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Origination of the concept of targeted, anti-evolutionary cancer therapy and identification of potential molecular targets for such therapy against clear cell renal cell carcinoma

PhD dissertation in medical sciences

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1. Abstract

In contrast to organismal evolution, human cancers are subjected to similar initial conditions and follow a limited range of possible evolutionary trajectories. Until now, the predictable patterns of how cancer progresses have not been utilized for therapeutic benefits.

Evolutionary trajectories of clear cell renal cell carcinoma (ccRCC) have been recently described. I proposed strategies to take advantage of the evolving nature of these tumors for patients' benefit.

One of these strategies is to modulate tumor's genomic instability. In search for the best candidates for molecular targeting, I identified two proteins, TRIP13 and KIF11, and explored the relationships between their expressions and clinical course of ccRCC using the tissue microarrays (TMAs).

The TMAs contained specimens from 90 patients followed up for 7 years. All the tumor samples were evaluated for TRIP13 and KIF11 expression using immunohistochemistry and the H-score method. The overall survival (OS) was analyzed using the Kaplan-Meier method and log-rank statistics. Univariate and multivariate analyses were conducted using Cox proportional hazard models.

Cytoplasmic expressions of TRIP13 and KIF11 in ccRCC tissues were lower than those in adjacent controls ($P < 0.05$). I dichotomized the cytoplasmic expressions of these proteins to low and high expression using the tool Cutoff Finder. Both the elevated expressions of TRIP13 and KIF11 served as independent unfavorable prognostic indicators of survival in ccRCC ($P < 0.05$).

Elevated expressions of TRIP13 and KIF11 predict poor clinical outcome in ccRCC patients. Our results may serve as a starting point for translational research, in which the modulation of TRIP13 and KIF11 expressions could provide new therapeutic strategies for ccRCC.

1. Streszczenie

W przeciwieństwie do ewolucji organizmów, nowotwory podlegają podobnym początkowym warunkom i podążają ograniczonym zakresem możliwych trajektorii ewolucyjnych. Do tej pory przewidywalne wzorce rozwoju nowotworu nie były wykorzystywane do uzyskania korzyści terapeutycznych.

Niedawno opisano trajektorie ewolucyjne raka jasnokomórkowego nerki (ccRCC). Zaproponowałem strategie wykorzystania ewoluującego charakteru tych nowotworów z potencjalną korzyścią dla pacjentów.

Jedną z tych strategii jest modulowanie niestabilności genomowej guza. W poszukiwaniu najlepszych kandydatów do celowania molekularnego zidentyfikowałem dwa białka, TRIP13 i KIF11, i zbadałem zależności między ich ekspresją a przebiegiem klinicznym ccRCC przy użyciu mikromacierzy tkankowych (TMA).

TMA zawierały próbki od 90 pacjentów, których obserwowano przez 7 lat. Wszystkie próbki guza oceniłem pod kątem ekspresji TRIP13 i KIF11 przy użyciu immunohistochemii i metody H-score. Całkowite przeżycie (OS) zanalizowałem przy użyciu metody Kaplana-Meiera i statystyki log-rank. Analizy jednowymiarowe i wieloczynnikowe przeprowadziłem przy użyciu modeli proporcjonalnego hazardu Coxa.

Cytoplazmatyczne ekspresje białek TRIP13 i KIF11 w tkankach ccRCC były niższe niż w sąsiednich kontrolach ($P < 0,05$). Podzieliliśmy cytoplazmatyczne ekspresje tych białek na niską i wysoką ekspresję za pomocą narzędzia Cutoff Finder. Zarówno podwyższone/a ekspresje/a TRIP13, jak i KIF11 służyły jako niezależne niekorzystne wskaźniki prognostyczne przeżycia w ccRCC ($P < 0,05$).

Podwyższone ekspresje TRIP13 i KIF11 przewidują złe wyniki kliniczne u pacjentów z ccRCC. Nasze wyniki mogą służyć jako punkt wyjścia do badań translacyjnych, w których modulacja ekspresji TRIP13 i KIF11 może zapewnić nowe strategie terapeutyczne dla ccRCC.

2. Abbreviations.

ccRCC	clear cell renal cell carcinoma
HR	homologous recombination
ITH	intratumor heterogeneity
NHEJ	non-homologous end joining
DDR	DNA damage response
PARP	poly(ADP)-ribose polymerase
OS	overall survival
SAC	spindle assembly checkpoint
DSBs	double-strand breaks
TMA	tissue microarray
wGII	weighted genome instability index
CIN	chromosomal instability
HDACi	histone deacetylase inhibitor

3. Introduction.

3.1. *Key cancer statistics and projections.*

In 2020, there were an estimated 19.3 million new cases of cancer and 10 million deaths from cancer worldwide. This accounted for 1 out of every 6 deaths. Breast cancer was the most commonly diagnosed cancer, with 2.3 million new cases, followed by lung, colorectal, prostate, and stomach cancers. Lung cancer was also the leading cause of cancer deaths, with 1.8 million deaths. The incidence of cancer was higher in transitioning countries compared to transitioned countries, but the mortality rate varied less. The global cancer burden is expected to increase by 47% by 2040, with a larger increase in transitioning countries due to demographic changes and increasing risk factors associated with globalization and economic growth [1].

3.2. *Kidney cancer and its exceptional resistance to systemic therapies.*

Kidney cancer is a prevalent form of cancer that is becoming more common. In 2022, it is estimated that 79,000 new cases and 13,920 deaths from kidney cancer will occur in the United States alone, and globally there will be over 400,000 new cases. It is among the 10 most common types of cancer for both men and women [2].

In only 10% of cases, kidney cancer presents with the typical symptoms of a mass on the flank, pain in the flank area and blood in the urine, which is known as the "classic triad" of symptoms [3]. Due to the kidney's ability to function even when part of it is damaged, it is usually not possible to detect kidney cancer early based on loss of function. This means that the cancer often goes unnoticed for a long time and is often not diagnosed until it has spread to other parts of the body. About one-third of patients are diagnosed with metastatic disease at the time of diagnosis. Even when the tumor is found early and removed surgically, there is still a significant chance (40%) of the cancer returning [4, 5].

The most well-researched type of kidney cancer is clear cell renal cell carcinoma (ccRCC), which starts in the lining of the proximal convoluted tubule and makes up 70% of all kidney cancer cases [6].

In the past two decades, there have been significant advances in the treatment of unresectable kidney cancer, including the development and improvement of targeted and immunotherapies that are now considered the standard of care. However, despite the relatively high response rates to these treatments, only a small percentage (up to 9%) of patients with poor- or intermediate-risk cancer may achieve a complete response, according to the results of a subgroup analysis of the CheckMate 214 clinical trial [7]. Most patients with metastatic ccRCC ultimately succumb to cancer progression within 1.5 years [8].

4. Aims of the doctoral thesis.

I. To propose a novel approach to cancer therapy on the example of kidney cancer.

II. To identify novel therapeutic targets for kidney cancer therapy.

5. Publications included in the dissertation.

5.1. ***Targeting the deterministic evolutionary trajectories of clear cell renal cell carcinoma.***

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Review

Targeting the Deterministic Evolutionary Trajectories of Clear Cell Renal Cell Carcinoma

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Simple Summary: In contrast to organismal evolution, human cancers are subjected to similar initial conditions and follow a limited range of possible evolutionary trajectories. Therefore, the repetitive nature of cancer evolution may prove to be its greatest weakness. Evolutionary trajectories of clear cell renal cell carcinoma (ccRCC) have been recently described. In this review, we will discuss the relevance of estimating the trajectory of ccRCC evolution as a readout for a response to therapy. Next, we will propose strategies to take advantage of the evolving nature of these tumors for patients' benefit.

Abstract: The emergence of clinical resistance to currently available systemic therapies forces us to rethink our approach to clear cell renal cell carcinoma (ccRCC). The ability to influence ccRCC evolution by inhibiting processes that propel it or manipulating its course may be an adequate strategy. There are seven deterministic evolutionary trajectories of ccRCC, which correlate with clinical phenotypes. We suspect that each trajectory has its own unique weaknesses that could be exploited. In this review, we have summarized recent advances in the treatment of ccRCC and demonstrated how to improve systemic therapies from the evolutionary perspective. Since there are only a few evolutionary trajectories in ccRCC, it appears feasible to use them as potential biomarkers for guiding intervention and surveillance. We believe that the presented patient stratification could help predict future steps of malignant progression, thereby informing optimal and personalized clinical decisions.

Keywords: clear cell renal cell carcinoma; ccRCC; RCC; kidney cancer; evolution; evolutionary trajectory; biomarker

1. Introduction

Renal cell carcinoma (RCC) is the eighth most commonly diagnosed cancer in the United States, with an estimated incidence of 74,000 new cases in 2020 [1]. The classic triad of flank pain, flank mass, and hematuria occurs only in 10% of cases [2]. Due to the ability of the kidney for functional compensation when part of it is destroyed, early detection from loss of function is usually impossible. As a result, RCC remains clinically occult for most of its course, and around one-third of patients present with metastatic disease at the time of diagnosis. Those with localized tumors have up to 40% risk of recurrence following complete resection [3,4]. Remarkable advances over the last decade contributed to the development of targeted therapies and immunotherapies that today represent a standard for unresectable RCC. Despite relatively high response rates to these agents, the vast majority of patients eventually experience cancer progression. The emergence of clinical resistance to currently

available systemic therapies represents a significant challenge and forces us to rethink our approach to RCC.

The best-studied histological subtype is clear cell renal cell carcinoma (ccRCC), which is derived from the proximal convoluted tubule and accounts for approximately 70% of all cases [5]. A series of next-generation sequencing studies led to a better understanding of the genetic background of ccRCC [6–10]. The results of these studies uncovered a near-universal inactivation of the von Hippel-Lindau disease (*VHL*) tumor suppressor gene. Other frequent alterations involve histone-modifying genes, SWI/SNF complex, and PI3K/AKT/mTOR pathway. Moreover, an integrated, genome-wide analysis of copy-number changes and gene expression profiles in ccRCC identified 7 chromosomal regions of recurrent arm level or focal amplifications (1q, 2q, 5q, 7q, 8q, 12p, and 20q) and 7 regions of losses (1p, 3p, 4q, 6q, 8p, 9p, and 14q) [8].

The evolutionary landscape in ccRCC is dominated by intratumor heterogeneity (ITH) at a genetic, transcriptomic, and functional level [9]. The exome sequencing performed on multiple, spatially separate ccRCC samples revealed that that two-thirds of the somatic mutations are not shared between all the primary tumor regions [10]. Hence, single-biopsy analysis is likely to miss the key genetic events or misclassify them as clonal. Apart from the direct impact on diagnostic procedures and biomarkers development, ITH has significantly hindered our understanding of ccRCC evolution.

