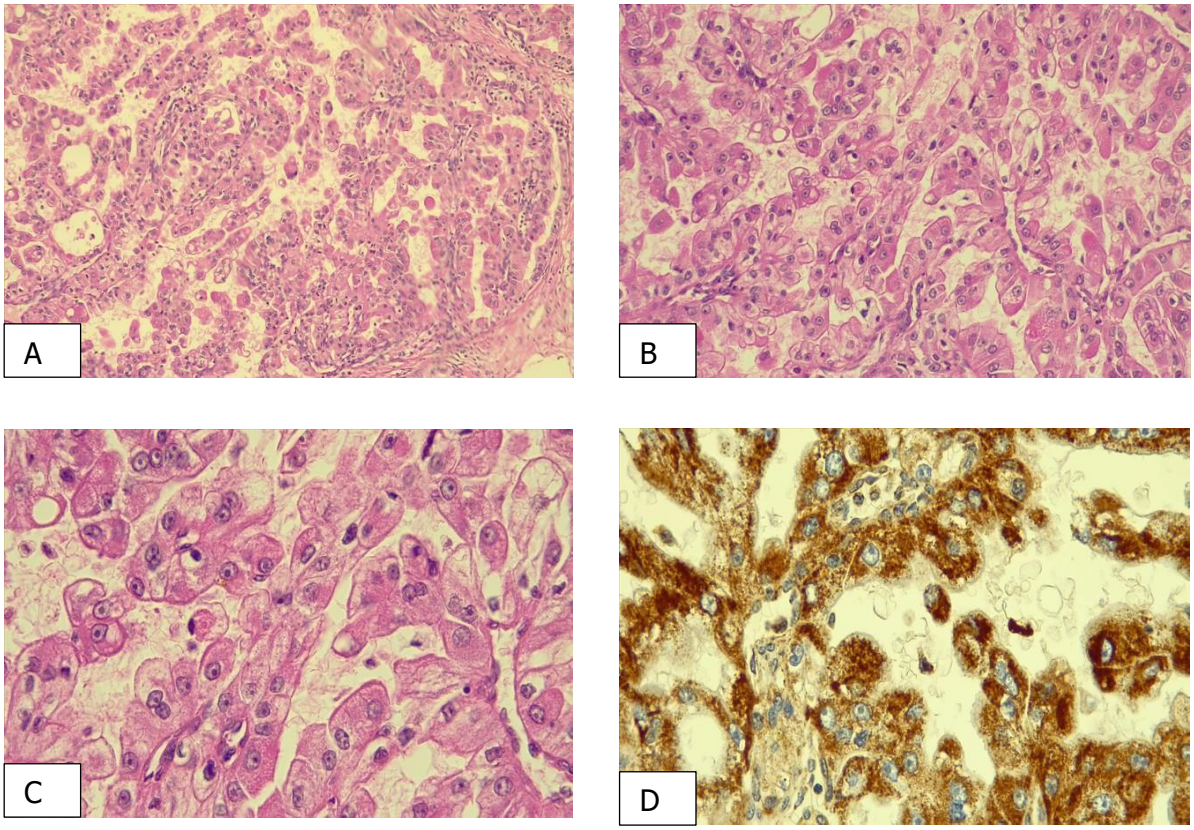

Figure 2.2: From the top left to right:

A: 10x magnification of case number 9, showing a multinodular tumour with primarily papillary architecture. B: 40x magnification of case number 9, showing the papillary structures lined by cells with hyperchromatic nuclei and prominent cell membranes with abundant eosinophilic and granular cytoplasm. C: 100x magnification of case number 9, showing the viral inclusion-like nucleoli. D: Immunohistochemical stain against fumarate hydratase, showing retained granular, cytoplasmic staining.

Discussion

Renal tumours associated with HLRCC syndrome are rare but are usually aggressive and tend to present at a high pathologic stage(14), with local and distant metastases at the time of diagnosis – even in the event of a small primary carcinoma(16). Many patients die of disease within five years of initial diagnosis(10,17). Renal cell carcinomas associated with HLRCC are usually unilateral, single lesions – in contrast to other hereditary renal tumours, which are often bilateral and multifocal(3,7) – and present at a younger age(6). It is therefore important to identify these cases as the diagnosis has implications for patient treatment, prognosis, and further work-up.

