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Analysis of Epigenetic Heterogeneity in Renal Tumors

Master's thesis

Genetics Master study program

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CONTENTS

ABBREVIATIONS

RCC	Renal cell carcinoma
ACTH	Adrenocorticotropic hormone
ADAMTS19	*ADAM metallopeptidase with thrombospondin type 1 motif 19* gene
ALDOB	*Aldolase, fructose-bisphosphate B* gene
BAP1	*BRCA1-associated protein 1* gene
BAG3	*BAG Cochaperone 3* gene
BHD	Birt-Hogg-Dubé Syndrome
ccRCC	Clear cell RCC
chRCC	Chromophobe RCC
CDKN2B	*Cyclin-dependent kinase inhibitor 2B* gene
CHST11	*Carbohydrate sulfotransferase 11* gene
CKD	Chronic kidney disease
CNS	Central nervous system
CT	Computed tomography
CpG	Cytosine-guanine dinucleotide
DNMT1	DNA (cytosine-5)-methyltransferase 1
DNMT3A	DNA (cytosine-5)-methyltransferase 3A
DNMT3B	DNA (cytosine-5)-methyltransferase 3B
ESRD	End-stage renal disease
ERβ	Estrogen receptor-β
FBN2	*Fibrillin-2* gene
FH	Fumarate hydratase
FLRT2	*Fibronectin leucine-rich transmembrane protein 2* gene
FLCN	*Folliculin* gene

HI Heterogeneity index

HIF Hypoxia-inducible factor

HIF1A Hypoxia-inducible factor 1 subunit alpha

IMRT Intensity-modulated radiation therapy

IGF-1 Insulin growth factor 1

INSR Insulin receptors

ITH Intratumoral heterogeneity

LC Local control

MAPK Mitogen-activated protein kinase

MC Methylated control

MET Mesenchymal-epithelial transition factor

MITF Microphthalmia-associated transcription factor

MRI Magnetic resonance imaging

mTOR Mammalian target of rapamycin

NTC No template control

NRT Non-tumor

PET-CT Positron emission tomography-computed tomography

pRCC Papillary RCC

PBRM1 *Polybromo 1* gene

PCDH8 *Protocadherin-8* gene

PTEN *Phosphatase and tensin homolog* gene

PUS1 *Pseudouridine synthase 1* gene

PI3K/AKT Phosphatidylinositol 3-kinase/protein kinase B

pT Pathohistological tumor stage

SAM S-adenosyl methionine

SBRT Stereotactic body radiation therapy

SETD2 *SET domain-containing 2* gene

SFRP1 *Secreted frizzled-related protein 1* gene

SSIGN Stage, Size, Grade and Necrosis.

TAC1 *Tachykinin-1* gene

TFAP2B *Transcription factor AP-2 beta* gene

TKI Tyrosine kinase inhibitor

TNF-α Tumor necrosis factor-alpha

TNM Tumor node metastasis

UC Unmethylated Control

US Ultrasound

VEGF Vascular endothelial growth factor

VHL *von Hippel-Lindau* gene

WHO/ ISUP World Health Organization/International Society of Urological Pathology

ZNF677 *Zinc finger protein 677* gene

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Nazrin Mammadova

Inkstų navikų epigenetinio heterogeniškumo tyrimas

Magistro baigiamasis darbas

SANTRAUKA

Inkstų ląstelių karcinoma (ILK) sudaro daugiau kaip 90 % visų inkstų navikų tipų ir pasižymi didžiausiu mirtingumu tarp visų urogenitalinės srities navikų. Dauguma ILK atvejų yra atsparūs gydymui ir tai ypač susiję su dideliu jų šių navikų heterogeniškumu. CpG salų hipermetilinimas yra dažnas ir esminis ILK pokytis, tačiau šių pokyčių tarpžidininis heterogeniškumas nėra išsamiai ištirtas. Pagrindinis šio tyrimo tikslas buvo nustatyti skirtingai metilintus genus inkstų navikų audinių mėginiuose ir įvertinti jų heterogeniškumą.

Aštuonių genų metilinimo būsena buvo tiriama metilinimui jautrios PGR metodu (MSP) prieš tai atliekant išskirtų DNR mėginių bisulfitinę modifikaciją. Iš viso buvo ištirta 40 mėginių (20 navikinių, 10 perinavikinių ir 10 nenavikinių). Visi mėginiai surinkti iš dešimties pacientų, kuriems buvo nustatyta ILK arba onkocitoma.

Metilinimo heterogeniškumas tarp dviejų tirtų naviko židinių buvo nustatytas septyniems iš aštuonių tirtų genų. Didžiausias heterogeniškumo indeksas (HI) apskaičiuotas *TAC1* genui ir siekė 0.5. Tuo tarpu *ZNF677*, *FBN2*, *PCDH8*, *ADAMTS19* ir *SFRP1* HI siekė 0.2 o *FLRT2* – 0.1. Genai *ZNF677*, *FBN2* ir *PCDH8* pasižymėjo didžiausiu metilinimo dažnio variacija (20%) tarp dviejų atsitiktinai pasirinktų naviko židinių. Didžiausias metilinimo dažnis perinavikiniuose mėginiuose nustatytas genams *ZNF677* (50%), *TAC1* (50%) *FBN2* (40%), ir *PCDH8* (30%). Visų pacientų atveju buvo nustatytas tirtų genų metilinimo būsenos inkstų audiniuose ir šlapimo mėginiuose neatitikimas.

Apibendrinant galima teigti, kad šis tyrimas atskleidė, jog ILK navikai yra heterogeniška. Dėl heterogeniškumo, specifinių genų metilinimo būseną naviko audiniuose sudėtinga naudoti kaip patikimą ligos biožymenį.

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SUMMARY

Renal cell carcinoma (RCC) constitutes more than 90% of all types of kidney tumors and represents the highest mortality rate among genitourinary neoplasms. Most RCC cases are treatment-resistant which is particularly related to their extensive phenotypic variability and inter-and/or intra-tumoral molecular heterogeneity (ITH). Hypermethylation of CpG islands is a frequent and pivotal alteration in RCC; however, interfocal heterogeneity of these changes has not been extensively investigated. The main aim of this study was to determine aberrantly methylated genes in renal tumor tissue samples and evaluate their heterogeneity.

The methylation status of eight genes was assessed through methylation-specific PCR (MSP), after the bisulfite conversion of the isolated DNA samples. A total of 40 (20 tumor samples, 10 peritumor samples, 10 non-cancerous samples) tissue samples were investigated from 10 patients diagnosed with renal cell carcinoma or oncocytoma.

Interfocal methylation heterogeneity was found in seven out of eight analyzed genes. The highest heterogeneity index (HI) was calculated for *TAC1* (0.5), while *ZNF677*, *FBN2*, *PCDH8*, *ADAMTS19,* and *SFRP1 HI* reached 0.2 and for *FLRT2* – 0.1. *ZNF677*, *FBN2,* and *PCDH8* demonstrated the highest variation in methylation frequency between the two randomly selected tumor foci (20%). The highest methylation frequency in peritumor samples was detected for *ZNF677* (50%), *TAC1* (50%) *FBN2* (40%), and *PCDH8* (30%). All individuals exhibited methylation disparities between urine and tissue samples.

Taken together, this study revealed that RCC is highly heterogeneous and highlights the challenge of using specific gene methylation statuses as reliable diagnostic biomarkers for early disease detection due to their heterogeneity.

INTRODUCTION

Renal cell carcinoma (RCC) is the most prevalent tumor within the kidney and constitutes more than 90% of all renal tumors. It is a heterogeneous group of tumors arising from the epithelial cells lining the inner surface of the renal tubules. The majority of patients diagnosed with localized RCC are typically treated through either partial or radical nephrectomy because chemotherapy has shown limited effectiveness against RCC compared to other treatments like targeted therapy and immunotherapy. Despite advancements in diagnostic and therapeutic approaches, RCC remains associated with considerable morbidity and mortality worldwide. One of the defining features contributing to the complexity of RCC is its inherent heterogeneity at the molecular level, which manifests in various forms, including genetic, epigenetic, and phenotypic diversities.

In recent years, exploring the significance and consequences of epigenetic diversity in RCC has garnered interest. Epigenetic mechanisms directly influencing gene expression are traditionally classified into two groups: chromatin modifications and DNA modifications. Among DNA modifications, DNA methylation plays a substantial role in modulating gene expression variability within tumors, notably impacting intratumor heterogeneity which refers to the presence of diverse cell populations within a single tumor and observed in various cancer types, such as renal cell carcinoma, hematological cancers, breast cancer, multifocal prostate cancer, and glioblastoma. However, despite numerous research conducted, specific mechanisms leading to hypermethylation in RCC are not yet definitively understood. Nevertheless, it has been speculated that the phenomenon may be attributed to the upregulation of DNA methyltransferase 3 beta splice variant 4 (DNMT3B4) and the consequent increase in oxidative stress. Tumor heterogeneity in RCC can be caused by clonal evolution, this condition involves the emergence of different sub-clones with unique DNA methylation profiles over time due to selective pressures in the tumor microenvironment, including intratumoral hypoxia, and immune responses. Therefore, intratumoral heterogeneity can impact the advancement of the cancer, the response to therapy, and the prognosis in individuals with RCC.

The methylation of cytosines in CpG dinucleotides is a prevalent epigenetic alteration observed in human biology and is commonly detected in various cancer types, including renal cell cancer. In accordance with some findings, the observation of hypermethylation in promoter regions is more common than genetic mutations in renal cell carcinoma.

Due to the differences in genetic loci used for methylation analysis among numerous studies on RCC, none of these findings have been implemented in a therapeutic context to date. Another acceptable reason may be attributed to tumor heterogeneity, which refers to the variation in the biomarker's methylation status across different foci. While biomarkers might be methylated in one

tumor foci but not in another. A potential solution to this problem is the use of urine samples because they can bypass the need for tumor tissue and thus overcome the issue of tumor heterogeneity.

In summary, the exploration of epigenetic diversity and the significance of DNA methylation alterations in RCC presents a promising way for understanding tumor heterogeneity and developing novel diagnostic and therapeutic strategies.

The **aim** of this study was to identify aberrantly methylated genes in two different renal tumor tissue foci and evaluate tumor tissue heterogeneity.
To achieve this aim, the following **tasks** have been undertaken:

1. To determine the methylation status of *ADAMTS19, FBN2, FLRT2, PCDH8, SFRP1, ZNF677, TAC1,* and *TFAP2B* genes in two different renal tumor and peritumor tissue samples by qualitative methylation-specific PCR(MSP).
2. Two calculate renal tumor heterogeneity index according to the investigated genes' methylation status.
3. To compare methylation differences in the tissue samples and the urine samples of the same patients at the regulatory regions of selected genes.

1. LITERATURE OVERVIEW

1.1. Kidney cancer- Epidemiology and Subtypes

Kidney or renal cancer stands as one of the most prevalent and aggressive malignancies within the urological tract, with a global diagnostic incidence. The prevalence of kidney tumors ranks 9th among men and 14th among women in terms of incidence rates within the common neoplasms (Turco et al., 2021a). Its incidence on a global scale has been escalating annually with higher rates observed in developed countries than in developing countries. Renal cell carcinoma (RCC) demonstrates a statistically significant difference in prevalence between genders, with a ratio of 1.5:1 in favor of men. Additionally, the mortality rate associated with RCC is higher for males compared to females. Moreover, the majority of RCC cases occur in individuals aged 60 to 70 years, after which the incidence declines, potentially due to the reduced use of aggressive diagnostic testing in this age group (Capitanio et al., 2019). Renal tumor predominantly affects individuals in European and North American populations, with a lower incidence rate observed in Asia. In 2022, a total of 179,368 deaths were recorded globally due to kidney cancer (Bukavina et al., 2022). Notably, Belarus, South America, and Latvia have demonstrated remarkable rates of renal malignancies **(Figure 1.1)**. Additionally, in 2022, the greatest incidence of kidney cancer occurred in Lithuania, followed by French Republic [According to 2022 data from Global Cancer Observatory, https://gco.iarc.fr/].