In comparison to other malignancies, ccRCC is characterized by a high prevalence of somatic copy number alterations (SCNAs) and a low burden of somatic substitutions [6,8,11,12]. The integrative analysis of the genetic and clinical data led to the identification of certain alterations with prognostic value, such as mutually exclusive mutations of *BAP1* and *PBRM1* [13–15]. These studies, although conducted on large cohorts of patients, did not determine the prognostic values of genetic alterations according to whether they were clonal or subclonal. Huang et al. were among the first to demonstrate the possibility of genomic subtyping of ccRCC [13]. Recently, Turajlic and colleagues provided a comprehensive model of ccRCC evolution [14], which might lay the foundation for the development of precision clinical management.

Cancer cells continuously undergo adaptive changes, and insensitivity to drugs arises due to genetic and epigenetic alterations that offer a survival advantage. While there is a number of pathways and networks a cancer cell has at its disposal, targeting individual components is likely to prove inadequate [15]. Instead, the ability to influence cancer evolution itself by inhibiting processes that propel it or manipulating its course might potentially put an end to cancer as a major health concern.

In this review, we will discuss the relevance of estimating the trajectory of ccRCC evolution as a readout for a response to therapy. Next, we will propose strategies to take advantage of the evolving nature of these tumors for patients' benefit.

2. The Origin, Evolution, and Routes to Metastasis of Clear Cell Renal Cell Carcinoma

2.1. The Origin of Clear Cell Renal Cell Carcinoma

Loss of the short arm of chromosome 3 is a nearly universal driver of ccRCC [16]. It occurs in childhood or adolescence, predominantly through chromothripsis. The deleted region encompasses at least four tumor suppressor genes, including *VHL*, *PBRM1*, *BAP1*, and *SETD2.* This earliest event produces a pool of a few hundred cells, which after decades of modest clonal expansion, acquire the necessary additional genetic alterations [17]. Chromosomal copies of deleted suppressor genes are often affected afterward, with inactivation of the second allele of *VHL* being the most common (65–80% of patients) [7,8,10]. In some cases, there are different driver mutations on the trunk of the phylogenetic tree, which, in contrast to 3p loss and *VHL* inactivation, trigger a substantial expansion [11,18].

2.2. The Evolutionary Trajectories of Clear Cell Renal Cell Carcinoma

On the basis of mutational ordering, timing, and co-occurrence, ccRCCs are classified into seven distinct evolutionary subtypes, or four groups, which correlate with clinical phenotypes [17,19].

These groups are distinguished by four features—variations in chromosomal complexity, ITH, model of tumor evolution, and metastatic potential. The variations in chromosomal complexity are measured as the fraction of the genome affected by SCNAs and expressed as a weighted genome instability index (wGII). ITH is measured as the ratio of subclonal drivers to clonal drivers [20].

Group 1 consists of primary tumors with *VHL* alteration as the sole driver event. They evolve in a "linear" fashion and are characterized by low both wGII and ITH. This mode of evolution is associated with indolent growth and low metastatic potential. Group 2 includes tumors in which early *PBRM1* mutation and subsequent *SETD2* mutation or PI3K pathway mutation or acquisition of SCNAs result in a "branched" evolutionary pattern. These are heterogeneous neoplasms with oligometastatic potential and attenuated progression. Clonal acquisition of multiple driver mutations (*VHL* plus ≥2 *BAP1*, *PBRM1*, *SETD2*, or *PTEN*) or the parallel *BAP1* mutation results in "punctuated" evolution. These tumors are characterized by high wGII but low ITH and belong to group 3. Punctuated evolution, driven mostly by high wGII, leads to rapid dissemination and is also observed among *VHL* wild-type tumors, which constitute the fourth group [14].

2.3. The Routes to Metastasis of Clear Cell Renal Cell Carcinoma

Metastasis competence is afforded by chromosome-level alterations that simultaneously affect the expression of hundreds of genes. These alterations provide a permissive genomic background for the selection of hallmark drivers of ccRCC metastasis and the loss of 9p and 14q [20]. Linear and branched evolution modes are analogous to Darwin's phyletic gradualism. On the other hand, punctuated evolution, as in punctuated equilibrium, is associated with rapid speciation events and considerable evolutionary changes. Thus, the acquisition of metastatic competence is far more likely through punctuated evolution.

3. Current Systemic Therapies for Renal Cell Carcinoma

Immunotherapy and/or tyrosine kinase inhibitors (TKI) constitute the standard of care for relapse or stage IV RCC. Appropriate clinical management depends on disease activity, according to the National Comprehensive Cancer Network (NCCN) Guidelines for Kidney Cancer. In favorable-risk patients, first-line treatments include a combination of axitinib plus pembrolizumab or monotherapy with pazopanib or sunitinib. For patients with poor- and intermediate-risk disease, the preferred regimen is ipilimumab with nivolumab or axitinib with pembrolizumab. Moreover, cabozantinib may be considered in a first-line setting, especially in cases with osseous metastatic RCC. Because of the significant toxicity of systemic therapies, a subset of asymptomatic patients with metastatic RCC may benefit from active surveillance.

A major advantage of immunotherapy is its potential to produce complete and durable responses in a subset of patients with advanced cancer, even after discontinuation of the drug. Indeed, despite the non-curative nature of systemic therapy in RCC, up to 9% of poor- and intermediate-risk patients may achieve a complete response, according to the results of subgroup analysis of CheckMate 214 clinical trial [18]. This rate could be further increased by introducing novel treatment modalities as well as better patient selection algorithms.

4. Strategies to Overcome the Evolution of Renal Cell Carcinoma

In the face of selective pressures, subpopulations of tumor cells with adaptive phenotypes emerge at the expense of others. The ability to predict the alterations in ITH along the temporal axis seems invaluable for the development of personalized therapy. In this section, we will provide a summary of recent strategies against RCC which, when contextualized within an evolutionary framework, could be significantly more effective.

4.1. Cytoreductive Nephrectomy

In select patients with metastatic RCC, primary nephrectomy is performed with cytoreductive intent. Apart from the alleviation of symptoms associated with larger masses, such intervention eliminates the reservoir of phenotypic tumor-cell diversity, minimizing the risk of further metastatic seeding from an evolving primary tumor [19]. While cytoreductive nephrectomy (CN) is associated with a significant risk of perioperative mortality (0–13%) and major complications (3–36%) [21], there is a great need to avoid unnecessary surgery in nonresponders.

Heng et al. examined the role of CN in metastatic RCC patients receiving targeted therapies in a retrospective study of data from the International Metastatic Renal Cell Carcinoma Database Consortium (IMDC). They found that patients with estimated overall survival (OS) of <12 months and those exhibiting fewer than 4 IMDC prognostic factors are not likely to benefit from CN [22]. From that time, several other observational studies demonstrated analogous results [23]. This data, however, must be treated with caution given the significant risk of selection bias inherent to their study designs, which potentially leads to misclassification of patients [24].

The role of CN continues to change amid a rapidly increasing armamentarium of systemic therapies. In the modern immuno-oncology era, CN is still a viable option, but careful patient selection is of paramount importance. The ongoing clinical trials are evaluating the use of deferred CN in patients receiving nivolumab and ipilimumab alone or alongside radiotherapy (NCT03977571, NCT04090710). These studies may help determine the most appropriate indications for CN.

4.2. Adaptive Therapy

In the case of disseminated cancer with no significant probability of cure, patient survival can be maximized if adaptive therapy is introduced. This strategy originates from mathematical models and aims at maintaining a stable tumor burden [25]. When drugs are administered sparingly and in a temporally dynamic fashion, a significant population of treatment-sensitive cells survives. These, due to their competitive advantage, suppress the proliferation of treatment-resistant populations under normal tumor conditions.

Adaptive therapy may play a role in metastatic RCC. Findings from a prospective phase II trial demonstrate active surveillance to be a viable initial strategy in patients with few adverse prognostic features [26]. Results from the SURTIME study, a randomized clinical trial comparing immediate vs deferred CN, revealed that deferred CN is a valid option for patients with the intermediate-risk disease and with general clinical conditions at baseline amenable to undergo surgery [27]. "Treatment-for-stability" may also be represented by an alternative schedule of sunitinib. The standard dosing schedule of sunitinib is 50 mg daily for 4 weeks, followed by 2 weeks off drug (schedule 4/2). However, according to a recent meta-analysis, the administration of sunitinib for 2 weeks followed by 1 week off (schedule 2/1) exhibited lower toxicity and lower rates of treatment discontinuation while maintaining comparable responses [28].

The full potential of adaptive therapy is yet to be witnessed. Frequency-dependent game-theoretic models of tumor evolution have enabled the introduction of three concepts to consider in the pursuit of designing a multi-drug adaptive approach [29]. These ideas focus on entrapping tumor evolution in periodic loops, limiting the evolutionary "absorbing region" reachable by the tumor and determining the optimal timing of drug administration. Each may contribute to the generation of new treatment schedules and comparisons to standards.

4.3. Targeting Trunk Mutations

The ability to target alteration present in all tumor cells is expected to diminish the odds of the escape of clonal branches. As previously described, inactivation of *VHL* constitutes the trunk event in ccRCC development while most of the other driver aberrations are subclonal. Apart from large chromosomal aberrations as in the cytogenetic 3p abnormalities, *VHL* inactivation may be

caused by small deletions affecting the locus, or promoter methylation and epigenetic silencing [30]. pVHL, a *VHL* gene product, is essential in the cell's normal response to ischemic stress. Decreased expression of *VHL* results in the accumulation of hypoxia-inducible factor alpha (HIFα). Among the three known HIFα subunits, HIF2α is thought to be the core ccRCC driver since it upregulates a series of hypoxia-responsive genes [31–33]. The net effect is the activation of various kinase-dependent signaling pathways, such as MAPK/ERK and PI3K/AKT/mTOR [34]. While the most significant targets of *VHL* loss are the production of VEGF and PDGF, HIF2α has been regarded as undruggable for years [35,36]. Eventually, a structure-based design approach led to the identification of PT2385, a first-in-class HIF2α antagonist [37]. In a phase I dose-escalation clinical trial, PT2385 was found to be well-tolerated and demonstrated clinical activity in extensively pretreated ccRCC patients [38]. Its efficacy and safety are currently being evaluated in a phase II trial (NCT03108066). The primary objective of this trial is to assess the overall response rate in patients with VHL disease-associated ccRCC.

According to the mathematical model presented by Bozic et al., in the case of metastatic disease, monotherapy with a targeted agent offers no hope for recovery. Instead, combinations of two or more agents given simultaneously offer a small chance of cure, especially in the absence of cross-resistance mutations [39].