The recommended treatment is usually partial or radical nephrectomy with wide surgical margins and retroperitoneal lymphadenectomy(7). Radiofrequency ablation or cryotherapy is not recommended for cancers in patients with HLRCC(7). Currently, there is no standard systemic treatment for metastatic FH deficient RCCs(7).

The incidence of RCC at NHLS Universitas Academic Laboratories was 10.2 cases per year, however, the incidence of FH deficient RCC at the same institution was 0.06 cases per year. One (0.58%) out of 172 cases of RCC diagnosed at NHLS Universitas Academic Laboratories, was confirmed to have a likely pathogenic variant in the FH gene by molecular analysis. These findings confirm that FH deficient RCC is, overall, an uncommon tumour. Cases associated with HLRCC syndrome have only been described in 200 families worldwide(4,5).

The tumour in our case was large, measuring 90mm in greatest dimension and involved the perirenal fat as well as the renal pelvis at the time of diagnosis, indicating an aggressive tumour with extensive local invasion. Unfortunately, no information regarding distant metastases at the time of diagnosis, was available. This finding correlates with the findings of previous studies, confirming that FH deficient RCC is aggressive and often at an advanced stage at diagnosis(14,15).

The characteristic nuclear features, i.e. viral inclusion-like macronucleoli and perinuclear halos, as well as pattern multiplicity in the same tumour(16,18), were present in our case of FH deficient RCC. FH expression was retained by immunohistochemistry but the morphological features prompted the authors to submit this case for molecular analysis.

Our case showed a missense variant in Exon 7 of the gene of interest and it is widely accepted that missense variants may result in retained expression of FH by immunohistochemistry since the capability to produce the fumarate hydratase protein has not been altered(3,10,20). The variant is a novel variant that has not been previously described.

The loss of FH staining is 100% specific in identifying FH deficient RCCs, however, its sensitivity is only about 87.5(3,18). The 2SC stain has been shown, by several authors, to be highly sensitive. Sensitivity and specificity for 2SC were both shown to be 91.7%. The combined sensitivity and specificity for FH negative and 2SC positive tumours was 100%(3,18). Our case also demonstrates the importance of using antibodies against both FH and 2SC to reliably predict the presence of a genetic variant in the FH gene. Hopefully the 2SC stain will become available commercially in the future.

Since no further information regarding the presence of uterine leiomyomas or a family history of RCC was present, our case was reclassified as FH deficient RCC. Due to the lack of clinical information with regards to the medical history of the patient and her family members, classifying this case as "FH deficient RCC" avoids labelling the patient with a possible genetic syndrome without adequate clinical history and work-up.

The findings in our study highlight the importance of the pathologist's role in recognizing the pattern multiplicity, rhabdoid/sarcomatoid morphology and the presence of viral inclusion-like nucleoli with perinuclear halos in a RCC, in order to prompt genetic testing of the tumour to exclude the presence of a pathogenic variant in the FH gene. In the presence of pathogenic variants, the patient's family members should be tested for the variant and follow-up of affected family members should occur according to the screening guidelines.

Until such time as both FH and 2SC are available for commercial use, the pathologist remains the lead role player in recognition of possible FH deficient RCC cases and should ask for molecular analysis in any cases with the typical phenotype, with or without loss of staining for FH by immunohistochemistry.

Conclusion

FH deficient RCC is a high-grade and aggressive variant of RCC and is associated with HLRCC syndrome.

The tumour has a wide variety of histological patterns, however, rhabdoid/sarcomatoid morphology and the presence of viral inclusion-like macronucleoli, even if only focally appreciated, should prompt genetic testing in order to identify mutations in the FH gene.

We recommend that all cases of RCC displaying the typical phenotype of FH deficient RCC be submitted for molecular testing in order to identify patients with HLRCC syndrome or confirm the diagnosis of sporadic FH deficient RCC to minimize morbidity and mortality in such patients and their families.