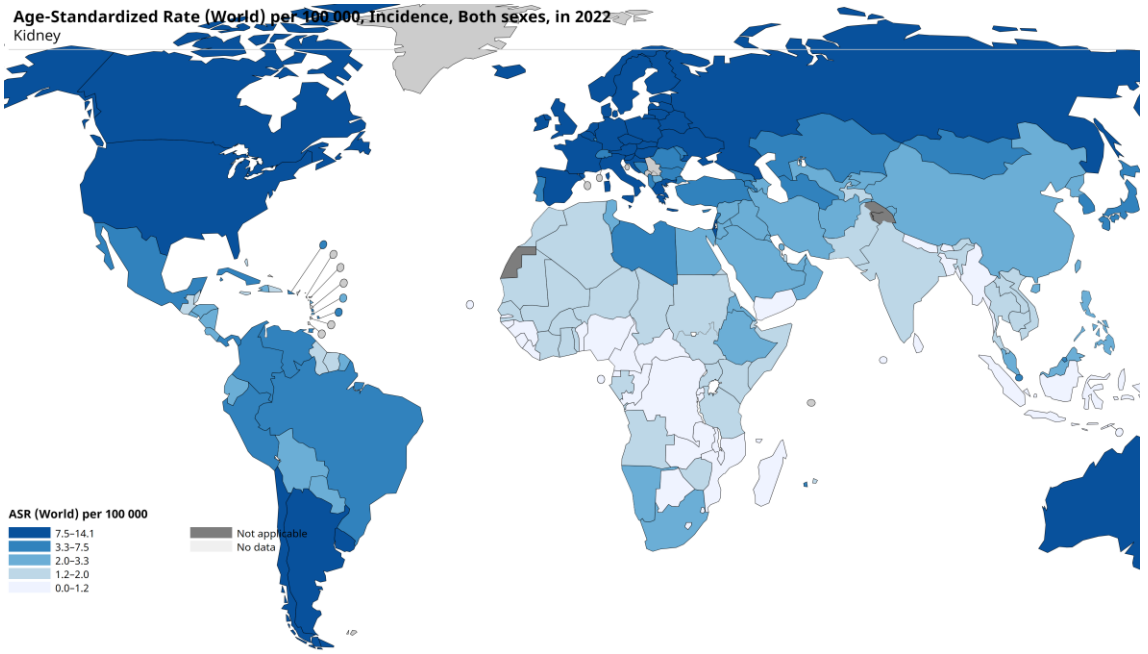


Fig. 1.1. In 2022, estimated age-standardized incidence rates (World) of kidney cancer in both sexes.

1.1.1. Renal Cell Carcinoma and its Histological Types

Kidney cancer originates from renal parenchyma. Renal cell carcinoma is a predominant variant of kidney cancer in adults, which represents approximately 90% of all renal neoplasm occurrences (Hsieh et al., 2017). RCC comprises approximately 3% of newly diagnosed cancer cases

in females and 5% in males, resulting in an estimated 400,000 cases worldwide. It is one of the top ten most prevalent cancers globally and has higher mortality rates, particularly in Europe (Jian et al., 2021). RCCs are derived from the epithelial cells lining nephron tubules within the kidney cortex. Urothelial carcinomas arise within the renal pelvis and additional uncommon epithelial tumors of renal parenchyma encompass generally benign tumors, such as oncocytomas and angiomyolipomas, however collecting duct carcinomas, and renal mesenchymal neoplasms are typically malignant tumors (LI et al., 2015; Wasifuddin et al., n.d.).

Renal cell carcinoma is typically incidentally identified through imaging techniques, commonly using Magnetic Resonance Tomography (MRT), ultrasound (US), or computed tomography (CT) scans. Diagnosis is frequently delayed as kidney cancer often remains asymptomatic in its early stages, however, some common symptoms associated with RCC include hematuria (dark red blood in the urine), abdominal bloating, anorexia, weight loss, anemia, and persistent fever of unknown origin (Bukhari et al., 2017). Furthermore, various endocrine abnormalities may be related to RCC. In some cases, only an increase in human chorionic gonadotropin (hCG) or adrenocorticotropic hormone (ACTH) may be observed, while at other times clinical conditions such as Cushing's syndrome may present. A group of researchers at the Mayo Clinic identified an association between RCC and Cushing's syndrome in 1961. Renal cell carcinoma has been shown to constitute 2% of the cases of Cushing's syndrome (Moradi et al., 2023)

RCC is a complex and diverse form of cancer. The 2022 World Health Organization 5th edition RCC classification recognizes 24 subtypes, where 16 subtypes were identified in the 2016 version. Previous categorizations have mainly emphasized malignancy morphology and immunostaining; nevertheless, more recent editions have focused on the importance of molecular and genetic factors in determining RCC subtype classification (Webster et al., 2022). Besides clear cell renal cell carcinoma (ccRCC), non-clear cell renal carcinomas are less common and considered rare. This diverse group is subsequently subdivided into different subtypes, notably papillary and chromophobe (comprising 10% to 5% of cases, respectively) and other types are uncommon (Hsieh et al., 2017). The studies have demonstrated that patients diagnosed with papillary and chromophobe subtypes of RCC have higher 5-year cancer-specific survival rates compared to those with ccRCC (Berglund et al., 2020).

Renal cell carcinomas of distinct histological phenotypes vary in terms of growth characteristics, aggressiveness, metastatic lesions, cell of origin, cytogenetics, and other factors **(Table 1.1**) (Perrino et al., 2018)).

Table 1.1. Pathologic classification of the most common types of renal cell carcinoma

Types	Features	Growth pattern	Cell of origin	Common cytogenetics
Clear cell	Most common, majority of sporadically	Acinar or sarcomatoid	Proximal tube	3p-, 5q+, 8p-, 9p-, 14q-
Papillary	Bilateral and multifocal	Papillary or sarcomatoid	Proximal tube	7+, 17, -Y, 12+, 16+, 20+
Chromophobe	Indolent course	Solid, tubular, or sarcomatoid	Distal convoluted tubes	Hypodiploid
Oncocytic	Rarely metastasize	Tumor nests	Cortical collecting duct	Undetermined
Collecting duct	Very aggressive	Papillary or sarcomatoid	Cortical collecting duct	Undetermined

p- short arm of chromosome; q- long arm of chromosome; +/- refers extra or loss genetic material of chromosome.

According to some studies, ccRCC originates in the proximal convoluted tubules. Also, the pRCC subgroup may derived from kidney proximal tubules, however, single-cell analysis suggested that this subgroup may also originate from kidney collecting duct cells and chRCC is believed to arise from distal convoluted tubules (Correia de Sousa et al., 2023).

RCC has been associated with a range of genetic changes, particularly the mutation in *von Hippel-Lindau* (*VHL*) gene that inhibits tumor; deletion of the short arm of chromosome 3 (3p) in the majority of ccRCC cases; and the occurrence of promoter hypermethylation or deletion (Razafinjatovo et al., 2016; C. J. Ricketts et al., 2018). Most patients diagnosed with *VHL* syndrome possess germline mutations; however, approximately 20% experience rare de novo mutations, and mutations to *VHL* are prevalent in sporadic ccRCC, with various chromosome 3p mutations being identified. Genetic modifications to chromosome 3p involving the tumor suppressors *SETD2, BAP1*, and *PBRM1* are also present in other cases, further contributing to carcinogenesis. Additionally, *VHL* mutations can occur in a mosaic fashion. *VHL* patients frequently acquire 5q and 8q amplifications along with 9p deletions (Webster et al., 2022).

1.1.2. Etiology of renal cell carcinoma

Numerous modifiable and unmodifiable (age and gender) factors have been established as potential risk factors for RCC **(Figure 1.2**). Tobacco smoking, obesity, and hypertension were established as the major modifiable risk factors. However, these associations may be influenced by the likelihood of routine imaging in such patients, potentially leading to the incidental discovery of renal masses (Capitanio et al., 2019). Besides age, gender is the most significant risk factor for RCC. There are several infrequent risk factors that may be linked to RCC involving adult-onset diabetes, chronic kidney disease (CKD), chronic use of palliatives, vitamin D level, intake of processed meat,

viral hepatitis infection, acquired renal cystic disease end-stage renal disease (ESRD), exposure to cadmium and trichloroethylene, elevated triglycerides. On the other hand, modifiable lifestyle factors such as alcohol consumption, obesity, hypertension, and genetic syndromes are more well-established determinants for RCC (Capitanio et al., 2019; Bahadoram et al., 2022). Nonetheless, the etiology of kidney cancer remains incompletely clarified, just like some other malignancies.

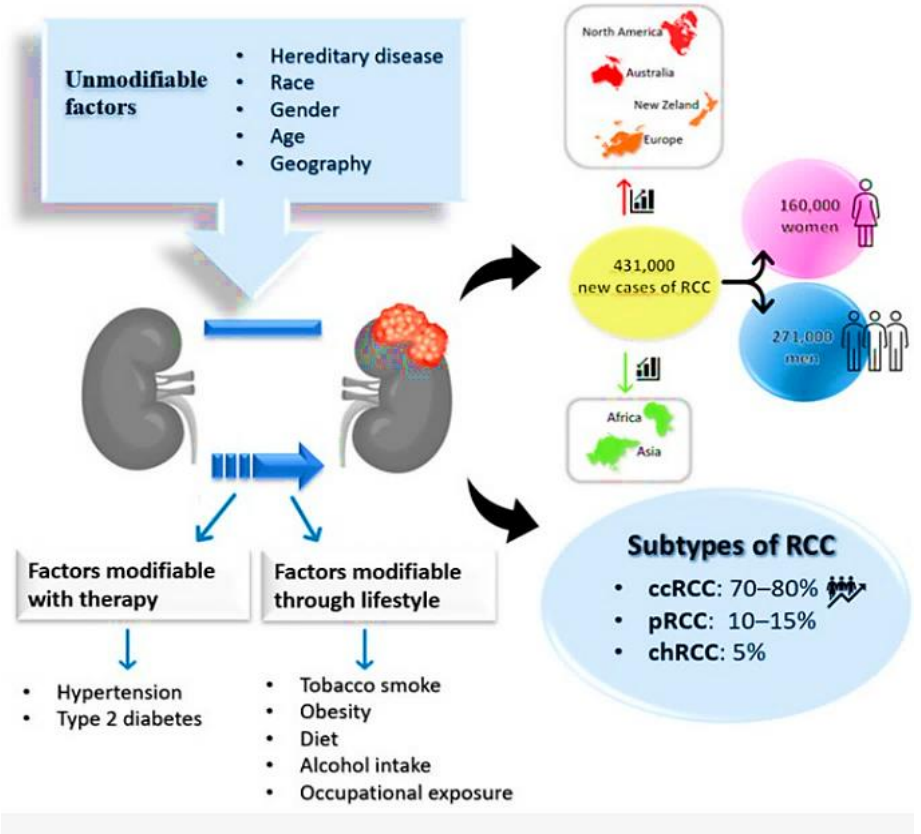


Fig. 1.2. Risk factors, epidemiology, and main subtypes of RCC. (Adopted from (Schiavoni et al., 2023)).