It is worth noting that the aforementioned drugs are directed against downstream effectors of *VHL*, hence, from an evolutionary point of view, there is a potential to better define the molecular target. Nicholson et al. found that inhibiting the cyclin-dependent kinases CDK4 and CDK6 impaired tumor growth in *VHL*-deficient ccRCC regardless of HIF2α dependency [40]. Abemaciclib, a CDK4/6 and PIM1 kinase inhibitor is currently being tested in phase I trial in combination with sunitinib in metastatic RCC (NCT03905889). Another compound that could represent a paradigm shift in targeted treatment is STF-62247. It has been shown to induce potent cytotoxic effects in *VHL*-deficient ccRCC cells, compared to their *VHL* wild-type counterparts [41]. The STF-62247-stimulated synthetic lethality occurs in a HIF-independent manner through autophagy; however, the mechanistic links between *VHL* and autophagy are incompletely understood [42].

4.4. Targeting Cancer Immune Evasion

Tumor cells interact with the immune system in a process called immunoediting, which consists of three phases: elimination, equilibrium, and escape [43]. Most of the tumor cells are destroyed in the first phase. Cells that cannot be eliminated enter the equilibrium in which they are selected through immune cell exhaustion and resistance to immune detection [44]. It is the longest of the three phases and, in ccRCC, manifests as a modest clonal expansion right after the 3p loss. The evolutionary pressure of immune predation may eventually lead to the development of mechanisms to escape immune responses. From that moment, malignant growth proceeds unrestrained. The ultimate goal of immunotherapy is to permanently reverse immune evasion strategies.

Recent phase III clinical trials led to the use of three immunotherapy-based combinations, including pembrolizumab, ipilimumab, and nivolumab, as a front-line for ccRCC [45]. These agents are highly effective, with a few patients achieving a durable complete response. The ongoing phase III clinical trials are currently testing different combinations of a checkpoint inhibitor plus a tyrosine kinase inhibitor (NCT02811861, NCT03937219) or IL-2 derivate (NCT03729245). Earlier phase studies are evaluating the potential of combining PD-1/PD-L1 inhibitors and antibodies directed against LAG-3 (NCT02996110, NCT03849469), TIM-3 (NCT02608268), or ICOS (NCT03693612, NCT03829501). An alternative approach is represented by the use of different cytokines (NCT02799095, NCT03063762) or personalized cancer vaccines (NCT03633110, NCT02950766).

ITH plays an essential role in shaping antitumor immune responses [43,44]. The highly heterogeneous tumors presumably escape immune surveillance because the reactive neoantigens undergo 'dilution' within the tumor, thereby leading to weaker antitumor immunity.

How do specific genomic features of ccRCC influence the clinical benefit from immunotherapy is under investigation. While tumor mutational burden (TMB) potentially increases ITH [46], a small

study on 25 metastatic ccRCCs failed to confirm the association between TMB and response to immunotherapeutics [47]. Miao and colleagues found that truncating mutations in *PBRM1* were associated with significantly extended progression-free survival (PFS) and OS of patients with metastatic ccRCC treated with immune checkpoint inhibitors [48]. The underlying mechanism is probably related to increased sensitivity to T-cell-mediated cytotoxicity of *PBRM1*-mutant tumor cells [49]. This association was confirmed in an independent ccRCC cohort by a post hoc analysis of the CheckMate 025 randomized phase III study [50]. On the other hand, the exploratory analyses from JAVELIN Renal 101 and CheckMate 214 do not support this hypothesis [51,52]. The discrepant results are presumably due to the different populations studied, such as treatment-naïve versus VEGF-refractory [53].

4.5. Modulating Genomic Instability

Genomic instability of cancer cells drives genetic diversity required for the natural selection of adaptive traits, but there is a threshold beyond which cells cannot replicate successfully [54]. Hence, it is tempting to alter (increase or decrease) the frequency of mutations within the cancer genome.

RCC is characterized by a moderate level of genomic instability and the absence of mutations in canonical DNA damage response (DDR) genes, such as *RAD9*, *BRCA1*, or *TP53* [6,49]. As a result, RCC patients are commonly unresponsive to DNA-damaging therapies, such as chemo- or radiotherapy. For that reason, reducing genetic instability could be a more suitable approach. It can be achieved, among others, by constitutive activation of the transforming growth factor β (TGF-β) axis. TGF-β has been shown to inhibit DNA double-strand breaks (DSB) repair mechanisms to heighten the genetic diversity and adaptability of cancer cells [55]. In ccRCC cell cultures, TGF-β enhances proliferative capacity and promotes metastatic growth [56]. Early phase Ib clinical trial (NCT00356460) investigated the use of a monoclonal antibody against TGF-β fresolimumab in RCC patients and showed preliminary evidence of antitumor activity [57].

On the contrary, particular ccRCC driver genes do influence DDR and there is preclinical evidence to support the poly(ADP)-ribose polymerase (PARP) inhibition in *VHL*- or *BAP1*-mutated ccRCC [54,58,59]. Moreover, cells harboring *SETD2* mutation undergo synthetic lethal interaction with WEE1 blockade due to the depletion of nucleotide pools [60]. AZD1775, an experimental inhibitor of WEE1, is currently being evaluated for patients with *SETD2*-deficient tumors, including RCC (NCT03284385).

4.6. Evolutionary Herding

The tumor is less likely to be resistant to multiple drugs simultaneously, hence the combination therapy allows for the extermination of resistant cells before the emergence of further adaptive mechanisms. However, the use of two or more drugs simultaneously is strictly limited by the toxicity to normal tissues.

While checkpoint inhibitor and the antiangiogenic combination is a standard of care for metastatic ccRCC, there is a significant overlap in the toxicity profile of these drugs, with diarrhea, hypertension, and hepatotoxicity being among the most commonly presented [55,61]. These and other adverse effects may all contribute to treatment discontinuation or dose reduction. Moreover, there is frequently a need for additional medications, such as loperamide secondary to axitinib or high-dose corticosteroids for autoimmune colitis and hepatitis in case of checkpoint inhibitors. Then, drug–drug interactions become even harder to predict. Despite the toxicity issue, in most cases, cancer cells eventually develop multidrug resistance.

Any biological adaptation often involves trade-offs. In cancers, the cost of one resistance mechanism is likely to induce a population to be sensitive to an alternative therapy [62]. Evolutionary herding exploits this weakness by administering a combination of drugs in a particular order which enables to control the tumor cell population. When a second drug is administered, the clonal structure

of the population is different from the start, and this may lead to enhanced sensitivity, or even complete tumor regression [63].

Since evolutionary herding alters the cellular composition of the tumor microenvironment, collateral drug sensitivity is likely to be persistent. Furthermore, this strategy is hardly influenced by stochastic perturbations and cell plasticity [64]. Acar et al. recently designed an experimental approach, in which evolution can be tightly controlled, monitored, and altered using drugs. It allows estimating evolutionary trade-offs and evaluating the effectiveness of patient-specific evolutionary herding strategies [65]. The suitability of evolutionary herding in RCC has not been tested yet.

5. Therapeutic Implications

As previously described, seven evolutionary trajectories can be distributed into four groups depending on the tumor's genomic characteristics, evolution mode, and clinical course. We suspect that each group has its own unique weaknesses that could be exploited. In Figure 1, we demonstrate the predicted effectiveness of evolution-targeted strategies against particular evolutionary trajectories of ccRCC.

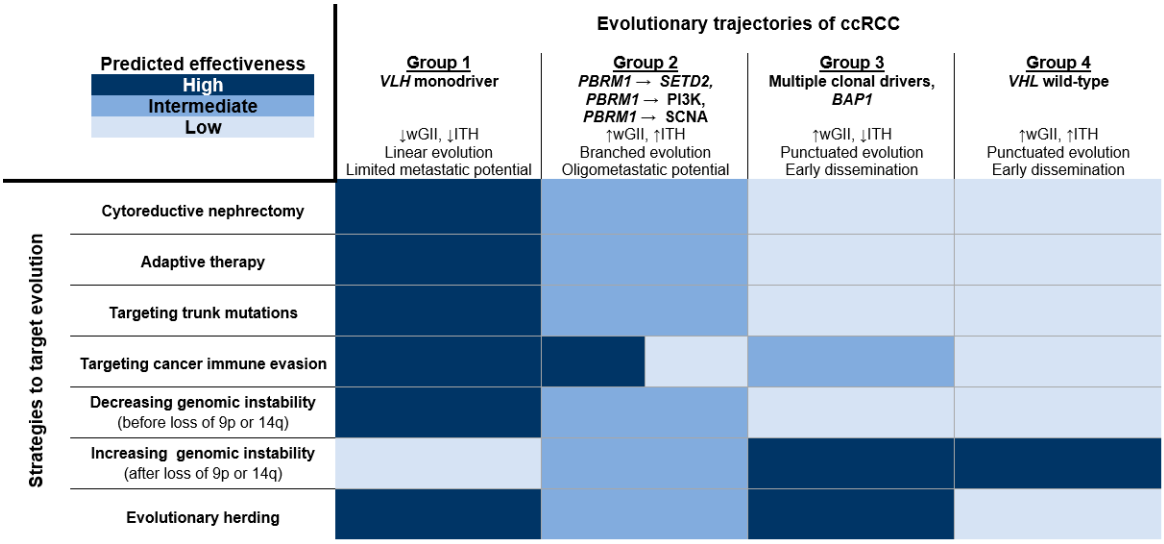

Figure 1. Predicted effectiveness of evolution-targeted strategies against particular evolutionary trajectories of clear cell renal cell carcinoma (ccRCC). Seven deterministic evolutionary trajectories are classified into four groups in terms of tumor's genomic characteristics, evolution mode, and clinical course. Loss of 9p or 14q represents the acquisition of metastatic competence. There are conflicting results regarding *PBRM1* mutation as a predictive biomarker of response to immunotherapy. The figure is based on assumptions about tumor biology and therapeutic options. ccRCC, clear cell renal cell carcinoma; wGII, weighted genome integrity index; ITH, intratumor heterogeneity.

While the benefit of upfront CN strictly depends on life expectancy, this procedure should be considered especially for Group 1 and, to a lesser extent, Group 2. Similarly, adaptive therapy that aims to enforce a stable tumor burden is expected to be highly effective against indolent cancers. Tumors from Group 1, in which *VHL* mutation is the sole driver event, are the best candidates for targeting trunk mutations. In Group 2, there is a limited number of trunk mutations, and this approach is still reasonable. As a general rule, ITH diminishes immune responses but tumors harboring *PBRM1* mutations (Group 2) could be highly vulnerable to immunotherapeutic agents. The predictive value of *PBRM1* mutation, however, is under debate and requires further investigation. Finally, decreased wGII is an indicator of a favorable response to immunotherapy, supporting its use in Group 1. In Figure 2, we illustrate how modulating genomic instability may affect ccRCC fitness. We suggest decreasing genomic instability before the loss of 9p or 14q, which represents the acquisition of metastatic

competence. This approach is particularly attractive in Group 1, characterized by low wGII. In contrast, Groups 3 and 4, due to high wGII and a punctuated evolution pattern, are expected to respond to increasing genomic instability. Modulating genomic instability in Group 2 could be unsuitable because of high wGII and branched mode of evolution. Evolutionary herding aims to decrease ITH with each subsequent therapy. Hence, it should be considered in Groups 1 and 3. This strategy may also be adequate in Group 2 due to its indolent nature in comparison to Group 4.