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Appendix A

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

[redacted]

Health Sciences Research Ethics Committee

12-Oct-2018

Dear **Dr Michelle Du Preez**

Ethics Clearance: **Fumarate hydratase deficient renal cell carcinoma: a retrospective study performed at NHLS Universitas Academic Laboratories, 2001 to 2017**

Principal Investigator: **Dr Michelle Du Preez**

Department: **Anatomical Pathology Department (Bloemfontein Campus)**

APPLICATION APPROVED

Please ensure that you read the whole document

With reference to your application for ethical clearance with the Faculty of Health Sciences, I am pleased to inform you on behalf of the Health Sciences Research Ethics Committee that you have been granted ethical clearance for your project.

Your ethical clearance number, to be used in all correspondence is:**UFS-HSD2018/1200/3010**

The ethical clearance number is valid for research conducted for one year from issuance. Should you require more time to complete this research, please apply for an extension.

We request that any changes that may take place during the course of your research project be submitted to the HSREC for approval to ensure we are kept up to date with your progress and any ethical implications that may arise. This includes any serious adverse events and/or termination of the study.

A progress report should be submitted within one year of approval, and annually for long term studies. A final report should be submitted at the completion of the study.

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

For any questions or concerns, please feel free to contact HSREC Administration: 051-4017794/5 or email EthicsFHS@ufs.ac.za.

Thank you for submitting this proposal for ethical clearance and we wish you every success with your research.

Yours Sincerely

Dr. SM Le Grange
Chair : Health Sciences Research Ethics Committee

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
IRB 00006240; REC 230408-011; IORG0005187; FWA00012784
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa

Appendix B

NATIONAL HEALTH LABORATORY SERVICE

Practice No. 5200296

Office of the Business Manager
UNIVERSITAS ACADEMIC LABORATORIES
PO BOX 339(G3)
C/O: CHEMICAL PATHOLOGY
1st FLOOR
BLOCK C
FACULTY OF HEALTH SCIENCES
UNIVERSITY OF FREE STATE
BLOEMFONTEIN
9301

REQUEST FOR APPROVAL OF LABORATORY RESOURCES FOR ACADEMIC PURPOSES

Date: 07 August 2018

Requestor: Dr M Du Preez

Project Name: **Fumarate Hydratase Deficient Renal Cell Carcinoma: a retrospective study performed at NHLS Universitas Academic Laboratories, 2001 to 2017**

Dear Dr Du Preez

Your request for use of laboratory facilities / data is hereby granted under following conditions:

1) That University Ethical Committee approval is obtained
2) All existing laboratory data remain confidential to the patient and doctor (anonymity is maintained)
3) This Office must be notified before any publication of any results / findings is made.
4) NHLS is recognised in all publications
5) Only existing data may be used.
6) Equipment may be used only upon approval of relevant manager and supply of own consumables.
7) Patient data can be extracted via NHLS Corporate Data Warehouse following submission and approval of a request on attached form.
8) You may need to have an NHLS account opened for your project (NHLS Trust or K-Project. The relevant forms are available at AARQA Research Office.

May your project be successful.

Regards,

Prof Henry Pieters
Business Manager

Appendix C

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

08th August 2018

Health Sciences Research Ethics Committee
Faculty of Health Sciences
The University of the Free State.

Dear Chair

I grant permission for the following retrospective cross-sectional MMed project to be conducted within the School of Pathology (**Department of Anatomical Pathology)**, Universitas Academic Laboratory Complex, Faculty of Health Sciences, Bloemfontein):

Fumarate Hydratase Deficient Renal Cell Carcinoma: a retrospective study performed at NHLS Universitas Academic Laboratories, 2001 to 2017

MMed Researcher: Dr.M du Preez
Supervisor: Prof. J Goedhals
Co-supervisors: Mr. J Oosthuizen, Prof. G Joubert

Permission also has to be obtained from NHLS management for the use of archival laboratory data.

I wish Dr. du Preez a successful outcome of his study.