Age. Age is an independent prognostic factor for RCC. The incidence rates of RCC tend to be higher for people between 65 and 74 in more rapidly developing countries. The risk of kidney cancer is low in individuals under the age of 45 (Padala et al., 2020). This type of tumor is found in only 3.4-7.5% of adults under the age of 40, and probably, a limited number of experiments performed on individuals within this age are responsible for this issue (Taccoen et al., 2007).

Gender. Epidemiological studies consistently report a consistent twofold higher lifetime risk of kidney cancer in males compared to females. Worldwide cancer incidence data from 1978 to 2007 consistently shows a 2:1 ratio of male to female cases, regardless of age, year, or region. The higher prevalence of modifiable determinants, such as cigarette smoking, obesity, and hypertension among males may partially explain this tendency (Capitanio et al., 2019; Peired et al., 2021). Studies have demonstrated that RCC cells exhibit higher levels of estrogen receptor-β (ERβ) expression compared to breast cancer cells, which suggests a potential protective function for ERβ as an anti-oncogene in

RCC. Stimulation of estrogen and ERβ activation leads to the suppression of RCC cell proliferation and induction of programmed cell death, also known as apoptosis. This protective effect is compromised when ERβ is downregulated. Furthermore, the involvement of ERβ in inhibiting RCC progression provides a plausible explanation for the sex-related disparity in RCC incidence, where men exhibit a higher susceptibility compared to women (Mancini et al., 2020). Most trials have shown that male gender was significantly associated with worse survival outcomes (Aron et al., 2008). Although various genetic and molecular biomarkers have been linked to this phenomenon, the specific mechanism has not yet been elucidated. Future experiments should shed more light on gaining a better understanding of the sex-related differences in kidney cancer, which can help in the development of individualized patient care (Lughezzani et al., 2019).

Smoking and alcohol consumption. Cigarette smoking is a considerable etiological factor in RCC, like other cancer types, such as lung carcinoma. The International Agency for Research on Cancer and the United States Department of Health and Human Services have classified tobacco consumption as a carcinogenic factor for the kidney. In developed countries, it is responsible for approximately 6% of kidney malignancy mortality are tobacco-related outcomes (Scelo & Larose, 2018). Numerous studies have indicated that renal cancer has also been linked to the presence of carcinogenic chemicals, such as aromatic amines or arylamines, and polynuclear aromatic hydrocarbons, which are produced during cigarette smoking (Kumar et al., 2023). According to recent findings, current and past tobacco users were more likely than non-smokers to have metastatic renal cancer, which is characterized by the presence of tumors in the lymph nodes or their spread to other areas of the body (Baral et al., 2023). In one retrospective study, individuals with a history of smoking, whether current or past, exhibited an increased risk of RCC, with a respective 1.5-fold and 1.6-fold enhancement in likelihood (Padala et al., 2020). Notably, multiple epidemiologic studies have reported that people who consume moderate amounts of alcohol are at decreased risk of developing RCC. In all these studies conducted, individuals who consumed alcohol showed a 20% decrease in risk compared to nondrinkers and light drinkers. Although the exact mechanism is not yet known, some researchers postulate that alcohol consumption has been demonstrated to enhance insulin sensitivity and potentially could lead to the reduction of kidney cancer risk via this indirect route (Scelo & Larose, 2018).

Hypertension. Hypertension is one of the causal risk factors in kidney cancer etiology that increases the susceptibility to RCC. That would be explained by the damage to its renal glomerulus and tubular apparatus (Padala et al., 2020). The potential biological mechanisms that explain the connection between hypertension and kidney cancer are not yet fully understood. Hypertension and RCC are likely influenced by numerous confounding modifiable factors, including smoking, obesity, and inadequate physical activity. These factors could provoke the enlargement of both RCC and

hypertension in similar ways, such as chronic inflammation, insulin, IGF-1, oxidative stress (like lipid oxidation), interleukin-6, leptin, and the VEGF pathway. A significant accumulation of hypoxia-inducible factors results in cell growth and angiogenesis dysregulation. Hypertension may also be linked to endothelial dysfunction and modified vascular remodeling, contributing to an elevated generation of reactive oxygen species in individuals with high blood pressure. These reactive oxygen species can potentially facilitate the growth of cancer cells by activating oncogenes through major biological mechanisms, such as angiogenesis, cell proliferation, and inflammation (Kim et al., 2020; Ba et al., 2022). Further comprehensive prospective studies that specifically investigate the impact of treating hypertension on this connection are required.

Obesity. Obesity is a well-known, recognized factor that increases the risk of developing RCC. Obesity induces insulin resistance, which results in the development of type 2 or adult-onset diabetes and subsequently causes excessive increases in insulin-like growth factor-1 (IGF-1) and insulin levels. The activation of insulin receptors (INSR) and IGF-1 receptors (IGF1R) by these hormones leads to the initiation of several signaling pathways, for example, PI3K/AKT, mTOR/HIF, and mTOR/cyclin D1 (Turco et al., 2021b). In addition, obese patients demonstrate leptin resistance and an increased level of leptin hormone, which stimulates cell proliferation, tumorigenesis, and metastatic progression through the activation of various pathways, including MAPK, Jak/Stat, and PI3K/AKT. Obesity is linked to chronic inflammation and contributes to the initiation and advancement of tumors, by facilitating the secretion and expression of inflammatory cytokines, such as TNF-α and IL-6. These cytokines stimulate the production of cyclooxygenase 2, leading to the generation of prostaglandin E2, thereby facilitating cancer progression (Turco et al., 2021b). One study revealed that a gain of weight about 5 kg elevated the probability of RCC by 25% in males and 35% in females (Padala et al., 2020). Furthermore, according to a meta-analysis, consistent engagement in regular physical exercise has been associated with a 22% reduction in the risk of RCC (Padala et al., 2020).

Genetic predisposition. Only 5% of all kidney cancers are attributed to hereditary factors (Testa et al., 2020). Hereditary cancer often presents at an early age, typically occurring before the age of 40. Hereditary RCC is characterized by familial predisposition, as evidenced by a family history of the disease, and the presence of bilateral and multifocal renal tumors (Schmidt & Linehan, 2016).VHL syndrome is the most widely recognized type of hereditary kidney cancer. If a parent has von Hippel-Lindau (VHL), there is a 50% (1 in 2) likelihood that their child will inherit the deleterious genetic alteration in the *VHL* gene. Von Hippel-Lindau is an inherited multisystem disorder characterized by autosomal dominant inheritance, which predisposes individuals to an increased likelihood of developing clear-cell kidney tumors and cysts. Additionally, those affected face the risk of developing adrenal gland tumors (pheochromocytomas), pancreatic cystic lesions, and islet cell cancers, as well as hemangioblastomas in the central nervous system (CNS) and retina (Schmidt &

Linehan, 2016). Some studies have shown that Birt-Hogg-Dubé Syndrome (BHD) increases the risk of different types of kidney cancer. An alteration in the *FLCN* gene causes it. It is a tumor suppressor gene; when the *FLCN* gene is mutated, unregulated cellular proliferation can result in cancer development (Haas & Nathanson, 2014). The development of RCC is associated with a range of additional conditions. For instance, hereditary papillary renal carcinoma (pRCC) is caused by a mutation in the *MET* proto-oncogene, and hereditary leiomyomatosis and renal cell carcinoma (encoding gene *FH*). Additionally, succinate dehydrogenase (SDH)-deficient renal cancer is caused by a mutation in another Krebs cycle enzyme, (encoding gene *SDH*) (C. Ricketts et al., 2008; Haas & Nathanson, 2014). In addition, some reports have demonstrated that *BAP1* gene mutations have an increased risk for ccRCC, and *MITF* gene mutations also lead to a risk of kidney cancer infrequently (Guhan et al., 2020; Feng et al., 2020). Other potential genetic risk factors for kidney cancer may be associated with mutations in *PBRM1, CDKN2B, PTEN, TSC1*, and *TSC2 genes.* Studies have revealed that as much as 40% of sporadic renal cell carcinoma (RCC) may be associated with *PBRM1*, while other cases show a connection to *CDKN2B* (Guhan et al., 2020; Naik et al., 2024).

Further comprehensive studies are necessary to explore the mechanisms underlying specific risk factors for kidney cancer. Nonetheless, there is no guarantee for entirely avoiding kidney cancer; however, individuals can reduce their risk by adopting strategies such as maintaining a healthy weight and, if applicable, quitting smoking (Tahbaz et al., 2018).

1.2. Genetic aspects of clear cell renal cell carcinoma

Clear cell renal cell carcinoma (ccRCC) arises from epithelial cells and is the predominant subtype of renal cell carcinomas, characterized by its malignant potential and clinical prevalence. Current genomic analyses have revealed a subset of clear cell renal tumor characteristic mutations of the tumor suppressor *VHL* gene, which is located on the chromosomal arm 3p and is a negative regulator of hypoxia-inducible factor (HIF) proteins. Functional loss of the VHL protein occurs in most ccRCC patients through non-germline (somatic) mutations and aberrant DNA methylation and plays a role as a major oncogenic factor and contributes to tumor development, progression, and metastasis by activating HIF proteins. In recent sequence analysis investigations, beyond the mutation observed in the *VHL*, mutations have emerged in genes critically involved in histone modification and chromatin remodeling, such as *PBRM1* (~45%), *SETD2* (10-15%), and *BAP1* (10-15%) (Shapiro et al., 2022). Furthermore, it is noteworthy that the activation of the HIF signaling pathway has been observed not only in ccRCC but also in several other subtypes of RCC, such as papillary RCC (Hsieh et al., 2018).

1.2.1. Genetic evolution of ccRCC

A significant proportion of individuals diagnosed with ccRCC exhibit chromosomal arm 3p deletion, resulting in the loss of the *VHL, PBRM1*, *SETD2*, and *BAP1* genes. This genetic event, which

typically occurs between early childhood and adolescence, is a distinguishing characteristic **(Figure 1.3)** (Beroukhim et al., 2010; C. J. Ricketts et al., 2018; Mitchell et al., 2018).

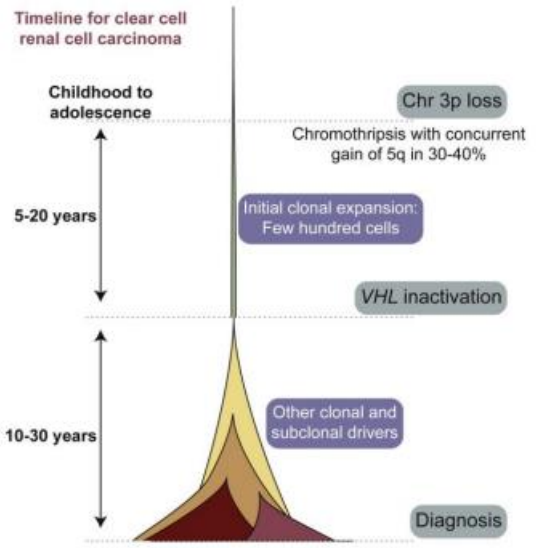


Fig. 1.3. Chronologic sequence of genetic events in ccRCC development. (Adopted from (Mitchell et al., 2018)).

In the early stages of ccRCC development, chromotrypsis events involving the short arm of chromosome 3 are frequently observed in both childhood and adolescence. These events result in the deletion of 3p, often leading to the loss of heterozygosity in numerous tumor inhibitors. Some investigations unveiled that the deactivation of the *VHL* allele predominantly manifests clonally in the majority of cancers, subsequently accompanied by subclonal alterations (Mitchell et al., 2018). The second most prevalent genetic alteration involves chromothripsis that occurs between the 3p and 5q regions, which is followed by the deletion of 3p and the gain of 5q. This chromosomal abnormality is present in around 65% to 75% of all ccRCC patients (Mitchell et al., 2018).