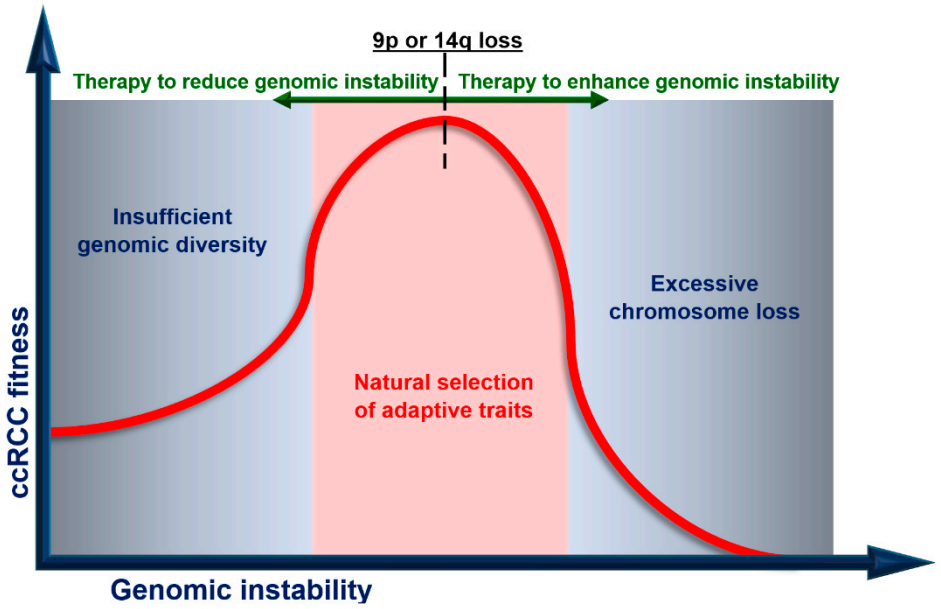

Figure 2. Modulating genomic instability to reduce ccRCC fitness. ccRCC fitness (vertical axis) is plotted against genomic instability (horizontal axis). There is an optimum range of genomic instability, in which ccRCC evolves. 9p or 14q loss represents the acquisition of metastatic competence and is a point of no return. Before this point is reached, decreasing genomic instability slows down cancer evolution. Once 9p or 14q is lost, increasing genome instability triggers extensive DNA damage and cell death.

6. Future Directions

Sequencing data obtained from spatial biopsies enable one to infer the phylogenetic tree structure and, in ccRCC, estimate the evolution trajectory. As a general rule, trunk alterations are found in all tumor cells and represent an ancestral event, while other modifications constitute the branches. The more regions sampled, the more branches will be found. In low-ITH cases, four biopsies would reflect the subclonal alteration with 75% accuracy. The gain in driver detection per additional sampling declines after eight, which is usually still not enough in cases with *PBRM1* mutation [14]. While molecular profiling of multiple specimens is not practical in the setting of clinical practice, the analysis of circulating tumor DNA (ctDNA) obtained from liquid biopsy represents a feasible alternative. Analysis of ctDNA enables identification of both clonal and subclonal tumor-specific mutations with high sensitivity and specificity, with detection rates comparable with those of traditional biopsies [61–66]. Furthermore, ctDNA has a relatively short half-life (approximately 2 h), allowing for the evaluation of tumor changes in real-time [67]. Finally, as minimally invasive, liquid biopsy eliminates the morbidity associated with the serial sampling of tumors. While qualitative and quantitative analyses of ctDNA have been extensively performed in RCC patients [68], liquid biopsy has not yet been used to capture RCC evolutionary trajectories.

The discovery of alternative evolutionary trajectories of RCC will provide a better insight into the underlying mechanisms of drug resistance. Some of these mechanisms may be closely related to geographic and environmental factors since patients from different regions have different genetic

backgrounds and are exposed to different carcinogens. Huang et al. identified mutational signatures and SCNAs specific to Chinese or Japanese ccRCC patients [13]. In the first group, the alterations could be due to exposure to aristolochic acid, a common ingredient in many Chinese herbs [69]. The cause of unique genetic alterations in the Japanese cohort remains unexplained.

Novel techniques to perform an in-depth analysis of datasets, as well as larger-scale studies, will greatly expand our knowledge on the development of RCC. Recently, an original computational method, CONETT (CONserved Evolutionary Trajectories in Tumors), enabled the detection of three additional directions of evolution among ccRCCs [70]. Two of them terminate with a sequence alteration in gene *KDM5C* and one in *TSC1*. The clinical significance of these findings is yet to be determined.

Identification of hidden evolutionary patterns is made possible by artificial intelligence (AI). Caravagna and colleagues devised a machine-learning method called repeated evolution in cancer (REVOLVER), which allows to overcome the stochastic effects of cancer evolution and information noise [71]. This technique uses transfer learning (TL) to achieve reproducible disease prognosis based on next-generation sequencing (NGS) count data [70–73]. As a result, it is possible to classify patients on the basis of how their tumor evolved, with implications for the anticipation of disease progression.

According to the NCCN Guidelines for Kidney Cancer, molecular profiling does not influence decision-making. The ongoing phase 2 clinical trials, A-PREDICT (NCT01693822) and ADAPTeR (NCT02446860), incorporate a multiregional sampling of metastatic RCC prior to and during therapy to evaluate biomarkers of treatment response. Whether evolutionary trajectories could reflect the effectiveness of a particular anti-RCC strategy, remains to be elucidated.

7. Conclusions

Many diseases are intimately tied to our evolutionary and genetic heritage. With our better understanding of these conditions, we gradually acquire the evolutionary perspective, which turns out necessary for both prevention and treatment [74,75]. In contrast to organismal evolution, human cancers are subjected to similar initial conditions and follow a limited range of possible evolutionary trajectories. Therefore, the repetitive nature of cancer evolution may prove to be its greatest weakness.

Genomic characterization is currently paving the way for clinical decision-making in RCC. The problem of exceptional ITH could be minimized by multiregion biopsy or liquid biopsy. These tools not only provide insights into cancer genetic architecture but also allow the measurement of clonal evolution. Recent studies resolved the evolutionary features and subtypes underpinning the diverse clinical phenotypes of ccRCC. In this review, we have summarized recent advances in the treatment of ccRCC and demonstrated how each strategy could be improved from the evolutionary perspective. Since there are only a few deterministic evolutionary trajectories in ccRCC, it appears feasible to use them as potential biomarkers for guiding intervention and surveillance. We believe that the presented patient stratification could help predict future steps of malignant progression, thereby informing optimal and personalized clinical decisions.

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5.2. ***TRIP13 predicts poor prognosis in clear cell renal cell carcinoma.***

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Original Article

TRIP13 predicts poor prognosis in clear cell renal cell carcinoma

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Abstract: What is the leading molecular mechanism that causes broad resistance to systemic therapies remains a key question in renal cancer related research. We explored associations of TRIP13 expression with the clinical course using the tissue microarray (TMA). The TMA contained specimens from 87 patients diagnosed with clear cell renal cell carcinoma (ccRCC). We performed immunohistochemistry to investigate TRIP13 protein expression levels. The overall survival (OS) was analyzed using the Kaplan-Meier method and log-rank statistics. Univariate and multivariate analyses were conducted using Cox proportional hazard models. Median follow up for the TMA cohort was 7.0 years. Tissues from 28.74% of patients demonstrated high TRIP13 expression. Mean TRIP13 expression in TRIP13-rich tumors was significantly higher comparing to adjacent normal tissues ($P < 0.05$). TRIP13 expression did not significantly correlate with stage nor tumor grade ($P > 0.05$). Elevated expression of TRIP13 served as an independent unfavorable prognostic indicator of survival in ccRCC ($P < 0.05$). TRIP13 overexpression predicts poor prognosis in ccRCC. Together with the emerging reports, this observation raises a suspicion that TRIP13 is a substantial driver of resistance to systemic therapies against kidney cancer.

Keywords: TRIP13, ccRCC, kidney cancer, renal carcinoma, expression, prognosis, survival, OS

Introduction

Renal cell carcinoma (RCC) is among the 10 most common cancers in both men and women, and its incidence is on the rise. In 2020, 73,750 new cases and 14,830 deaths due to RCC will occur in the US and over 400,000 new cases will occur worldwide [1]. Up to 30-40% of RCC cases are present as metastatic disease, either initially or after curative treatment [2]. The most common subtype, clear cell renal cell carcinoma (ccRCC), arises from the proximal convoluted tubule cells and accounts for approximately 70% of all cases [3]. Despite significant improvements in the clinical management over the last decade, most patients with metastatic ccRCC succumb to cancer progression within 1.5 years [4].

The exceptional intratumoral heterogeneity of RCC represents a considerable challenge limiting the efficacy of established systemic therapies [5]. Such treatment often further exacerbates the heterogeneity and leads to outgrowth of tumor cell subclones with resistance properties, including the resistance to apoptosis [6, 7]. The accumulating alterations found in both intrinsic and extrinsic apoptotic pathways aberrantly extend cells viability and eventually contribute to cancer progression [8]. Since apoptosis causes negligible damage to adjacent tissues [9], the apoptotic pathway-targeted therapies emerge as particularly promising strategy for RCC treatment.

TRIP13 is a protein encoded by *TRIP13* gene. Recent evidence implicates TRIP13 in various cell cycle phases, including meiosis, G2/Prophase and during the mitotic spindle assembly checkpoint (SAC) activation. TRIP13 is required for the development of higher-order chromosome structures and contributes to synaptonemal complex formation. It also promotes early steps of the DNA double-strand breaks (DSBs)

repair process. The latest reports together with in silico analysis, indicate its prominent role in driving tumorigenesis.

The Human Pathology Atlas is based on a systems-based analysis of the transcriptome of 17 main cancer types using data from 8,000 patients [10]. A national supercomputer center was used to analyze more than 2.5 petabytes of underlying publicly available data from the Cancer Genome Atlas (TCGA) to generate 900,000 survival plots describing the consequence of RNA and protein levels on clinical survival. All the data in the knowledge resource allows exploration of the human proteome. In this study, we explore the clinical association of TRIP13 with ccRCC histology and oncologic outcomes using the tissue microarray (TMA) ccRCC cohort, and validate these findings in TCGA.