Yours Sincerely

Jocelyn Naicker
Head: School of Pathology
Faculty of Health Sciences
Tel: 051 405 2914 | Cell: 0829071925
jocelyn.naicker@nhls.ac.za | www.nhls.ac.za

NATIONAL HEALTH LABORATORY SERVICE

Appendix D

Fumarate Hydratase Deficient Renal Cell Carcinoma: a retrospective study performed at NHLS Universitas Academic Laboratories, 2001 to 2017

Researchers:

1. **Dr M du Preez (MMed Candidate)**
   Department of Anatomical Pathology – Registrar
   University of the Free State and NHLS
   Tel: 084 580 4506
   e-mail: mflooi429@gmail.com

2. **Prof J Goedhals (Supervisor)**
   Department of Anatomical Pathology – HOD
   University of the Free State and NHLS
   Tel: 083 701 1179
   e-mail: gnmbjg@ufs.ac.za

3. **Mr J Oosthuizen**
   Division of Medical Genetics
   University of the Free State and NHLS
   Tel: 051 405 3047
   e-mail: 2008000198@ufs4life.ac.za

4. **Prof G Joubert**
   Department of Biostatistics
   University of the Free State
   Tel: 051 401 3117

Contents

Introduction

Hereditary leiomyomatosis and renal cell carcinoma syndrome (HLRCC), also known as Reed syndrome is an uncommon disorder and has been found in approximately 200 families worldwide, since the first published report in 2001 (Valencia et al, 2016). Even though the penetrance of syndrome-associated malignancies is variable in carriers, timely identificationof syndromic patients allows for regular surveillance, better treatment and appropriate prophylaxis (Buelow et al, 2016).

HLRCC is inherited in an autosomal dominant pattern with incomplete penetrance and is clinically characterized by three main features:

1. Multiple cutaneous leiomyomas (associated with the arrector pili muscle), which present as hard, erythematous skin papules or nodules, often involving the trunk and limbs,
2. The early onset, usually before 40 years of age, of multiple and, in many cases, symptomatic uterine leiomyomas which may lead to early myomectomies or hysterectomy, and
3. Onset of aggressive renal cell carcinoma before age 40, in 20 to 30% of these patients, which tend to metastasize early (Menko et al, 2014).

In the early literature, type II papillary renal cell carcinoma and occasionally collecting ductcarcinoma were described as HLRCC-associated tumours. Recently, however, a spectrum of architectural patterns has been suggested, including tumours with papillary, tubulopapillary, tubular, solid and cystic elements. It was suggested, by Merino et al, that the morphologichallmark of HLRCC uterine leiomyomas and renal tumours are typical nuclear features, i.e.:big nuclei with a viral inclusion-like, eosinophilic macronucleoli, surrounded by perinucleolar halos (in uterine leiomyomas and renal tumours) and eosinophilic cytoplasmic inclusions (in uterine leiomyomas). If the architectural and nuclear features are taken into account, the differential diagnosis of HLRCC renal tumours could include a wide range of high grade renal cell carcinomas of different histologic subtypes, including the sporadic type II papillary renalcell carcinoma, collecting duct carcinoma, or high grade renal cell carcinoma, unclassified (Chen et al, 2014). HLRCC syndrome associated renal cell carcinoma is currently recognized as a separate entity, and is included in both the 2013 International Society of Urological Pathology Vancouver Classification of renal tumours and the 2016 WHO classification (Trpkovet al, 2016).

HLRCC is caused by heterozygous germline mutations in the fumarate hydratase (FH) gene on chromosome 1q43.2-q43. This gene encodes the enzyme fumarate hydratase, a vital component of the Krebs cycle, which catalyses the conversion of fumarate to malate (Alrashdi et al, 2009). The mechanism of carcinogenesis in FH mutated cells remains incompletelyunderstood, however, bi-allelic inactivation of fumarate hydratase results in the accumulation of fumarate and S-(2-succino)-cysteine (2SC) (Reyes et al, 2014). It is believed that thesemetabolites modulate the activity of transcription factors, notably HIF1a, NRF2 and AMPK,leading to increased cell proliferation and resistance to programmed cell death/apoptosis (Buelow et al, 2016).