The frequent minimal deletion of chromosome 3p results in the loss of heterozygosity (LOH) in Polybromo-1 (*PBRM1*), SET domain containing 2 (*SETD2*), and BRCA1-associated protein *(BAP1*) genes which are lead to inactivation mutations (Zhu et al., 2020).

The *VHL* gene is a multi-domain protein, the best-characterized function of the von Hippel-Lindau protein (pVHL) is its role as a substrate recognizer in the E3 ubiquitin ligase complex, the principal regulator of HIF. It facilitates degradation of HIF-1α and HIF-2α proteins. Under normal oxygen tensions, HIFα undergoes prolyl hydroxylation by Egl-9 Family Hypoxia Inducible Factor (HIF) 1 (EGLN), ubiquitination by VCB-Cul2, and degradation by 26S proteasome. However, in the presence of hypoxia, hydroxylation of HIFα is temporarily inhibited; hence, it is not targeted for degradation by tumor suppressor pVHL (Hsieh et al., 2018). Instead of this, HIF-1α forms an active HIF-1 complex by dimerizing with structurally expressed HIF-1β and activates the transcription of genes that promote glycolytic metabolism and angiogenesis (in the long term) (Semenza, 2011;

Schito & Semenza, 2016). The result is ccRCC tumors lacking functional pVHL, thus permissive for aggressive tumorigenesis. Notably, somatic mutations of VHL are identified in around 92% of individuals diagnosed with ccRCC, and they are absent in non-clear cell RCC (Ferro et al., 2023).VHL disease is categorized as type 1 and type 2. Type 1 is associated with a significant risk and is usually caused by a complete lack of protein. Type 2 is linked to missense mutations, which increase the likelihood of developing the syndrome of pheochromocytoma or paraganglioma (Chappell et al., n.d.). Moreover, vascular endothelial growth factor (VEGF) holds considerable significance in ccRCC, and these family genes are the most extensively investigated. Some studies have shown that healthy renal parenchyma and ccRCC exhibit constitutive expression of vascular endothelial growth factor (VEGF), an angiogenic cytokine. While VEGF is uniformly expressed in normal renal parenchyma, its expression is heterogeneous and elevated in ccRCC. Therefore, overexpression of this angiogenic factor is an unfavorable histologic prognostic indicator since it is associated with higher nuclear grade, increased proliferation, and larger tumor size (Djordjevic et al., 2007). HIFα activation also causes a swift rearrangement of the central metabolic pathways in ccRCC. This includes a decrease in oxidative phosphorylation, an increase in aerobic glycolysis (known as the "Warburg effect"), and the production of fatty acids and glycogen, resulting in the accumulation of lipids and glycogen (Pescador et al., 2010; Wise et al., 2011; Semenza, 2013).

Recent genome sequencing studies have shown that mutations in *PBRM1*, a gene encoding the BAF180 protein, a component of the SWI/SNF-B (PBAF) chromatin remodeling complex, have been observed in approximately 40% of ccRCC cases (Hsieh et al., 2018). The BAF180 protein plays a role in multiple DNA repair mechanisms, and it is also essential for cohesion between centromeres, contributing to the maintenance of genomic stability (Carril-Ajuria et al., 2019). The precise processes via which mutations in the *PBRM1* gene facilitate the development of cancer and malignant progression have not yet been completely understood. The majority of *PBRM1* mutations result in loss of function, and the absence of its expression is linked to enhanced cell proliferation and migration in ccRCC. Additionally, it enhances the response to hypoxia-inducible factor alpha (HIFα). Therefore, in ccRCC, the role of *PBRM1* as either a tumor suppressor or an oncogene depends on the specific context (Carril-Ajuria et al., 2019).

BAP1 (BRCA1-related protein) is a tumor suppressor gene that was initially identified through its interaction with *BRCA1*. Sporadic and germline mutations of *BAP1* were subsequently discovered in various tumor types, including uveal melanoma, mesothelial, and renal tumors. *BAP1* serves as a ubiquitin C-terminal hydrolase and regulates essential cellular processes, such as the cell cycle, cell differentiation, apoptosis, gluconeogenesis, and DNA damage repair with homologous recombination (Hsieh et al., 2018). Furthermore, it has been demonstrated that somatic *BAP1* mutations are present in various tumors, including melanoma skin cancer, mesothelioma, and occasionally other types of

malignancies as well. Furthermore, *BAP1* mutation was related to many cancer-related pathways, like the mTOR signal pathway and the WNT signal pathway (Tan et al., 2020). Notably, *BAP1* does not induce tumorigenesis by accelerating cell proliferation; instead, it facilitates tolerant and slow G1/S cell cycle checkpoint, resulting in slower yet uncontrolled tumor expansion (Bott et al., 2011).

*SETD2 (*Histone methyltransferase SET-domain-containing 2) is one of the most prevalent mutated genes in ccRCC, and it holds significant functions in the epigenetic regulation of functional pathways during the development and progression of ccRCC. According to previous study, the frequency of mutations in the *SETD2* gene is increased to 47% in metastatic clear cell ccRCC tumors (Xie et al., 2022). In that case, ccRCC is triggered by alters in the epigenetic profile resulting from the depletion of H3K36me3, thus reducing DNA methylation (Xie et al., 2022). *SETD2* plays crucial role in chromatin-related biological processes, encompassing transcriptional regulation, DNA damage repair, interplay of histone modification, alternative mRNA splicing, and methylation of non-histone targets (Rao et al., 2023).

In addition, despite the genes mentioned above, less frequently, mutations in *TP53, MTOR, TSC1, TSC2, PIK3CA, KDM5C*, and *SMARCA4* are detected in ccRCC (Ferro et al., 2023).

Most mutations linked to the *BAP1, PBRM1,* and *SETD2,* genes are found in a particular subcluster of cells that have an inactive *VHL* gene (Bihr et al., 2019) and in accordance with one hypothetical pattern, *PBRM1* is considered the second driver mutation in renal carcinogenesis following the loss of *VHL* (Nargund et al., 2017). Certain findings have demonstrated that mutated *BAP1* and *SETD2*, rather than *VHL* and *PBRM1*, are correlated with poorer prognosis among patients with ccRCC, indicating that dysfunction or alteration in *VHL* and *PBRM1* may contribute to the initiation of tumor growth. However, *SETD2* and *BAP1* genes play a significant role in cancer progression (C. J. Ricketts et al., 2018).

1.2.2. Role of epigenetic modifications in ccRCC

Many studies demonstrated that epigenetic regulation plays a significant role in ccRCC development and progression. Epigenetic processes refer to a variety of mechanisms that cause alterations in gene expression without modifying the nucleotide sequence of the DNA.DNA methylation, histone modification, and non-coding RNA (ncRNA) expression are critical mechanisms involved in epigenetic regulation.

In addition, several studies have suggested that specific epigenetic modifier genes' expression levels or mutations may serve as biomarkers for ccRCC (Angulo et al., 2021). DNA methylation is widely recognized as a crucial epigenetic modification that occurs in eukaryotic organisms. Hypermethylation of CpG islands in the promoter regions of genes can result in gene silencing or gene expression is inhibited. It involves the covalent binding of a methyl group (-CH3) from the amino acid S-adenosyl methionine (SAM) to the 5th carbon of the pyrimidine ring of cytosine in CpG

dinucleotides, resulting in the formation of 5-methylcytosine. This process is catalyzed by DNA methyltransferase (DNMTs) enzymes. DNMT1 serves as the primary enzyme responsible for maintaining the methylation patterns that persist following DNA replication. This is achieved by the addition of methyl groups to hemimethylated CpG sites. Conversely, DNMT3A and DNMT3B are responsible for methylating new CpG sites and are highly expressed during embryonic development, with minimal expression in adult tissues (Peters et al., 2020; Angulo et al., 2021). Furthermore, the hypermethylation of KLF5, a regulatory element responsive to epidermal growth factor, by DNMT1 is closely linked to an unfavorable prognosis for ccRCC patients. In RCC, DNMT1 demonstrates an oncogenic function, whereas DNMT3A plays a tumor-suppressive role. Notably, DNMT3A contributes to the silencing of HIF2α through methylation. This silencing mechanism imposed by DNMT3A restricts the proliferative capacity of cells, particularly under hypoxic conditions (Hong et al., 2020).

The onset and progression of cancer disrupt regular epigenetic processes, leading to widespread alterations in the DNA methylation pattern across the genome (Kubiliūtė et al., 2022). CpG island hypermethylation is frequently found in different carcinomas, including renal cell carcinoma, and is commonly linked to the silencing of tumor suppressor genes and their associated signaling pathways (**Figure 1.4**) (Kubiliūtė et al., 2022).

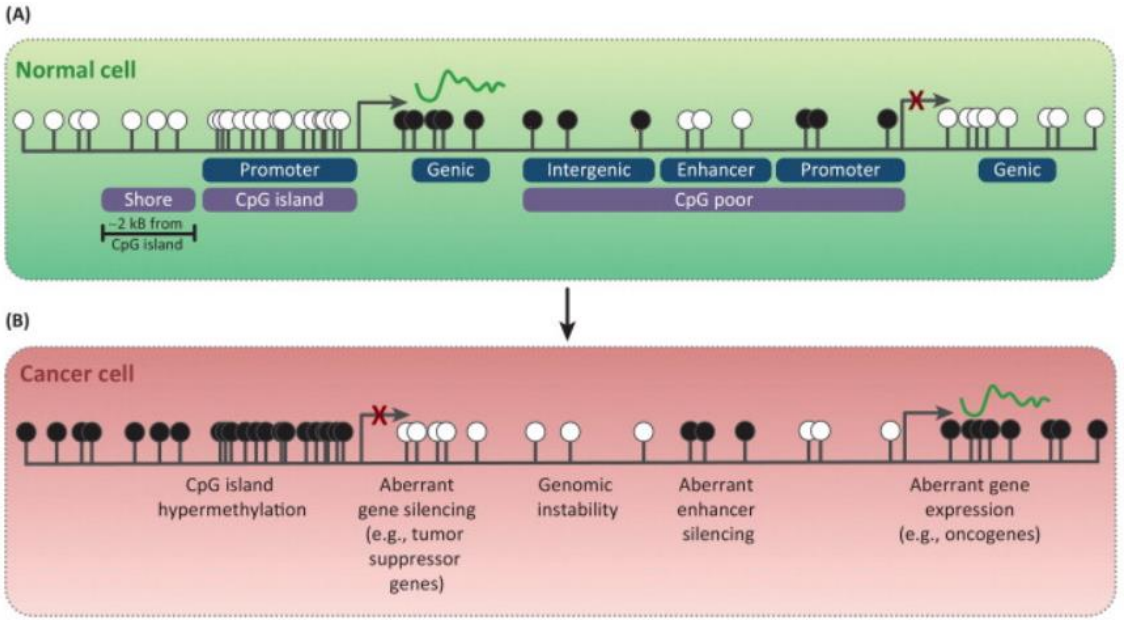


Fig. 1.4. DNA methylation patterns in the normal and cancer cells**.** (Adapted from (Stirzaker et al., 2014)). The white circles depict unmethylated CpG sites, while the black circles – depict methylated CpG.