Materials and methods

Tissue microarray

Tissue microarray (TMA) slide was obtained from a commercial supplier (US Biomax, Rockville, MD; TMA catalog number HKid-CRC180-Sur-01). The TMA (HKid-CRC180Sur-01) contained specimens from 92 patients, tumor and matched normal adjacent tissue (1 core/case), followed up for 7 years. Cores derived from 3 patients were missing, therefore these patients were excluded from the analysis. Retrievable patient data included age, pathology diagnosis, TNM, grade, stage and overall survival. The quality of the TMA was additionally approved by our pathologist. The study follows the principles of the Declaration of Helsinki. The tissues were collected under the highest ethical standards and HIPPA approved protocols with the donor being informed completely and with their consent. Since the tissues were commercially purchased, the study has been exempted from requiring ethical approval.

Immunohistochemistry

The TMA slide was processed at the Department of Clinical Pathology. The primary rabbit polyclonal anti-TRIP13 (HPA005727) antibody (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was applied to estimate the expression of TRIP13 protein. The protocol has been standardized using a series of positive and negative control reactions. The positive control reaction was performed on a tissue model selected according to reference sources (The Human Protein Atlas: http://www.proteinatlas.org) and the antibody data-sheet. TRIP13 positive control reaction was performed on pancreatic cancer tissue showing cytoplasmic and nuclear expression. All negative control reactions were performed on additionally analyzed tissue sections, by substituting the primary antibody with a solution of 1% BSA (bovine serum albumin) diluted in PBS (phosphate buffered saline). Immunohistochemical staining was performed using primary rabbit polyclonal anti-TRIP13 (1:200) antibody and visualization system EnVisionFlex+ Anti-Mouse/Rabbit HRP-Labeled Polymer (Dako, Agilent Technologies) on an Autostainer Link48 platform. Finally, tissue sections were dehydrated in ethanol of increasing concentration (from 80% to 98%), then cleared in a series of xylenes (from I to IV) and coverslipped in a medium (Dako, Agilent Technologies, USA).

IHC analysis and scoring

Initially, two experienced pathologists blinded to the clinical data evaluated the immunostained slides using the light microscope ELIPSE E800 (Nikon Instruments Europe, Amsterdam, Netherlands) at 20× and 40× original objective magnification. IHC revealed cytoplasmic and nuclear TRIP13 expression.

The cytoplasmic staining intensity of cells and percentage of cells at each staining intensity level were determined for each fixed core in the TMA. Staining intensity was graded as 0 (negative), 1+ (weak), 2+ (modarate), and 3+ (strong). The H-score was assigned using the following formula: [1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)], obtaining a value from 0 to 300.

The nuclear expression evaluation was scored on a two-point scale: 0 (negative IHC reaction result) and 1 (positive IHC reaction result).

Statistical analysis

All the statistical analyses were performed using Statistica version 10 (StatSoft) and Microsoft Excel 2019. The comparative studies were analyzed statistically using the nonparametric chi-square test. The p value < 0.05 was considered statistically significant.

Table 1. Baseline characteristics of TMA (n = 87) patient cohort

Clinical information	*n* (%)
Age, yr	
Mean	59.0
Range	29-83
Stage	
I	58 (66.67)
II	17 (19.54)
III	3 (3.45)
IV	2 (2.30)
Unknown	0 (0.00)
T Stage	
T1	62 (71.26)
T2	17 (19.54)
T3	4 (4.60)
Unknown	4 (4.60)
Lymph nodes	
N1	1 (1.15)
N0/Nx	84 (96.55)
Unknown	2 (2.30)
Metastasis	
Yes	2 (2.30)
No	85 (97.70)
Grade	
G1	32 (36.78)
G1-G2	14 (16.09)
G2	27 (31.03)
G2-G3	4 (4.60)
G3	9 (10.34)
G3-G4	1 (1.15)
Median follow up time	7.0
Disease course	
Alive	59 (67.82)
Dead	28 (32.18)

Results

The location and expression of TRIP13 protein in ccRCC TMA cohort

IHC was performed on 87 pairs of ccRCC and corresponding normal tissues. Five cores of corresponding tissues were lost during IHC staining procedure. **Table 1** summarizes the characteristics of the TMA cohort. The mean age of patients was 59 years (range: 29-83 years) and the median follow-up was 7.0 years.

Cytoplasmic TRIP13 staining was observed in 77 (88.51%) of 87 ccRCC tissues and the median expression was 100 (interquartile range 0-215). Among adjacent controls, 70 (85.37%) of 82 cores were positive and the median expression was 115 (interquartile range 70-200). Cytoplasmic expressions of TRIP13 in ccRCC tissues were lower than those in adjacent controls ($P < 0.05$, **Figure 1A**). Next, we dichotomized the cytoplasmic expressions of TRIP13 to low expression and high expression. Using the tool *Cutoff Finder,* we set the best cutoff at 105 [11]. Mean TRIP13 expression in TRIP13-rich tumors was significantly higher comparing to adjacent normal tissues ($P < 0.05$, **Figure 1B**). Similarly, adjacent normal tissues were characterized by elevated TRIP13 expression when compared to TRIP13-depleted tumors ($P < 0.05$) (**Figure 1C**).

Nuclear TRIP13 staining was observed in 14 cancer tissues (16.28%) (**Figure 2A**) and only in 2 adjacent normal tissues (2.44%) (**Figure 2B**). This difference was statistically significant ($P < 0.05$).

Clinical course and TRIP13 protein expression in ccRCC TMA cohort

TRIP13 protein expression did not significantly correlate with TNM stage nor tumor grade (both $P > 0.05$). Univariate analysis revealed that patients with high cytoplasmic TRIP13 protein expression had significantly shorter OS comparing to those with low expression ($P < 0.05$, HR = 2.88 [1.35-6.15]) (**Figure 3**). We found no significant association between the presence of TRIP13 nuclear expression and OS (**Figure 4**). In conclusion, TRIP13 overexpression predicts poor prognosis in ccRCC. Together with the emerging reports, this observation raises a suspicion that TRIP13 is a substantial driver of resistance to systemic therapies against kidney cancer.

Discussion

TCGA ccRCC cohort analysis

We found that cytoplasmic TRIP13 protein overexpression significantly correlates with poor survival in ccRCC patients. To evaluate whether the expression of TRIP13 mRNA was also associated with the clinical course of the disease, we accessed TCGA database. All transcriptomics information were obtained employing the Human Pathology Atlas, the separate part of The Human Protein Atlas available from www.

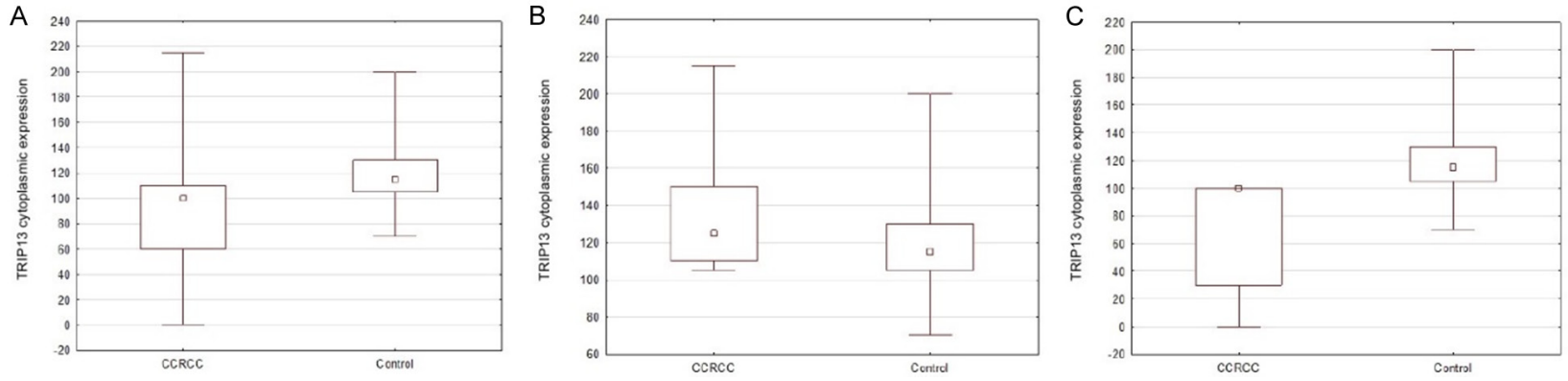

Figure 1. A. Cytoplasmic expression of TRIP13 in Clear Cell Renal Cell Carcinoma (CCRCC) and adjacent normal tissue (Control). B. Cytoplasmic TRIP13 expression in TRIP13-rich CCRCC and control. C. Cytoplasmic TRIP13 expression in TRIP13-depleted CCRCC and control.

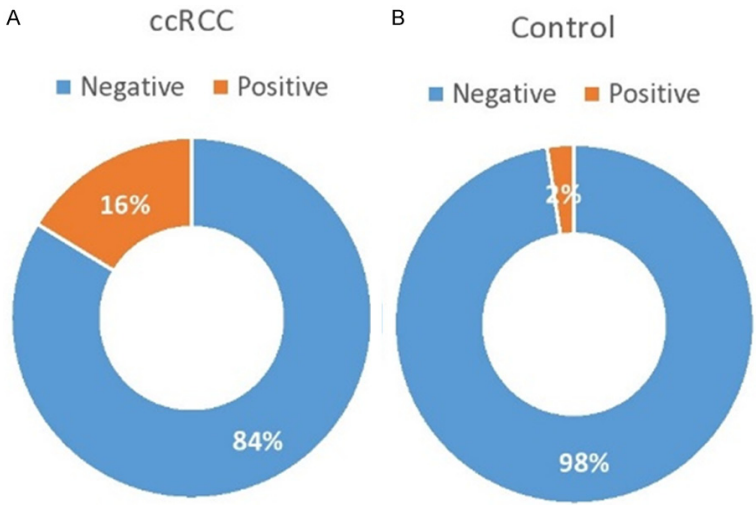

Figure 2. Prevalence of positive TRIP13 nuclear expression among (A) clear cell Renal Cell Carcinoma tissues, and (B) adjacent normal tissues (Control).