The renal cell carcinomas associated with HLRCC syndrome are usually at an advanced stage at presentation, they are aggressive and generally have poor clinical outcomes. In a study conducted by Toro et al, 9 out of 13 patients demised of metastatic renal cell carcinoma within 5 years of diagnosis. Therefore, these patients would benefit from early identification and appropriate surveillance (Joseph et al, 2015).

It has been suggested by Joseph et al that a combination of morphological features of renal cell carcinomas (prominent eosinophilic macronucleoli with perinucleolar halos and eosinophilic cytoplasmic inclusions), positive immunohistochemical staining for S-(2-succino)- cysteine (2SC) and loss of Fumarate Hydratase (FH) staining correlate well with FH mutation analysis by Sanger DNA sequencing (Joseph et al, 2015). It has been found by Buelow et al that the 2SC immunohistochemistry stain demonstrates superior sensitivity and specificity compared with FH immunohistochemistry for FH gene mutation associated renal cell carcinomas. However, at present the S2C antibody is not commercially available.

There is currently no published research regarding Fumarate Hydratase deficient renal cell carcinoma and its association with hereditary leiomyomatosis and renal cell carcinoma syndrome in Africa and we plan to determine whether this form of renal cell carcinoma occurs in our local population.

Aim

The aim of this study is to determine the number and profile of patients with fumarate hydratase deficient renal cell carcinoma seen by the Department of Anatomical Pathology, University of the Free State and NHLS.

Objectives

1. To determine the number of cases of renal cell carcinomas seen with specific morphological features and immunohistochemical staining patterns suggesting HLRCC and their correlation with FH mutation analysis by DNA sequencing over a 16-year period from January 2001 to December 2017 by the Department of Anatomical Pathology, University of the Free State and NHLS.
2. To evaluate the demographic and pathological features of the patients diagnosed with Fumarate Hydratase deficient renal cell carcinoma.

Methods

A retrospective cross-sectional study will be performed. It will not be possible to perform a prospective study due to the small number of patients diagnosed with renal cell carcinoma each year. This study will include cases of renal cell carcinoma diagnosed by the Department of Anatomical Pathology at the University of the Free State and NHLS over a 16 year period. The department provides histopathological services to all government hospitals and clinics in the Free State Province and at times from the Northern Cape and North West Provinces. A

manual search of archived pathology request forms will be used to identify cases between 1 January 2001 and 31 March 2004. A SNOMED search of the NHLS electronic databases will be used to identify cases diagnosed between 1 April 2004 and 31 December 2017. The DISA system will be searched for the years 2004 to 2014 and the Labtrak system will be searched for the years 2015 to 2017. We estimate there will be approximately 200 cases during thistime period.

Inclusion criteria are as follows:

1. All ages.
2. Males and females.
3. Sufficient tissue available in archived wax blocks.
4. Renal cell carcinomas diagnosed by the Department of Anatomical Pathology at the University of the Free State and NHLS from 1 January 2001 to 31 December 2017.

Exclusion criteria:

1. Metastatic malignant tumours to the kidneys.
2. Primary renal tumours in patients known with Von Hippel-Lindau syndrome.
3. Benign kidney tumors, eg. renal oncocytoma.

Once the cases have been identified, the slides and wax blocks will be retrieved from the departmental archives and the slides will be reviewed by Dr Du Preez and Prof Goedhals. If the cases meet the inclusion criteria a representative wax block will be chosen. The wax blocks will be sectioned at 4 micrometres and the sections placed on glass slides. Unfortunately, there is no commercially available antibody against 2SC. We will thus only stain the slides for Fumarate Hydratase. The slides will be stained for Fumarate Hydratase byusing the rabbit polyclonal antibody from Abcam. Slides will be stained using a BenchmarkXT automated slide stainer. All reagents are pre-diluted and ready to use. The slides will be counterstained with Mayers Haematoxylin, dehydrated and cover slipped. Prof Goedhals and Dr Du Preez will then evaluate the slides to determine whether they are positive or negative. Stains will be categorised as negative if there is complete loss of staining in the tumour cells. All other cases with any degree of staining will be regarded as positive.