Indeed, DNA methylation has numerous advantages as a favorable epigenetic biomarker, not only due to its easy detectability in bodily fluids but also because it accurately reflects tumor heterogeneity. Tissue specimens from primary and metastatic cancer regions exhibit considerable heterogeneity, containing genetically and/or epigenetically distinct cancer cell populations, whereas

these populations may not be captured by needle biopsy. In both tissue and liquid biopsy samples, DNA demonstrates greater stability and resilience against degradation caused by formalin fixation and freezing procedures compared to RNA. Besides, DNA has the potential to amplify, enhance sensitivity, and identify biomarkers, even in cases with limited sample amounts or quality. Due to the inherent limitations of a single biopsy of ccRCC that does not capture the entire spectrum of (epi)genetic alterations, the utility of DNA methylation-based biomarkers across various clinical samples, especially body fluids, bypasses the tumor and the limitations associated with tumor-specific assessments. Nowadays, numerous methods to easily detect DNA methylation by bisulfite sequencing, either target-based (such as methylation-specific polymerase chain reaction (MSP), pyrosequencing methylation-specific restriction endonucleases, etc.) or genome-wide approaches (Tavares et al., 2022). The efficacy of DNA methylation for identifying biomarkers in ccRCC in practical medical applications is greatly enhanced by its diverse range of characteristics.

1.3. Management of Renal Cell Carcinoma

In the evaluation of patients' survival within most classification systems, there is a lack of differentiation between various kidney cancers and histological subtypes. The following subchapters summarize the most common systems in clinical practice.

1.3.1. Diagnosis, stages and grades of RCC

Renal cell carcinomas (RCCs) are frequently asymptomatic and typically detected incidentally during abdominal examinations. Currently, computed tomography (CT), ultrasound or ultrasonography (US), and magnetic resonance imaging (MRI) are major techniques for assessing RCC. US is useful as a first-line imaging modality and can be used to evaluate low-risk patients with microscopic hematuria and simple and complex renal cysts with guidance provided by Bosnian classification (Silverman et al., 2019; Usher-Smith et al., 2020). CT with intravenous contrast is better than US. MRI is recommended for analyzing the extent of disease in cases where contrast usage is inappropriate or when US or CT scans provide unclear results. MRI and positron emission tomography (PET) are the preferred diagnostic methods for detecting metastatic RCC. CT evaluates the entire urinary system and has a better capacity to diagnose kidney tumors than intravenous urography (Rossi et al., 2018)

The tumor stage refers to the progression of cancer cells. The most commonly used staging system for ccRCC is the American Joint Committee on Cancer (AJCC) TNM system, which indicates primary tumor (T), node (N), and the presence of distant metastasis (M). The 8th edition of the TNM classification uses the system presented in **Table 1.2** (Shao et al., 2018).

Table 1.2. 8th edition of the AJCC TNM classification system for kidney cancer. (Adopted from (Swami et al., 2019; Elkassem et al., 2021))

T	TX	Tumor size of the primary tumor cannot be evaluated
	T0	No evidence of primary tumor
	T1a	Primary Tumor diameter < 4cm
	T1b	Primary Tumor diameter > 4cm but ≤ 7cm
	T2a	Primary Tumor diameter > 7cm but ≤ 10cm
	T2b	Primary Tumor diameter > 10cm, limited to the kidney
	T3a	Primary tumor extends into the renal vein or its segmental branches, or invades the pelvicalyceal system, or invades perirenal and/or renal sinus fat but not beyond Gerota's fascia
	T3b	Primary tumor extends into vena cava below the diaphragm
	T3c	Primary tumor grossly extends into vena cava above diaphragm or invades wall of vena cava
	T4	Tumor invading beyond Gerota's fascia
N	NX	Lymph node involvement cannot be evaluated
	N0	No regional lymph node metastases
	N1	Single or multiple regional lymph nodes involved (regional lymph nodes: hilar, caval, aortic)
M	M0	No distant metastases
	M1	Distant metastasis, including noncontiguous adrenal involvement

The TNM classification system is used to give a predictive cancer stage to kidney tumors (as shown in Table 1.2). This stage is essential in determining which therapy is suitable and is considered one of the most accurate indicators of treatment success.

ccRCC are also classified according to the microscopic analysis of nuclear morphology. In 1982, Fuhrman *et al.* classified tumors as grade 1 to grade 4 based on nuclei characteristics, such as shape and size of nuclei, and nuclear pleomorphism (Lin et al., 2019). Although used for a long time, the Fuhrman grading system has several methodological problems. The main difficulty is caused by the requirement that grading implies simultaneous evaluation of three parameters. The Fuhrman system dictates that grading is determined by the highest-grade area, regardless of whether it is the focal point. This means that small areas with higher grade cancer and tumor regions adjacent to necrotic areas are considered for grading. These issues are likely factors contributing to variations in the assessment of tumor grade among both the intra-observer and inter-observer. The current Fuhrman grading system is declared not applicable because of these limitations (Delahunt et al., 2019).

The conventional Fuhrman grading system, traditionally used to evaluate the aggressiveness of neoplastic cells in renal cell carcinoma (RCC), faces challenges related to limited interobserver reproducibility and uncertain prognostic value. Consequently, the International Society of Urological Pathology (ISUP) has introduced a novel grading system known as ISUP grading. This revised classification, incorporated in the 2016 edition of the WHO "Blue Book" on urogenital tumors, is applicable to clear cell and papillary RCC (**Table 1.3)**. The presence of nucleolar prominence is

indicative of clear cell and papillary RCCs in grades 1 to 3, while excessive nuclear pleomorphism, giant cells in tumors, sarcomatoid and/or rhabdoid differentiation characterize grade 4 tumors. As nucleolar grading has been shown to have superior prognostic predictive value compared to the traditional Fuhrman grading, the current ISUP grading is now a necessary inclusion in the pathological findings (Rabjerg et al., 2021).

Table 1.3. The World Health Organization/International Society of Urological Pathology grading system for clear cell and papillary renal cell carcinoma. (Adopted from (Rabjerg et al., 2021)).

Grade	Description
1	Nucleoli absent/indistinct and basophilic at x400 magnification
2	Nucleoli are prominent and eosinophilic at x400 magnification, not prominent but visible at x100 magnification
3	Nucleoli conspicuous and eosinophilic at 100x magnification
4	Extreme nuclear pleomorphism, anaplastic giant cells, rhabdoid morphology, and sarcomatoid differentiation.

To predict the outcome of ccRCCs following radical nephrectomy, Frank *et al*. introduced a scoring system known as the Stage, Size, Grade, and Necrosis (SSIGN) score, one of the most common predictive models for ccRCC. This score is determined by considering factors such as TNM stage, tumor size, nuclear grade, and the presence of histological tumor necrosis (Parker et al., 2017). Zigeuner *et al.* conducted a validation study of the SSIGN score using an external cohort from a single center and recommended applying the system routinely (Zigeuner et al., 2010).

1.3.2. Treatment of ccRCC

The most widely accepted and effective treatment for early stages of renal cell carcinoma involves local surgical resection, which has the potential for curative outcomes. The two most frequently used nephrectomy approaches for RCC are radical nephrectomy (RN) and partial nephrectomy (PN), depending on factors such as size, stage, and location of tumor, and the patient's general condition. Furthermore, systematic treatments involving targeted medicines or immune checkpoint inhibitors have been performed for metastatic RCC (Hsieh et al., 2017; Yang et al., 2023). Radical nephrectomy refers to removing the entire kidney, whereas partial nephrectomy only removes part of the diseased kidney tissue, aiming to preserve as much of the normal kidney unit on the same side as possible. However, the partial nephrectomy procedure is time-consuming and carries the risk of common complications, such as hematuria, perirenal hematoma, and urinary tract fistula. In light of these considerations, the recommendations suggest that, due to feasibility, partial nephrectomy should be prioritized for T1 and T2 stage tumors (Yang et al., 2023).

A new era for the treatment of RCC is based on targeted drugs or immune checkpoint inhibitors. In 2005 and 2006, sorafenib and sunitinib became the initial orally administered targeted agents approved by the FDA, specifically targeting the VEGF receptor **(Figure 1.5)** (Kase et al.,

2023). Moreover, Sorafenib was a broad-acting tyrosine kinase inhibitor (TKI). Interleukin 2 (IL-2), a cytokine involved in the development and differentiation of T lymphocytes, is used in the treatment of RCC. The use of immune checkpoint inhibitors, such as antigen-associated antigen-4 (CTLA-4), has significantly advanced the treatment of RCC. Pembrolizumab and nivolumab are monoclonal antibodies that selectively bind to programmed death ligand-1 (PD-L1) and block its interaction with PD-L2. Avelumab is another monoclonal antibody that selectively binds to PD-L1. Lastly, ipilimumab is a monoclonal antibody that targets CTLA-4 (Ross & Jones, 2017; Wojtukiewicz et al., 2021; Yang et al., 2023).

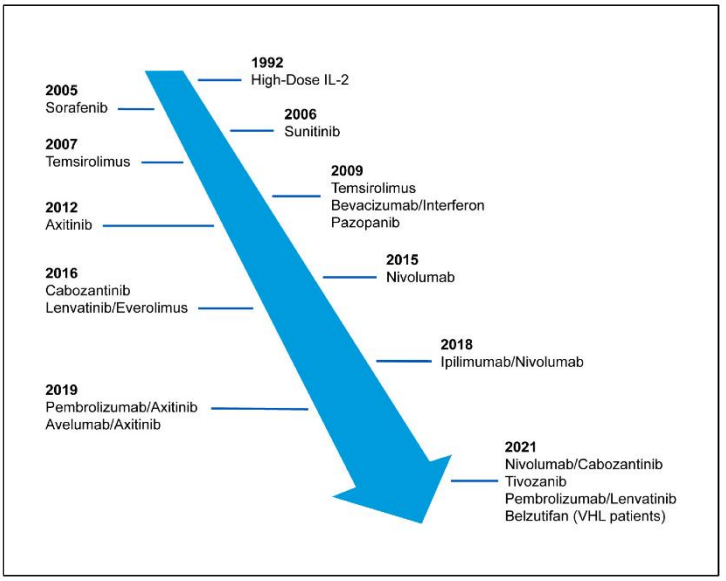


Fig. 1.5. Timeline of FDA-approved agents and combination treatments for metastatic RCC. (Adopted from (Kase et al., 2023)).

In the following years, regulatory authorities approved increasing tyrosine kinase inhibitors (TKIs), with sunitinib and pazopanib specifically emerging as the primary choices for first-line indications of advanced renal cell carcinoma. While their effectiveness is comparable, pazopanib may exhibit a slightly superior safety profile (Amaro et al., 2022). In addition, other TKIs that have received approval include tivozanib, axitinib, cabozantinib, and lenvatinib and each of these agents possesses slightly distinct capabilities in binding to tyrosine kinases (Michaelis et al., 2022).

Chemotherapy and radiotherapy have received significant skepticism over the past few decades. Historically, RCC was contemplated as a radio-resistant malignancy. However, in recent years, new methods such as intensity-modulated radiation therapy (IMRT), and stereotactic body radiation therapy (SBRT) have been used. These techniques can achieve effective local control (LC) and symptom relief in metastatic cases while minimizing toxicity to surrounding normal tissues (Spyropoulou et al., 2021). Despite the ongoing use of chemotherapy, it has not proven effective for treating all subtypes of RCC (Aweys et al., 2023).