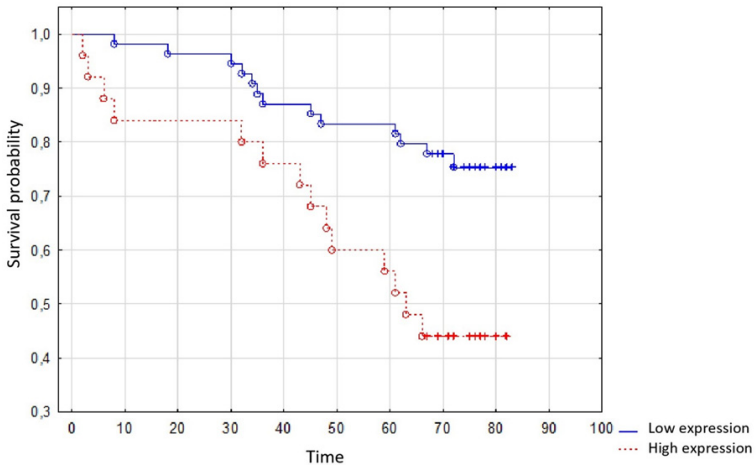

Figure 3. The survival curve of clear cell Renal Cell Carcinoma patients according to TRIP13 cytoplasmic expression.

proteinatlas.org. TCGA cohort consisted of 528 patients diagnosed with ccRCC [12]. The available characteristics of study subjects are summarized in **Table 2**. The mean age of patients was 60.5 years (range: 26-90 years) and the median follow-up was 3.28 years. The TCGA RNA-seq data was mapped using the Ensembl gene id available from TCGA, and the FPKMs (number Fragments Per Kilobase of exon per Million reads) for *TRIP13* were subsequently used for quantification of expression with a detection threshold of 1 FPKM. Based on the FPKM value of *TRIP13*, patients were classified into two expression groups. To choose the best FPKM cutoff for grouping the patients most significantly, all FPKM values from the 20th to 80th percentiles were used to group the patients, significant differences in the survival outcomes of the groups were examined and the value yielding the lowest log-rank *P* value (3.4e-11) was selected. 109 of 528 (20.64%) patients had higher expression than the established cutoff. The prognosis of each group of patients was examined by Kaplan-Meier survival estimators and the survival outcomes of the two groups were compared by log-rank tests. The five-year survival was reached by 70% of patients with low *TRIP13* expression and 39% of those with high expression. Taken together, TRIP13 mRNA expression is prognostic and its high expression is unfavourable in RCC ($P < 0.05$), according to TCGA.

The Human Protein Atlas tissue repository could not be used to evaluate TRIP13 protein expression in RCC because it showed low or negative immunoreactivity to both recommended antibodies (HPA053093 and HPA005727). Also the group of patients in this trial was small (n = 12).

Role of TRIP13 in cancer

TRIP13 takes part in a variety of cellular activities, including cell cycle regulation, DNA repair and apoptosis. Study on multiple myeloma cells revealed that overexpression of TRIP13 abrogates SAC. The underlying mechanism includes activation of PI3K-Akt signaling pathway that induces proteasome-mediated degradation of MAD2, the key component of SAC [13]. Dysfunctional SAC contributes to chromosomal instability (CIN), aneuploidy, and eventually facilitates cancer progression [14-16]. Moreover, one of major downstream effectors of AKT is the mammalian target of rapamycin (mTOR), which induces cell growth, proliferation, survival, and motility, as well as angiogenesis [17].

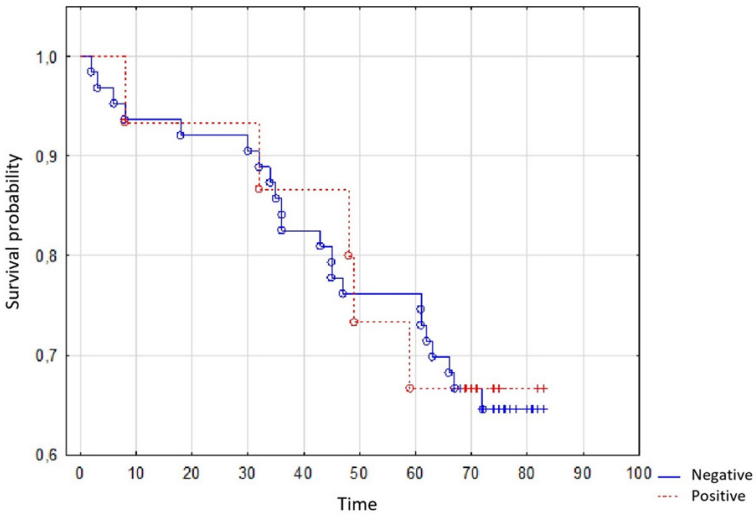

Figure 4. The survival curve of clear cell Renal Cell Carcinoma patients according to TRIP13 nuclear expression.

Table 2. Baseline characteristics of TCGA (n = 528) patient cohort

Clinical information	*n* (%)
Age, yr	
Mean	60.5
Range	26-90
Sex	
Male	344 (65.15)
Female	184 (34.85)
Race	
White	459 (86.93)
Black or African American	54 (10.23)
Asian	8 (1.52)
Unknown	7 (1.33)
Stage	
I	263 (49.81)
II	57 (10.80)
III	123 (23.30)
IV	82 (15.53)
Unknown	3 (0.57)
Median follow up time	3.28
Disease course	
Alive	355 (67.23)
Dead	173 (32.77)

According to study of Yao et al., TRIP13 promotes growth and metastasis of hepatocellular carcinoma through inhibition of TGF-β1/Smad3 signaling [18]. Repressed Smad3 activity has been associated with breast cancer bone metastasis by its effects on tumor angiogenesis, and epithelial-mesenchymal transition (EMT) [19]. Zhou et al. revealed that TRIP13 enhances the proliferation and invasion via activation of the NOTCH signaling and induction of EMT [20]. In damaged cells, TRIP13 functions to favor non-homologous end joining (NHEJ) over homologous recombination (HR). Both are the major pathways for DNA DSBs repair. While HR results in accurate repair, NHEJ is an intrinsically error-prone pathway and may lead to CIN and eventually carcinogenesis [21].

Study of Banerjee et al. demonstrated significant impact of TRIP13 in chemoresistance development among head and neck cancers. Cells with downregulated TRIP13 expression, treated with cisplatin were characterized by better response rate and slower growth [22]. TRIP13 might enhance the resistance to systemic therapy in RCC as well. The systemic therapy (targeted therapy, immunotherapy or chemotherapy) could constitute a selective pressure acting on TRIP13 expression in RCC cells. During the course of the disease, the population of cells with higher expression of TRIP13 would rise because of its protective properties. Ultimately, the cancer tissue would become irreversibly resistant to applied treatment. Our results do not support this hypothesis, because we did not find significant relationships between protein expression and grade or stage of the disease. On the other hand, this approach might be worth pursuing, since the relatively small cohort of advanced ccRCC within our TMA could not ensure a valid representation.

Although the detrimental effect of TRIP13 has been confirmed in biologically diverse neoplasms [13-25], the exact mechanisms and their relative importance are not yet clear. While, as previously described, TRIP13 promotes malignant properties of ovarian cancer cells [20], elevated TRIP13 mRNA expression is associated with favorable outcomes in ovarian

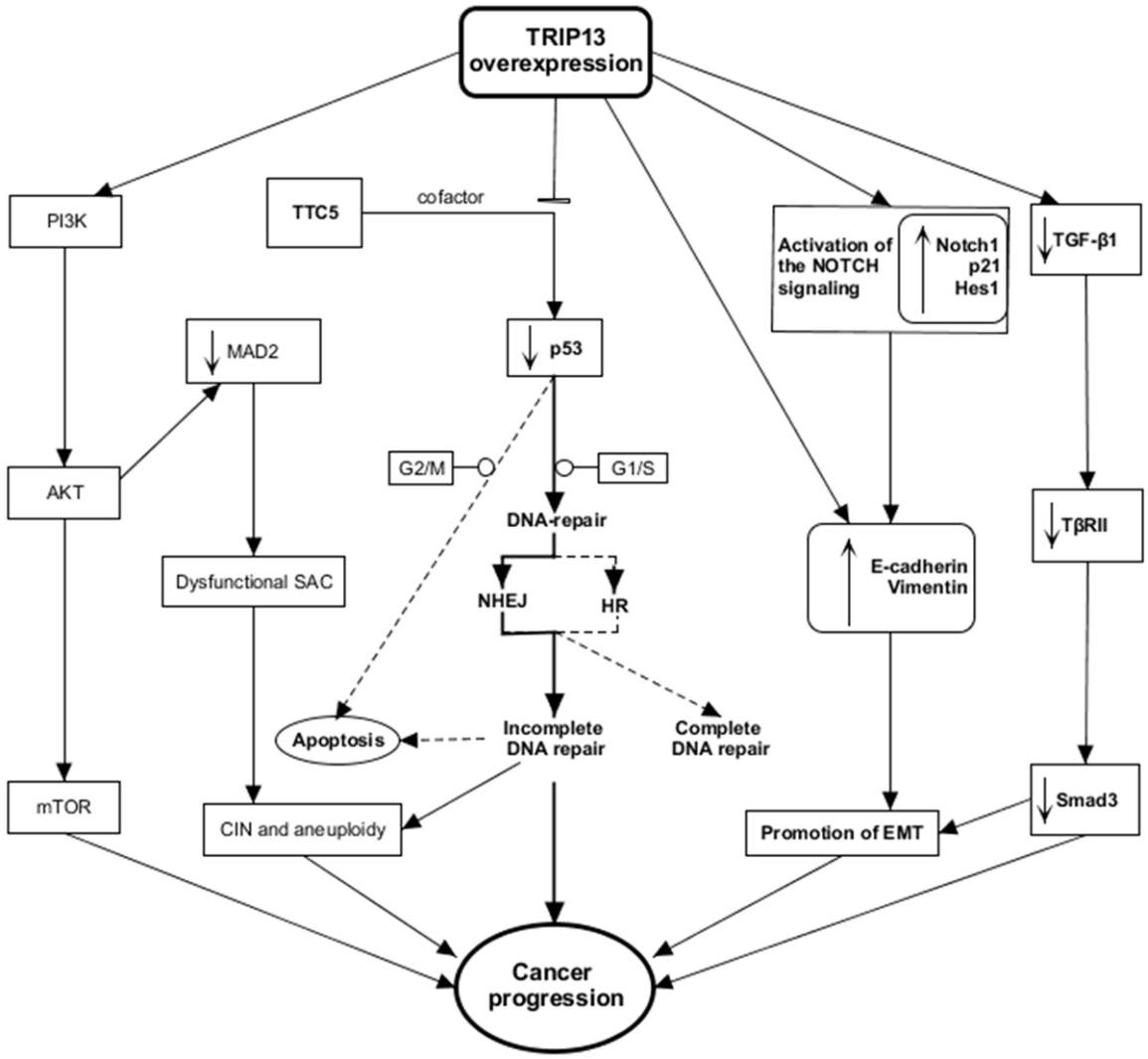

Figure 5. Probable interactions among TRIP13 and other molecules within clear cell Renal Cell Carcinoma. Overexpression of TRIP13 activates PI3K/AKT/mTOR pathway. AKT induces proteasome-mediated degradation of MAD2, the key component of SAC. Dysfunctional SAC leads to CIN and aneuploidy, which, together with mTOR, mediate cancer progression. TRIP13 together with TTC5 as a cofactor, inhibits p53 signaling and, consequently, suppresses the apoptosis. In cells with damaged DNA, TRIP13 functions to favor NHEJ over HR. NHEJ as more likely to be inaccurate, may contribute to cancer progression. TRIP13 also induces the expressions of E-cadherin and vimentin directly or through activation of the NOTCH signaling. The net effect is the promotion EMT, which is directly associated with gain of migratory and invasive capabilities. TRIP13 reduces the expressions of TGF-β1, TβRII and Smad3, the mediators of cellular senescence. The inhibition of TGF-β1/Smad3 signaling supports tumor growth. Decreased Smad3 activity promotes EMT. SAC-spindle assembly checkpoint; CIN-chromosomal instability; NHEJ-non-homologous end joining; HR-homologous recombination; EMT-epithelial-mesenchymal transition.

cancer patients [12]. It demonstrates that the actual contribution of TRIP13 to tumorigenesis could be far more complex.