Any cases which are negative for FH or which have the nuclear features suggestive of HLRCC will undergo molecular testing as not all cases will actually have the mutation when sequenced. Molecular analysis will be done by extracting DNA from tumour obtained fromthe wax blocks using a QIAamp DNA FFPE Tissue Kit according to the manufacturer's instructions. DNA sequencing will then be performed using previously described primers of10 coding exons.

In addition, the patients' age, sex, race, type of renal cell carcinoma, results of other immunohistochemical stains and clinical presentation including the presence of leiomyomas will be noted. This information will be obtained from the pathology report. The data will becaptured in an Excel spreadsheet for statistical analysis.

Time frame

The study should take approximately 12 months to complete once funding has beenobtained.

August 2018: Submission for Ethics approval. September –

November 2018: Identification of cases.

An application for funding will be made as soon as the ethics approval has been obtained.
As it can take up to one year to obtain the funds an exact time line is not possible. However,the following is estimated:

Six months for laboratory work.

Six month for analysis and write up.

Statistics

Prof Gina Joubert from the Department of Biostatistics at the University of the Free Statewill perform the statistical analysis. Results will be summarized by frequencies and percentages (categorical variables) and means, standard deviations or percentiles (numerical variables).

Financial Implications

Menzel Glazer superfrost slides	R200.00
Blank labels	R500.00
LCS	R550.00
EZ prep	R1 100.00
SSC3	R1 100.00
Reaction buffer	R700.00
Inview kit	R8 700.00
FH antibody (4 kits will be required at R7000 each)	R28 000.00
CC1	R1 400.00
Scotts tapwater	R100.00
Haematoxylin	R400.00
96% alcohol	R650.00
100% alcohol	R650.00
Xylene	R200.00

Coverslips	R4 000.00
DNA extraction kit	R 6 000.00
DNA sequencing	R20 000.00
Total	**R74 250.00**

A funding application will be made to the NHLS Research Trust once approval has been obtained from the Health Sciences Research Ethics Committee as the Research Trust will notaccept any applications without an attached ethics approval letter.

Ethical Aspects

Prof J Goedhals will allocate a unique study number to each case to ensure confidentiality.None of the other investigators will have access to any identifiers. Immunohistochemical stains, data collection and data analysis will be performed using only the unique study numberin order to protect the patients' identities. Permission has been obtained from the NHLS only, since only data from the NHLS pathology reports will be used. It is thus not necessary to obtain permission from the Free State Department of Health.

Proposed outcome

The proposed project will be completed in fulfilment of the requirements for an MMeddegree for Dr M du Preez under the supervision of Prof J Goedhals. The results will be submitted for publication in a peer reviewed journal.

Should cases of fumarate hydratase deficient renal cell carcinoma be identified, the FH immunohistochemical stain will be added to the Department of Anatomical Pathology diagnostic platform to be used is future cases of renal cell carcinoma.

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Appendix E

Study no.	Age	Race	Sex	Type of RCC	Previous IH stains	FH IH	Light microscopic features suggestive of HLRCC	Clinical presentation

Appendix F

American Journal of Surgical Pathology

Online Submission and Review System

Author Resources

Instructions for Authors (this page)
Reprint Ordering
Permissions Requests
Reprints

SCOPE

The *American Journal of Surgical Pathology* has achieved worldwide recognition for its outstanding coverage of the state of the art in human surgical pathology. In each monthly issue, experts present original articles, review articles, detailed case reports, and special features, enhanced by superb illustrations. Coverage encompasses technical methods, diagnostic aids, and frozen-section diagnosis, in addition to detailed pathologic studies of a wide range of disease entities.

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Patient Anonymity and Informed Consent

It is the author's responsibility to ensure that a patient's anonymity be carefully protected and to verify that any experimental investigation with human subjects reported in the manuscript was performed with informed consent and following all the guidelines for experimental investigation with human subjects required by the institution(s) with which all the authors are affiliated. Authors should mask patients' eyes and remove patients' names from figures unless they obtain written consent from the patients and submit written consent with the manuscript.