In conclusion, since RCC and its all subtypes are resistant to chemotherapy and radiotherapy, immunotherapy and target-based therapies are considered more effective treatment methods for RCC. Ongoing technological advancements will result in wide range of personalized approaches in RCC

1.4. Tumor heterogeneity

The proliferation of tumor cells often leads to genetic diversification, which has resulted in tumor heterogeneity becoming a significant challenge in molecular oncology and the treatment of tumors (Ricketts & Linehan, 2014). Tumor heterogeneity originates primarily from the clonal proliferation of genetically distinct cells, influenced by interactions within the evolving tumor microenvironment, resulting in the modulation of cancer phenotypic properties at the epigenetic, transcriptional, protein, and environmental levels (Proietto et al., 2023). Intratumor heterogeneity (ITH) characterizes the presence of genetically different clones in various subpopulations of the same tumor at a genetic level. Renal cell carcinoma is a heterogeneous tumor, and the occurrence of intratumoral heterogeneity in RCC was identified over two decades ago (Beksac et al., 2017). Numerous investigations have highlighted the presence of ITH across primary and metastatic neoplastic sites, discerned through disparities in the prevalence of von Hippel-Lindau gene (*VHL*) mutations and alterations in chromosomal copy numbers. These genomic differences play a crucial role in determining the responsiveness of cancer cells to pharmacological treatments and resistance to therapy (Vaziri et al., 2012). Moreover ITH presents a considerable obstacle to the discernment of driver mutations in RCC (Beksac et al., 2017). Gerlinger et al. demonstrated that the greater the number of tissues sampled, the more heterogeneity was detected, implying that the depth of genomic heterogeneity in ccRCC might be underestimated (Gerlinger et al., 2014)

Vogelstein et al. classified genetic heterogeneity in tumors into four categories: intratumoral, intermetastatic, intrametastatic, and among patients. Due to intratumoral heterogeneity, it is not possible to observe two genetically identical cells in a tumor (Vogelstein et al., 2013). The cause of this phenomenon is the introduction of new mutations during each cell division. Intermetastatic heterogeneity is observed between different metastatic lesions in the same patient. Metastatic RCC is considered one of the most challenging tumors to treat due to this heterogeneity (Gerlinger et al., 2012).

Considering the functions of epigenetic mechanisms in modulating gene expression and responding to environmental cues, they play a significant role in guiding intratumor heterogeneity. Indeed, it has been reported that dysregulation of epigenetic mechanisms can also increase plasticity of cancer cells which leads to tumor heterogeneity (Wang et al., 2021). Consistent with this notion, variance in cellular transcriptional programs across tumor types is often independent of genetic-level ITH but is associated with variability in 5mC patterns within genetically homogeneous cell populations. 5mC patterns positively support expression when localized in gene bodies or suppress

transcription when present in promoters and enhancers. In such study, it was found that epigenetic ITH is considerably higher in metastatic lesions compare to primary tumors in ccRCC (El Khoury et al., 2023). Hence, epigenetic ITH is correlated to different tumor characteristics and poses a number of challenges for potential biomarkers.

It has been suggested that one of the tumor microenvironmental conditions is hypoxia, which is related to epigenetic heterogeneity in RCC. Under low oxygen levels, a number of significant signaling pathways are activated, which leads to hypermethylation in cancer cells and decreases enzymatic activation of several histones (Choudhry & Harris, 2018; Terry et al., 2020).

According to the study by Turajilic et al., characterized seven different evolutionary subcategories of ccRCC, each distinguished by distinct levels of intratumor heterogeneity (ITH) and genomic instability (GI). It was determined that the majority of tumors exhibited low levels of ITH and GI were caused by *VHL* mutation and characterized by restricted branching. Intermediate levels of ITH were evident across five additional subtypes, characterized by medium to high levels of GI. The first subtype of tumors contained a variety of genetic alterations, including at least two clonal mutations in *BAP1*, *SETD2, PBRM1*, or *PTEN*. A subsequent subtype comprised cases where *BAP1* served as the sole mutational driver alongside *VHL*. *VHL-wild-type* tumors exhibiting sarcomatoid differentiation were included in the third subtype. The fourth and fifth subtypes were delineated by sequential events of *PBRM1* mutation leading to activation of either the PI3K-mTOR pathway or subclonal somatic copy number alterations (SCNAs), respectively, and were predominantly associated with T1 stage tumors. Lastly, the evolutionary subtype driven by *PBRM1* mutation followed by *SETD2* histone alterations exhibited remarkably high ITH, with approximately ten clones per tumor, frequent instances of parallel evolution, and metastatic stage (Turajlic, Xu, Litchfield, Rowan, Chambers, et al., 2018).

As a result of research conducted in metastatic clear cell renal cell carcinoma (ccRCC), it was revealed that the metastatic regions exhibited greater homogeneity and fewer somatic changes compared to the primary cancer. Just 5.4% of driver events were detected to be new mutations in metastatic lesions, indicating that most hereditary changes occur in the initial neoplasm (Turajlic, Xu, Litchfield, Rowan, TRACERx Renal Consortium, et al., 2018).

To conclude, RCC is highly heterogeneous and has a number of distinct evolutionary forms that limit its treatment methods. Therefore, fully understanding the mechanisms of tumor tissue heterogeneity is very significant in order to determine the most accurate therapeutic options.

2. STUDY COHORT AND METHODS

The current study of DNA methylation analysis in multifocal renal cell carcinoma consisted of three stages **(Figure 2.1)**. In the first stage, DNA was extracted from two different tumor foci tissue samples using a standard phenol-chloroform technique. The next stage encompassed isolated DNA modification with bisulfite. In the third stage, Methylation-specific PCR (MSP) was used to determine the methylation patterns of the investigated genes.

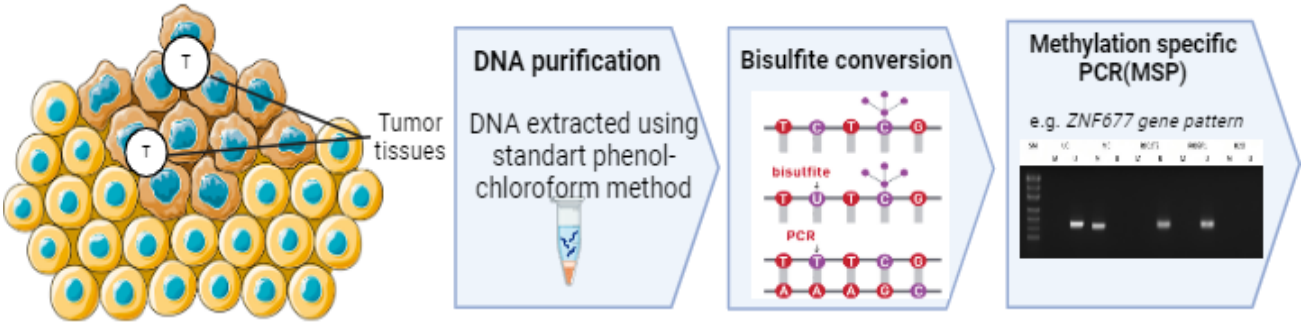


Fig. 2.1. The workflow of present research.

2.1. Patients and samples collection

Approval to conduct biomedical research (Nr. 158200-18/12-1077-585) was obtained from the Lithuanian Bioethics Committee, and written informed consent was provided by all patients.

Human kidney tissue samples were collected from 10 patients; 8 out of 10 were primarily diagnosed with clear cell renal cell carcinoma (ccRCC), and 2 patients were diagnosed with oncocytoma (OCT). Among the 10 patients, 6 were female and 4 were male. In the current research, 40 tissue samples were investigated, in total 20 tumor, 10 peritumor, and 10 non-tumor samples (10 of the 20 tumor and 10 non-tumor samples were previously examined). After the neoplastic kidney tissue was surgically removed, that was stored at -80°C. Tumors were categorized based on pathological stage and histological subtype, and nuclear differentiation was graded according to the Fuhrman and World Health Organization/International Society of Urological Pathology grading systems. The median age of patients was 62.9. The necrotic zone was detected in just one tissue sample. Detailed information on these patients is summarized in **Table 2.1.**

Table 2.1. Clinicopathological characteristics of the patients.

Patient's ID	Age	pT stage	Histology	Tumor size, mm	Fuhrman grade	PSO/ISUP grade	Intravascular invasion	Lymphovascular invasion	Fat invasion
2T	27	pT1a	ccRCC	28	2	2	No	No	No
3T	62	pT2a	ccRCC	75	3	3	No	No	No
4T	66	pT2a	ccRCC	73	3	3	No	No	No
5T	71	pT3a	ccRCC	50	2	2	Yes	No	No
6T	75	pT1a	OCT	34	No	No	No	No	No
7T	51	pT1b	ccRCC	42	3	3	No	No	No
8T	73	pT1b	ccRCC	50	3	2	No	No	No
9T	55	pT1b	OCT	45	No	No	Yes	No	No
10T	75	pT1b	ccRCC	45	2	2	No	No	No
13T	74	pT3a	ccRCC	130	3	3	Yes	Yes	Yes

2.2. DNA extraction and bisulfite conversion

In the laboratory settings, renal tissue samples were homogenized in liquid nitrogen. Homogenate tissue powder was digested for up to 18 h at 55 °C with 10-25 µl of proteinase K (Thermo Scientific™, Thermo Fisher Scientific, Wilmington, DE, USA) and 500 µl of lysis buffer, consisting of 50 mM Tris-HCl pH 8.5, 1 mM EDTA, 0.5% Tween-20 (all from Carl Roth, Karlsruhe, Germany). Reliable and conventional techniques such as phenol-chloroform purification and ethanol precipitation were used for DNA extraction.

The purity and concentration of the extracted DNA were analyzed by NanoDrop™ 2000 spectrophotometer (Thermo Scientific™, Thermo Fisher Scientific, Wilmington, DE, USA).

In the targeted DNA methylation analysis, modification with sodium-bisulfite was performed by EZ DNA Methylation™ Kit (Zymo Research, Irvine, CA, USA) using up to 400 ng of purified DNA and following the manufacturer's instructions, except for the initial incubation of samples at 42°C for 15 min as detected better results compared to 37 °C.

2.3. Targeted methylation analysis by methylation-specific PCR

After DNA is bisulfite modified, it is amplified using two distinct primer pairs: methylated (M-reaction) and unmethylated (U-reaction) specific primers. This method is called Methylation-Specific PCR (MSP). MSP is used to analyze the status of regulatory-sequences methylation of *ADAMTS19, FBN2, FLRT2, PCDH8, SFRP1, ZNF677, TAC1,* and *TFAP2B* genes. DNA methylation data were obtained from tissue and urine samples. The particular data in the urine samples were obtained previously in the laboratory using quantitative methylation-specific PCR (qMSP). The sodium bisulfite-treated DNA served as a substrate for MSP. Primers specific for methylated and unmethylated DNA were designed with Methyl Primer Express® Software v1.0 (Applied

Biosystems™, Thermo Fisher Scientific, Carlsbad, CA, USA) and procured from Metabion (Martinsried, Germany). Each sample's DNA was amplified in two separate tubes, with the only difference being the primers used. The reaction is carried out in a volume of 25 µL. The components of the reaction mixture and their respective quantities per reaction are provided in **Table 2.2**.

Table 2.2. The reaction mixture for methylation-specific PCR.

No.	Component	Per reaction, µL
1	Nuclease-free water (H2O)	15
2	10×PCR buffer	2.5
3	25mM MgCl2	2.5
4	16 mM dNTP	2.5
5	Primers (s + as)	0.125 + 0.125
6	GC Enhancer	1
7	DNA Polymerase	0.25
TOTAL MIX VOLUME		**24.0**

For reactions M and U, the mixtures are prepared individually, thoroughly combined, and then dispensed into 24 µL aliquots, with 1 µL of modified DNA added to each tube. The prepared samples are centrifuged at 13000 rpm for 1 min and placed in an Eppendorf Mastercycler®pro* thermocycler. All runs for each primer pair included a methylated control (*in vitro* fully methylated human leukocyte DNA, MC), an unmethylated control (human leukocyte DNA, UC), and a no-template control (NTC). Prior to the study, thermocycling conditions were altered as necessary and comprised 10 min at 95 °C, 35-38 cycles of 45 s at 95 °C, and primer annealing for 45 s at 58-63 °C (**Table 2.3 and Table 2.4**).