Role of TRIP13 in kidney

It remains unexplored whether TRIP13 plays a significant role in renal cell carcinoma. However, Pressly et al. recently shed new light on the antiapoptotic role of TRIP13 in renal tubules. As they reported, TRIP13 interacts with Tetratricopeptide Repeat Domain 5 (TTC5) and inhibits p53. Insufficient TRIP13 consequently increases the susceptibility of damaged tubular epithelial cells to progress towards apoptotic cell death [31].

Interestingly, biallelic loss-of-function mutations in TRIP13 have been shown to predispose to Wilms tumor, a kidney cancer that primarily

affects children [32]. The authors of this report indicate a substantial impairment of SAC, which eventually leads to a high rate of chromosome missegregation in these patients. This study supports the existence of a close relationship between TRIP13 and SAC, which when disturbed, increases cancer risk or drives its progression.

Potential role of TRIP13 in renal cell carcinoma

In **Figure 5**, we summarized probable interactions among TRIP13 and other molecules within kidney cancer cell. This pathway diagram illustrates how TRIP13 may affect survival in these patients, and therefore may serve as a starting point for translational research.

It is tempting to speculate, that the ability of TRIP13 to inhibit apoptosis is of paramount importance in RCC patients. Firstly, it has been confirmed in renal tubular epithelial cells, the same that give rise to ccRCC. Secondly, there is accumulating evidence of positive responses to apoptosis inducers in RCC [33-40].

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Disclosure of conflict of interest

None.

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5.3. ***Overexpression of KIF11 is a poor prognostic factor in clear cell renal cell carcinoma.***

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Overexpression of KIF11 is a poor prognostic factor in clear cell renal cell carcinoma

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Unresectable renal cell carcinoma continues to be a great challenge due to our limited understanding of its underlying pathophysiology. We explored the relationship between KIF11 protein expression and the clinical courses of clear cell renal cell carcinoma (ccRCC) using a tissue microarray.
Material and methods: The tissue microarray contained specimens derived from 90 patients, cancer and matched adjacent non-cancerous tissue (2 cores per case), followed up for 7 years. Tumour samples were evaluated for KIF11 expression using the H-score, and their correlations with clinicopathological data and survival data were analysed.
72.7% of ccRCC tissues presented KIF11 cytoplasmic expression with a median value of 20 (interquartile range 0–200). The nuclear staining was positive in 36.36% of ccRCC tissues. Among controls, nuclear KIF11 expression was absent, but cytoplasmic expression was identified in all cases, with a median value of 230 (interquartile range 45–290). Cytoplasmic KIF11 expression in ccRCC tissues was lower than in the control tissues and was positively correlated with tumour grade and mortality ($p < 0.05$). KIF11 nuclear expression did not correlate with overall survival.
Elevated expression of KIF11 predicts poor clinical outcome in ccRCC patients. Downregulation of KIF11 may provide a new therapeutic strategy for ccRCC.

Key words: KIF11, ccRCC, kidney cancer, renal carcinoma, expression, prognosis, survival, OS.

Introduction

Renal cell carcinoma (RCC) is in the top 10 most common cancers, and its incidence is on the rise. Despite significant advances in medical management, the American Cancer Society estimates that in 2020 in the US, 14,830 people will die from this disease [1]. The most common subtype of RCC that accounts for 65–70% of cases, is the clear cell renal cell carcinoma (ccRCC). It originates from the proximal tubular epithelial cells of nephrons [1, 2]. The extraordinary heterogeneity of this tumour poses a great challenge

for its effective treatment [3]. Thus, the establishment of novel molecular targets is an attractive approach.

KIF11, as a motor protein encoded by the *KIF11* gene, assists in spindle dynamics. Among its main functions are chromosome positioning, centrosome separation, and establishing a bipolar spindle during mitosis [4]. Its overexpression reflects poor prognosis in various carcinomas including gastric, laryngeal, breast, prostate, and pancreatic [5–9]. Recent reports together with *in silico* analysis suggest that KIF11 may also contribute to ccRCC progression. We explored associations of KIF11 expression with the clinical course using a tissue microarray (TMA) and validated these findings in The Cancer Genome Atlas (TCGA).

Material and methods

Tissue microarray

The tissue microarray was purchased from a commercial supplier (US Biomax, Rockville, MD). The tissue microarray (HKid-CRC180Sur-01) contained specimens derived from 90 patients, cancer and matched adjacent non-cancerous tissue (2 cores per case), followed up for 7 years. Samples were consecutively collected from July 2006 to February 2008, following informed consent and under approval of the Ethics Committee. All of the specimens were obtained prior to any therapeutic manipulation. The diagnosis was made by at least 2 different evaluators in accordance with up-to-date World Health Organization guidelines. Two cores with ccRCC and 5 cores with normal adjacent tissue were missing and therefore were excluded from the analysis. Retrievable clinicopathological data included age, pathological diagnosis, TNM, stage, grade, and overall survival (OS). The quality of each specimen was additionally approved by our pathologists.

Immunohistochemistry

The tissue microarray slides were processed at the Department of Clinical Pathology. Primary rabbit polyclonal anti-KIF11 (HPA010568; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) antibody was used to estimate the expression of KIF11 protein. The standardization of the protocol was achieved using a series of control reactions: positive and negative. The positive control reaction was performed in accordance with reference sources (Human Protein Atlas: http://www.proteinatlas.org) and the antibody datasheet. KIF11-positive control reaction performed on pancreatic cancer tissue presented cytoplasmic and nuclear expression. Furthermore, all negative control reactions were performed on additionally analysed tissue sections by substituting the primary antibody with a solution of 1% bovine serum albumin diluted in phosphate-buffered saline. Immunohistochemical (IHC) staining was performed using primary rabbit polyclonal anti-KIF11 (1 : 200) antibody and visualization system EnVisionFlex+ Anti-Mouse/Rabbit HRP-Labelled Polymer (Dako, Agilent Technologies) on an Autostainer Link48 platform. Lastly, dehydration of tissue sections was performed in ethanol at increasing concentrations (80–98%), then cleared in a series of xylenes (I–IV) and cover-slipped in a medium (Dako, Agilent Technologies, USA).

Immunohistochemical analysis and scoring

All immunostained samples were evaluated by 2 experienced pathologists blinded to the patients' clinical data. The level of KIF11 cytoplasmic and nuclear expression were assessed using the light microscope at 20× and 40× magnification. The extent of cytoplasmic immunoreactivity was assessed by H-Score. In this case, we distinguished 3 levels of expression intensity (1+ = 'low'/2+ = 'moderate'/3+ = 'high'). The percentage of those cells were applied to the following formula:

$1 \times (\%$ cells $1+) + 2 \times (\%$ cells $2+) + 3 \times (\%$ cells $3+) =$ H-score

The final score ranged 0–300. The nuclear expression of KIF11 was evaluated using a two-point scale (0 = 'negative IHC result'/1 = 'positive IHC result').

Statistical analysis

All the statistical analyses were performed using Statistica version 10 (StatSoft) and Microsoft Excel 2019. We used the log-rank test to compare the survival distributions of patients with different protein expression patterns. The Kaplan-Meier estimator was performed to estimate the survival functions from lifetime data. We used the Mann-Whitney *U* test to compare the protein expressions between cancerous and adjacent normal cells. Cox Proportional Hazards for analysing ccRCC survival data were considered. The data were divided into 4 groups according to patients' ages (age ≤ 65 and age > 65 years), grade (G1 and G2, G3), stage (T1 and T2, T3) and KIF11 expression level (KIF11 ≤ 42.5 – low and KIF11 > 42.5 – high). The p-value < 0.05 was considered statistically significant.

Ethical review and approval were waived for this study due to the lack of access to identifiable private information. Informed consent was obtained from all subjects involved in the study

Results

The study included 88 pairs of ccRCC and corresponding non-cancerous tissue. During the IHC staining procedure, 5 cores of corresponding tissue and 2 cores of ccRCC were lost. Summarized charac-

Table I. Baseline characteristics of the tissue microarray ($n = 88$) patient cohort

Clinical information	*n* (%)
Age [years]	
Mean	59.09
Range	29–83
Stage	
I	60 (68.20)
II	17 (19.30)
III	3 (3.40)
IV	2 (2.30)
Unknown	6 (6.81)
T stage	
T1	63 (71.59)
T2	17 (19.32)
T3	4 (4.55)
Unknown	4 (4.55)
Lymph nodes	
N1	1 (1.11)
N0/Nx	85 (96.59)
Unknown	2 (2.30)
Metastasis	
Yes	2 (2.28)
No	86 (97.72)
WHO/ISUP grade	
G1	33 (37.5)
G2	41 (46.59)
G3	13 (14.77)
G4	1 (1.14)
Median follow-up time [years]	7.0
Disease course	
Alive	60 (68.18)
Dead	28 (31.82)

ISUP – International Society of Urological Pathology, WHO – World Health Organization

teristics of the TMA cohort are presented in Table I. The median follow-up was 7.0 years.

64 of 88 ccRCC tissues (72.7%) presented KIF11 cytoplasmic expression with the median value of 20 (interquartile range 0–200). The nuclear staining was positive in 32 of 88 ccRCC tissues (36.36%). Among controls, nuclear KIF11 expression was absent, but cytoplasmic expression was identified in all cases, with a median value of 230 (interquartile range 45–290). Cytoplasmic KIF11 expression in ccRCC tissues was lower compared to control tissues ($p < 0.05$) (Fig. 1A).