Conflicts of interest

Authors must state all possible conflicts of interest in the manuscript, including financial, consultant, institutional and other relationships that might lead to bias or a conflict of interest. If there is no conflict of interest, this should also be explicitly stated as none declared. All sources of funding should be acknowledged in the manuscript. All relevant conflicts of interest and sources of funding should be included on the title page of the manuscript with the heading "Conflicts of Interest and Source of Funding:". For example:

Conflicts of Interest and Source of Funding: A has received honoraria from Company Z. B is currently receiving a grant (#12345) from Organization Y, and is on the speaker's bureau for Organization X – the CME organizers for Company A. For the remaining authors none were declared.

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MANUSCRIPT SUBMISSION

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All manuscripts must be submitted on-line through the new Web site at **https://www.editorialmanager.com/ajsp**. **First-time users:** Please click the Register button from the menu above and enter the requested information. On successful registration, you will be sent an e-mail indicating your user name and password. Print a copy of this information for future reference. Note: If you have received an e-mail from us with an assigned user ID and password, or if you are a repeat user, do not register again. Just log in. Once you have an assigned ID and password, you do not have to re-register, even if your status changes (that is, author, reviewer, or editor). **Authors:** Please click the log-in button from the menu at the top of the page and log in to the system as an Author. Submit your manuscript according to the author instructions. You will be able to track the progress of your manuscript through the system. If you experience any problems, please contact Nancy Kriigel, Managing Editor, njk3q@virginia.edu , 434-924-9136, fax 434-924-8767.

Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals

Authors should read and follow the ICMJE general guidelines for conducting and reporting medical studies, as well as the specific guidelines outlined here for AJSP submissions. http://www.icmje.org/icmje-recommendations.pdf

PREPARATION OF MANUSCRIPT Manuscripts that do not adhere to the following instructions will be returned to the corresponding author for technical revision before undergoing peer review.

Title Page

Include on the title page (a) complete manuscript title; (b) authors' full names, highest

academic degrees, and affiliations; (c) name and address for correspondence, including fax number, telephone number, and e-mail address; (d) address for reprints if different from that of corresponding author; and (e) all sources of support, including pharmaceutical and industry support, that require acknowledgment.

The title page must also include disclosure of funding received for this work from any of the following organizations: National Institutes of Health (NIH); Wellcome Trust; Howard Hughes Medical Institute (HHMI); and other(s).

Unstructured Abstract and Key Words

Abstract must be submitted as a separate file. Limit the abstract to 250 words. It must be factual and comprehensive. Limit the use of abbreviations and acronyms, and avoid general statements (eg, "the significance of the results is discussed"). List three to five key words or phrases.

Text

Organize the manuscript into four main headings: Introduction, Materials and Methods, Results, and Discussion. References and figure legends should be placed after the Discussion section. Define abbreviations at first mention in text and in each table and figure. If a brand name is cited, supply the manufacturer's name and address (city and state/country).

Abbreviations

For a list of standard abbreviations, consult the Council of Biology Editors Style Guide (available from the Council of Science Editors, 9650 Rockville Pike, Bethesda, MD 20814) or other standard sources. Write out the full term for each abbreviation at its first use unless it is a standard unit of measure.

References

The authors are responsible for the accuracy of the references. Enter the references (double-spaced) in order of citation after the Discussion section. Cite unpublished data—such as papers submitted but not yet accepted for publication and personal communications, including e-mail communications— in parentheses in the text. If there are more than three authors, name only the first three authors and then use et al. Refer to the List of Journals Indexed in Index Medicus for abbreviations of journal names, or access the list at http://www.nlm.nih.gov/tsd/serials/lji.html. Sample references are given below:

Journal article

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Book chapter

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by Michelle Du Preez

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T: +27(0)51 405 3058
F: +27(0)51 444 0616
E: gnmbjg@ufs.ac.za

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