Table 2.3. The thermal cycling conditions of MSP

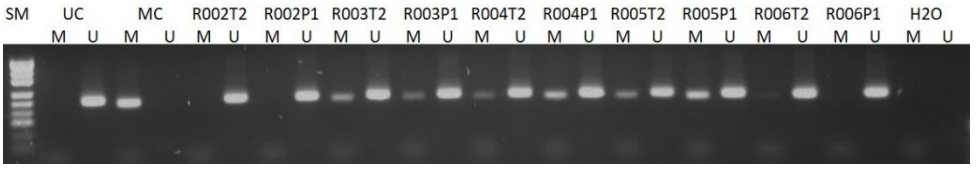
Step	Temperature	Time	Number of MSP cycles
Polymerase activation	95 °C	10 min	1
Initial denaturation	95 °C	45 s	According to gene
Primers annealing	According to gene	45 s	
Primers extension	72 °C	45 s	
Final extension	72 °C	10 min	1
Storage	4 °C	∞	1

Table 2.4. Amplification conditions of each selected gene

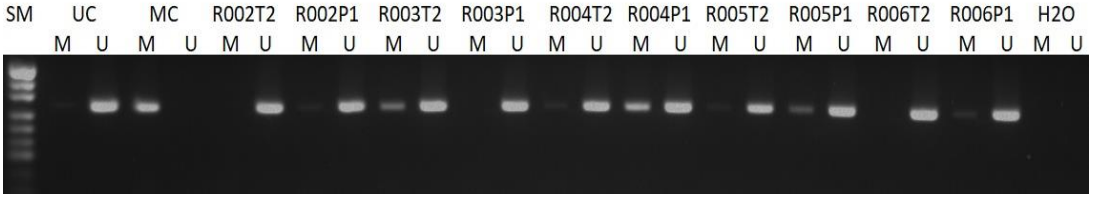
Gene name	Primers annealing temperature	Number of MSP cycles
ZNF677	58 °C	37
FBN2	58 °C	37
PCDH8	58 °C	37
TFAP2B	58 °C	35
FLRT2	63 °C	38
ADAMTS19	58 °C	36
SFRP1	58 °C	36

The reaction products were analyzed on 3% agarose gels (3.6 g of agarose dissolved in 120 mL of 1× TAE buffer) and visualized under UV light after adding 7 µL of ethidium bromide (10 mg/mL)) (Carl Roth GmbH, Co., KG). 2 µL of pUC19 DNA/MspI (HpaII) Marker, 23, ready-to-use (Thermo Scientific) is added to the first well. The products was fractionated in a 90 V electric field for 40 min. An example of MSP analysis results is shown in **Figure 2.2**. The imaging of the gel is accomplished using the GelDoc-It™ 310 Imaging System from UVP. These steps were performed by my lab colleagues.

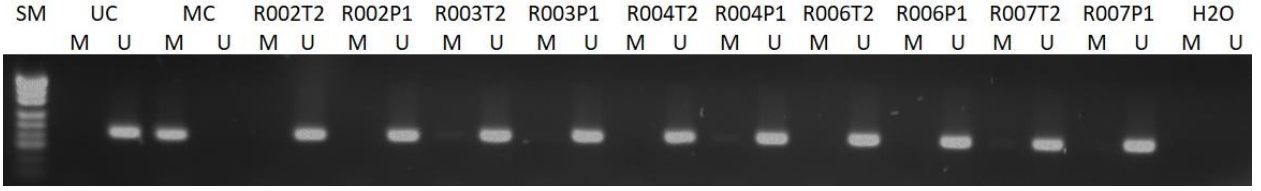
A) *FBN2*



B) *FLRT2*



C) *ADAMTS19*



D) *TFAP2B*

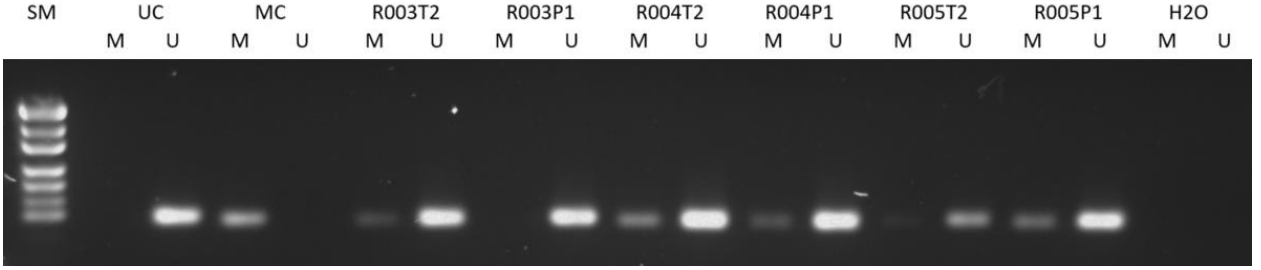


Fig. 2.2. An example of methylation-specific PCR results for some selected genes. The specific gene is indicated in the above. SM – DNA size marker (26-501 bp), UC – unmethylated control, MC – methylated control samples, R000T– samples of renal tumor tissue, R000P- samples of the peritumor tissue, NTC – no template control, M/U – amplification products with primers specific for methylated/unmethylated DNA.

Individual biomarkers were classified as methylated when the sample detected amplification products using primers designed for methylated DNA. Conversely, a biomarker was categorized as

unmethylated if amplification products were detected only with primers designed for unmethylated DNA, and no products with primers specific for methylated DNA were observed.

2.4. Statistical analysis

Statistical analyses were performed using Excel Software (Microsoft, Redmond, WA) and STATISTICA™ v8.0 (StatSoft, Tulsa, OK, USA). The statistical significance level was accepted as $P<0.05$.

Data visualization was performed using GraphPad Prism v8 software (GraphPad Software, Inc., La Jolla, CA, USA).

The heterogeneity index (HI) was determined as a proportion of differentially methylated paired foci for each gene. If a gene exhibits methylation in one locus of tumor tissue while being unmethylated in another, the identification of such discrepancy indicates the presence of heterogeneity in the methylation pattern of that gene. If the heterogeneity of the particular gene was found in 1 out of 10 patients, the heterogeneity was accepted as 0.1. value and so on.

3. RESULTS

The methylation patterns in different renal tumor foci and peritumor tissues were analyzed for *ADAMTS19, FBN2, FLRT2, PCDH8, SFRP1, ZNF677, TAC1,* and *TFAP2B* **(Table 3.1)**. The selection of these genes was based on previous research conducted in a particular laboratory, and the methylation frequency in these genes was found to be notably higher in tumor samples than in non-tumor samples, and all genes demonstrated statistical significance (P<0.050) (**Figure 3.1**). However, the current study determines the methylation differences of these genes in different tumor foci and peritumor tissue samples.

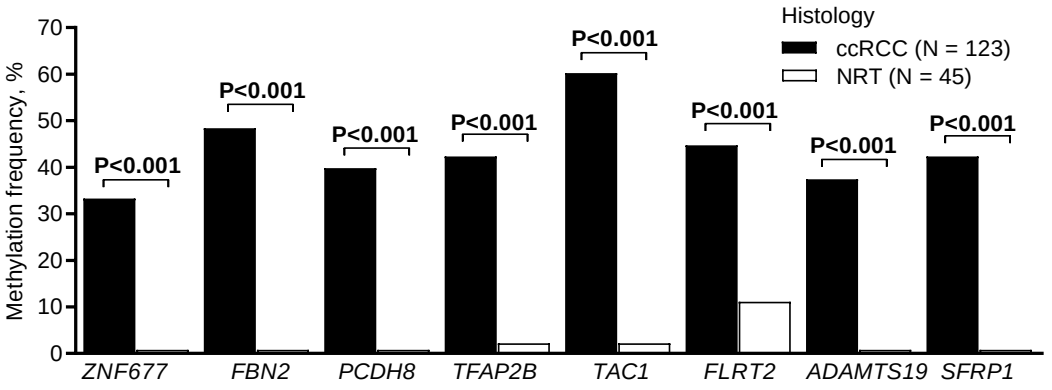


Fig. 3.1. Methylation frequencies of the investigated genes in renal tissues. The results were obtained by qualitative methylation-specific PCR (MSP). ccRCC – clear cell renal cell carcinoma, NRT – noncancerous renal tissues. Significant P-values are in bold. (Results were obtained prior study).

Table 3.1. Genes are selected for methylation analysis.

Gene symbol	Gene name	Chromosomal location(strand)	Relevance to cancer
ADAMTS19	ADAM metallopeptidase with thrombospondin type 1 motif 19	5q23.3 (+)	Cell migration, proliferation
FBN2	fibrillin 2	5q23.3 (-)	Invasion, tumor metastasis
FLRT2	fibronectin leucine-rich transmembrane protein 2	14q31.3 (+)	Cell adhesion, invasion
PCDH8	protocadherin 8	13q14.3 (-)	Cell adhesion, EMT
SFRP1	Secreted frizzled-related protein 1	8p11.21 (-)	WNT pathway dysregulation
ZNF677	zinc finger protein 677	19q13.4 (-)	Transcriptional regulation
TAC1	tachykinin precursor 1	7q21.3 (+)	Inflammatory response
TFAP2B	transcription factor AP-2 beta	6p12.3 (+)	Transcriptional regulation

EMT – epithelial-mesenchymal transition; WNT – Wingless and Int-1.

3.1. Targeted DNA methylation analysis of the selected genes in the renal tissue samples

The DNA methylation status of *ADAMTS19*, *FBN2*, *FLRT2*, *PCDH8*, *SFRP1*, *ZNF677*, *TAC1*, and *TFAP2B* was analyzed qualitatively at regulatory regions of the genes in 10 pairs of multifocal tumor, 10 peritumor, and 10 non-tumor samples. The paired tumor tissue samples were obtained from different parts of the renal tumor. The methylation status of *FBN2*, *PCDH8*, *ZNF677*, and *TAC1* varied from 20% to 50% in two randomly selected tumor foci (**Figure 3.2**). The variability in methylation frequencies of *ADAMT19* and *SFRP1* was undetermined between two tumor foci The variability of methylation frequency of *FLRT2* was 20% in one foci and 10% in the other. The *TFAP2B* gene exhibited the same methylation pattern in both tumor foci (30%). *TAC1* and *ZNF677* were observed as the highest level of methylation (50%) in peritumor tissue. *FBN2*, *PCDH8*, *TFAP2B*, *SFRP1*, and *FLRT2* exhibited a moderate methylation frequencies (10%-40%) in peritumoral tissue, whereas *ADAMTS19* showed no methylation pattern (0%).

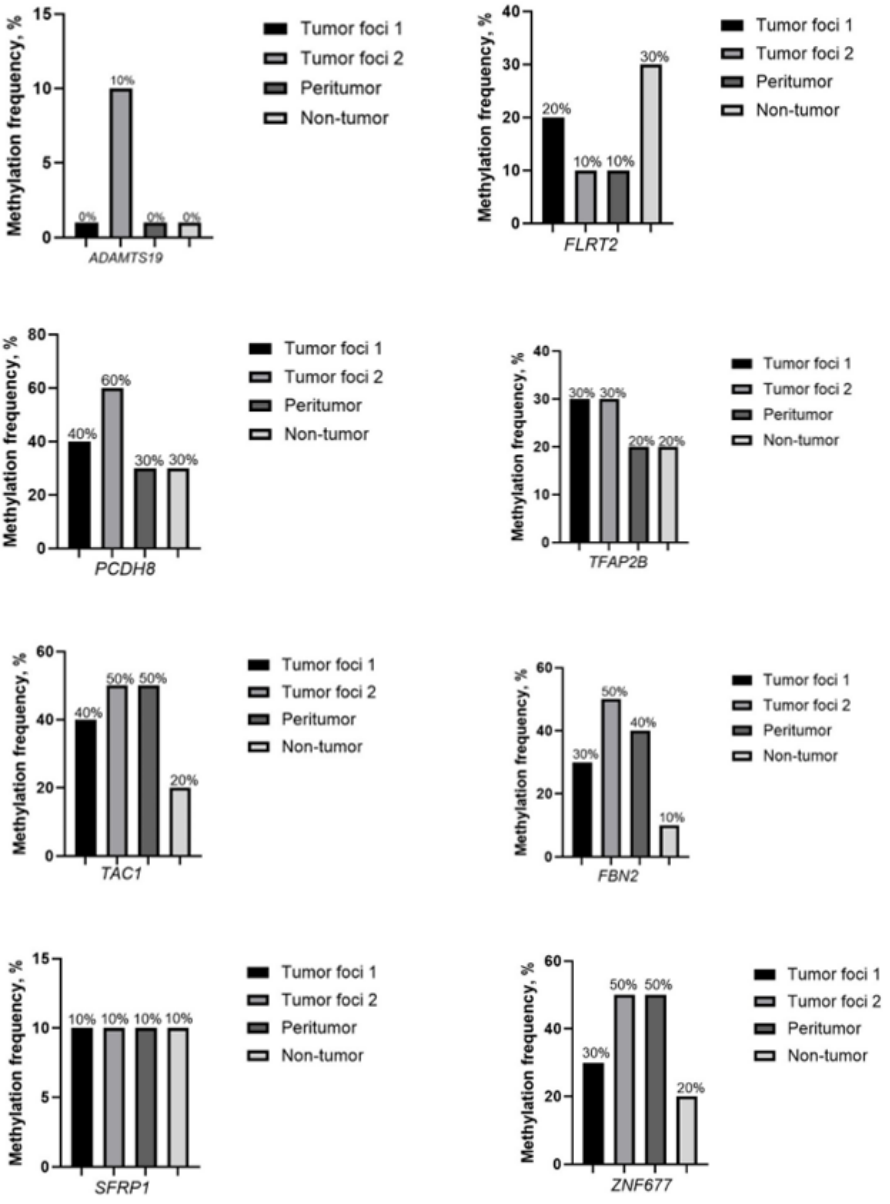


Fig. 3.2. Methylation frequencies of selected genes in two RCC tumor foci, peritumor and non-tumor samples.

3.1.2. Tumor Heterogeneity Index of Tissue Samples

The heterogeneity index (HI) for the *TAC1* was 0.5 (**Figure 3.3**), however for the *FBN2*, *ZNF677*, *SFRP1*, *FLRT2*, *ADAMTS19*, and *PCDH8*, it ranged from 0.1-0.2. Only interfocal heterogeneity was undetected in the *TFAP2B*.

Patient ID	*FBN2*		*FLRT2*		*ADAMTS19*		*PCDH8*		*TFAP2B*		*TAC1*		*ZNF677*		*SFRP1*	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
R002T								■								
R003T	■	■	■	■				■	■	■	■			■		■
R004T		■					■	■	■	■	■	■	■	■		
R005T	■	■					■	■	■	■		■	■	■		
R006T							■	■				■				
R007T												■				
R008T											■	■		■	■	
R009T																
R010T		■				■					■					
R013T	■	■	■				■	■					■	■		
HI	0.2		0.1		0.1		0.2		0		0.5		0.2		0.2	

Fig. 3.3. Methylation status variability in tumor tissue samples. Pink color indicates methylated status of particular gene and white indicates unmethylated status. HI-heterogeneity index. **a, b** Randomly coded paired tumor foci.

3.1.3. Comparison of the methylation status of selected genes in tissue and urine samples

Based on the previous observation in the particular laboratory, we compared the DNA yOmethylation status of *ZNF677, FBN2, PCDH8, TFAP2B, TAC1,* and *FLRT2* genes in both tissue and urine samples. Overall, all cancer patients were methylation positive in urine sediment DNA for at least one of the 6 genes tested **(Figure 3.4**).

Patients	*ZNF677*		*FBN2*		*PCDH8*		*TFAP2B*		*TAC1*		*FLRT2*	
	T	U	T	U	T	U	T	U	T	U	T	U
2T		■			■							
3T	■	■	■		■		■		■		■	
4T	■	■	■	■	■		■		■			
5T		■	■		■	■	■		■	■	■	■
6T		■			■		■		■		■	■
7T									■			
8T	■	■		■				■	■	■		
9T	■							■				
10T	■		■			■		■				
13T	■	■	■	■	■				■		■	

Fig. 3.4. Summary of the methylation states of *ZNF677*, *FBN2*, *PCDH8*, *TFAP2B*, *TAC1,* and *FLRT2* in tissue (*T*) and matched urine (*U*) samples. Pink boxes represent methylated samples; white boxes represent unmethylated samples.

According to results, *ZNF677* is found to be more methylated in urine compared to renal tissue samples. However, *FBN2*, *PCDH8*, *FLRT2*, and *TAC1* exhibited higher methylation patterns in tissue samples than in urine sediments. Furthermore, no identical methylation status were observed in the *TFAP2B* gene between the tissue and matched urine DNA samples in the same patients.

4. DISCUSSION OF RESULTS

Renal cell carcinoma (RCC) is the second most prevalent urologic neoplasm in both genders, accounting for approximately 5% of adult cancer cases in men and 3% in women (Vig et al., 2020). The majority of patients with RCC are asymptomatic in the initial stages when the disease is detected incidentally on imaging studies performed for unrelated conditions. Only about 10% of patients diagnosed with RCC present with the typical symptoms, including flank pain, erythrocyturia, and palpable mass in the abdominal. The presence of fatigue, fever, weight loss, and classic symptoms may indicate metastasis (Pullen, 2021).

RCC presents with multiple and distinct cancer cell populations within tumors, which contribute to intratumor heterogeneity (ITH). ITH and specific genetic/epigenetic alterations play a role in poor prognosis, evident as RCC progresses to an advanced stage. Changes in the patterns of epigenetic modifications, particularly DNA methylation can introduce more complexity to tumor heterogeneity. Therefore, it is difficult to identify reliable biomarkers for RCC (El Khoury et al., 2023).

On the other hand, a variety of DNA methylation biomarkers have been shown to detect RCC in tissues and urine. These biomarkers offer better sensitivity and specificity, low invasiveness and risk assessment compared to typical molecular and biochemical tests for RCC (Kashyap et al., 2005; Kubiliute & Jarmalaite, 2021; Gupta & Kanwar, 2023). Consequently, they can be utilized to monitor treatment response and the efficacy of various treatment options (Tanvir et al., n.d.; Lommen et al., 2021).

The novelty of our research is the analysis of methylation in second tumor foci and peritumoral tissue samples in selected genes that are considered as potential biomarkers for renal cancer. We identified considerable variance in methylation patterns of almost all investigated genes between two different separate foci of RCC and peritumoral samples.

While *ZNF677* and *TAC1* were detected more frequently methylated in peritumor tissue (50%) and was the same as the tumor foci 2 (50%). While the exact mechanisms by which *ZNF677* contributes to RCC progression are still being elucidated, some studies suggested that aberrant methylation of the *ZNF677* gene has been implicated in the development of RCC (Li et al., 2022). As has been found in previous studies of other carcinomas, such as squamous cell carcinoma of the lungs (Du et al., 2024), we found that peritumor tissue exhibits approximately the same DNA methylation patterns as in distinct two tumor foci in RCC. These alterations in peritumoral tissues may create a microenvironment that promotes the proliferation and recurrence of cancer. However, the main mechanisms underlying the impact of peritumoral tissue on renal carcinogenesis have not yet been fully elucidated.

One of the demonstrating methylation heterogeneity genes, *FBN2* has been shown in genome-wide methylation analysis and was found to be hypermethylated in RCC (Morris et al., 2011). By confirming this result, our data showed that *FBN2* hypermethylation remarkably in two tumor foci and peritumor samples. This suggests that could be an early and common epigenetic modification in the development of RCC. Interestingly, *PCDH8* methylation patterns in peritumor and non-tumor samples were the same, and the theory of field cancerization provides a possible explanation for these results. Field carcinogenesis is associated with the presence of specific preneoplastic genetic and epigenetic markers in normal tissue adjacent to the tumor, which favors its progression to second primary tumors (Bansal et al., 2020). The low detection of methylation events in *ADAMTS19*, *SFRP1*, and *FLRT2* genes may suggest that these are not the primary "driving" epigenetic changes in RCC carcinogenesis. Their methylation may be more of a "passenger" event rather than playing a critical functional role in tumor progression (Hoffman & Cairns, 2011; Sawan et al., 2008).

The inconsistent methylation patterns observed in tissue and urine samples are controversial and may be related to several factors. The most plausible reason may be due to the heterogeneity of the renal tumor, and selected foci in the tumor tissue may not fully reflect this phenomenon, whereas the urine samples may provide a more comprehensive representation of the genomic/epigenomic landscape (Costa et al., 2010; Xin et al., 2016). In addition, the qMSP method was used for urine samples, and MSP was used for tissue samples, and since the sensitivity and specificity of these techniques are different, this may cause distinctions (Daniūnaitė et al., 2019). Nevertheless, investigation of methylation profiles in renal cancer is of great importance for recognizing potential epigenetic alterations that contribute to tumor development and progression at an early stage.

In summary, the aforementioned finding is further supported by the fact that the methylation status of selected genes, particularly *ZNF677, ADAMTS19, TAC1, SFRP1, TFAP2B, PCDH8, FLRT2,* and *FBN2* exhibited variable levels of DNA methylation in RCC tumor paired foci and peritumor samples. Variability in methylation patterns among different tumor foci underscores the heterogeneity of RCC biology and challenges the potential utility of DNA methylation biomarkers for RCC diagnosis and prognosis in tumor tissue samples. It is essential to confirm the study findings on a considerable number of clinical samples. Further research is needed to elucidate the molecular mechanisms underlying these alterations and their clinical implications.

CONCLUSIONS

1. *ZNF677*, *FBN2*, and *PCDH8* demonstrate a 20% and *TAC1*, *FLRT2* 10% difference in methylation frequency between the two randomly selected tumor foci. The methylation frequency of all investigated genes (except of *ADAMTS19*) in peritumor samples ranged from 10 to 50%.

2. The renal tumor heterogeneity index ranged from 0.1 to 0.2 for the *ZNF677*, *SFRP1*, *FLRT2*, *ADAMTS19*, *PCDH8,* and *FBN2* genes while for *TAC1*, it was detected 0.5. *TFAP2B* demonstrated no interfocal heterogeneity.

3. In all investigated genes, methylation differences at the regulatory regions of selected genes were observed between tissue and urine samples of the same patients.

PERSONAL CONTRIBUTION

I conducted a comprehensive literature analysis relevant to the research problem and performed detailed calculations on the obtained results. Additionally, I contributed to the preparation of figures and tables illustrating key findings from our analyses. I wrote the results and discussion sections, synthesizing experimental results with relevant literature and proposing future research directions in renal cancer epigenetics.

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I extend my sincere appreciation to the all the patients who participated in this research study. Their willingness to provide informed consent and generously share their time, experiences, and personal information has been essential to the success of this project. Without their cooperation and dedication, this study would not have been possible.

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