Cytoplasmic KIF11 expression positively correlated with tumour grade ($p = 0.0013$) and mortality (HR 2.17; 95% Cl: 0.99–4.73; $p = 0.047$) (Figs. 1B, 2A). Cox Proportional Hazard was statistically significant only for T1 tumours. HR estimates of 0.19 (95% Cl: 0.04–0.96; $p = 0.045$) for low KIF cytoplasmic expression and 0.42 (95% Cl: 0.15–1.16; $p = 0.049$) for high KIF cytoplasmic expression were calculated. KIF11 nuclear expression did not correlate with OS ($p = 0.72$) (Fig. 2B). KIF11 cytoplasmic or nuclear expression did not correlate with tumour stage.

Discussion

Surgical resection is the best therapeutic strategy for localized RCC [10]. However, around 30% of patients experience tumour recurrence following complete resection [11, 12]. Immunotherapy and/or targeted therapy represent a standard of care for stage IV and recurrent RCC. Despite relatively high response rates to these agents, most patients eventually succumb to cancer progression. Versus KIF11 has been shown to promote the epithelial-mesenchymal transition and activate many molecular mechanisms involved in cancer progression, including Wnt/β-catenin, PI3K/AKT/mTOR, and MAPK/ERK pathway [9, 13, 14]. Targeting KIF11 inhibits invasion, proliferation, and self-renewal in glioblastoma cell lines [15]. A similar effect was observed in breast cancer and prostate cancer cells [9, 16–20]. Filanesib, a potent KIF11 inhibitor, has recently demonstrated clinical efficacy in patients with multiple myeloma [21].

The metastatic competence of ccRCC is afforded by chromosome complexity, in particular 9p and 14q loss [22]. We found that high KIF11 cytoplasmic (but not nuclear) expression correlates with poor survival in patients with ccRCC. KIF11 within the cytoplasm contributes to centrosome separation and bipolar spindle formation and can provoke vital chromosome-level alterations. Hence, it could play a significant role in driving ccRCC evolution and metastatic spread. Then, targeting KIF11 would be an attractive complement to evolution-targeted therapy. Evolution-targeted therapy in ccRCC is a novel concept that relies on patient stratification according to the deterministic evolutionary trajectory of the tumour [23]. Currently there are 7 well described evolutionary trajectories in ccRCC according to the tumour's genomic characteristics, evolution mode, and clinical course [24]. While the evolutionary trajectory could be used as a biomarker for guiding the intervention, inhibition of KIF11 could further curb cancer evolution, making this approach more effective.

According to the cBioportal for Cancer Genomics, a database with genome sequencing and comparative genome hybridization, KIF11 overexpression is driven by epigenetic alterations in 99.61% of cases. The remaining causes include genetic amplifications

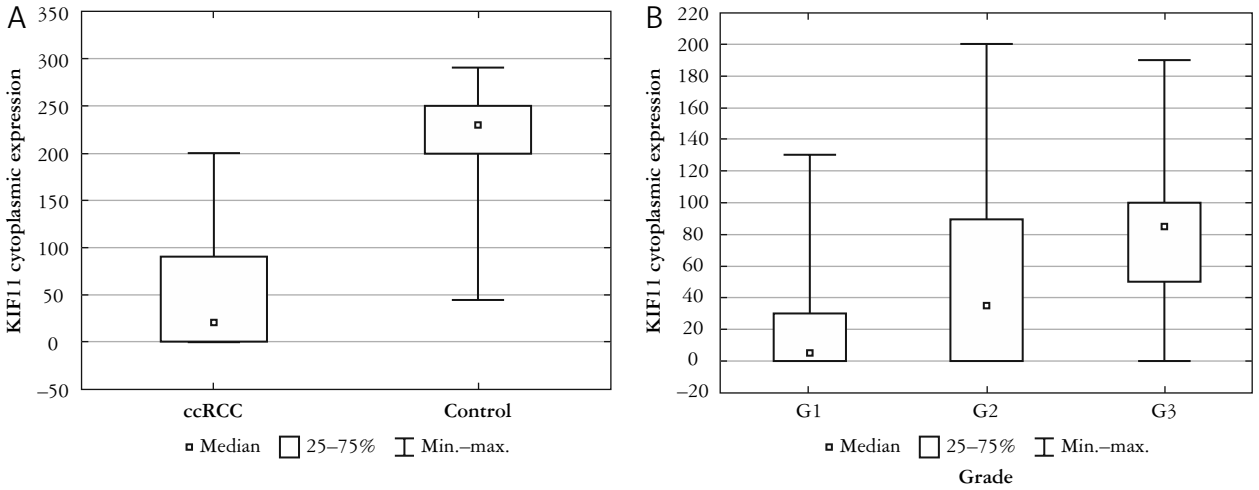

Fig. 1. A) Cytoplasmic KIF11 expression in clear cell renal cell carcinoma (ccRCC) and adjacent normal tissue (control). B) KIF11 expression according to ccRCC grade

ccRCC – clear cell renal cell carcinoma

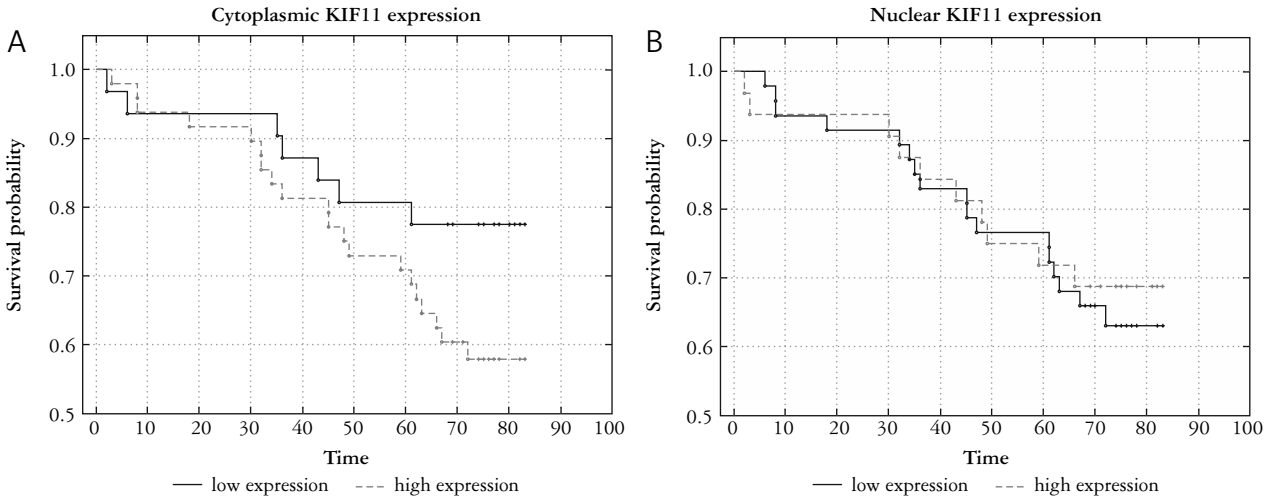

Fig. 2. A) The survival curve of clear cell renal cell carcinoma (ccRCC) patients according to cytoplasmic KIF11 expression. B) The survival curve of ccRCC patients according to nuclear KIF11 expression

and missense mutations. The epigenetic alterations are reversible and play a central role in renal carcinogenesis [25]. Hence, specifically targeting these alterations could restore a normal epigenetic pattern and potentially cure the disease. Currently, targeted epigenetic therapies are under investigation. Their combination with antiangiogenic or immune checkpoint treatments represents a particularly promising paradigm that could overcome frequent monotherapy resistance [26–29]. Epigenetic therapeutics are classified into agents that have a targeted effect, such as anti-miRNA oligonucleotides, and agents that have a more broad effect and lead to large-scale changes in gene expression, such as HDAC inhibitors (HDACi) [25]. The principal problem with the first group of agents is their difficult delivery to cancer cells [30]. The second group of agents, on the other hand, activate genes that are normally repressed, leading to adverse off-target effects that influence numerous processes in the body [31]. As a result, no epigenetic alteration can be both safely and precisely targeted, and therefore successful clinical translation of epigenetics in RCC remains to be seen.

To evaluate the association between KIF11 mRNA expression and the clinical course of ccRCC we accessed the TCGA database [32, 33]. In TCGA, patients were classified into 2 expression groups based on the KIF11 FPKM (number fragments per kilobase of exon per million reads) value. To choose the best FPKM cut-off for grouping the patients, significant differences in the OS of the groups were analysed, and the value yielding the lowest log-rank *p*-value (1.5e–8) was selected. KIF11 expression among 119 of 528 (22.54%) patients was higher than the established cut-off. The Kaplan-Meier survival estimators evaluated the prognosis of each group. The survival outcomes of the 2 groups were compared by log-rank tests. The five-year survival was reached

by 69% of patients with low KIF11 expression and 44% of patients with high KIF11 expression. According to data from the TCGA database, elevated KIF11 mRNA expression is associated with poor prognosis in ccRCC ($p < 0.05$). These results are in accordance with our findings.

Our study cohort comprised mainly low-grade and low-stage cases. Therefore, further research incorporating advanced, unresectable tumours is needed to translate our results toward a future potential clinical intervention.

Conclusions

Elevated expression of KIF11 predicts poor clinical outcome in ccRCC patients. Downregulation of KIF11 may provide a new therapeutic strategy for ccRCC.

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The authors declare no conflict of interest.

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6. Overview of the publications included in the dissertation.

6.1. *Origination of the concept of targeted, anti-evolutionary cancer therapy.*

Cancer treatments that target specific cells or pathways often stop working because cancerous cells adapt and change. A new approach to treating cancer may involve disrupting the evolution of cancer cells, rather than just targeting individual cells or pathways. This could potentially put an end to cancer as a major health concern.

Scientists have recently discovered different types of clear cell renal cell carcinoma (ccRCC) based on the way the cancer cells change and evolve. These types can be grouped into seven distinct evolutionary subtypes or four groups, and they are related to how the cancer behaves and responds to treatment [9, 10]. These groups are distinguished by four features - variations in chromosomal complexity, intratumor heterogeneity (ITH), model of tumor evolution, and metastatic potential.

Group 1 consists of primary tumors with VHL alteration as the sole driver event. They evolve in a “linear” fashion and are characterized by low both weighted genome instability index (wGII) and ITH. This mode of evolution is associated with indolent growth and low metastatic potential. Group 2 includes tumors in which early PBRM1 mutation and subsequent SETD2 mutation or PI3K pathway mutation or acquisition of SCNAs result in a “branched'' evolutionary pattern. These are heterogeneous neoplasms with oligometastatic potential and attenuated progression. Clonal acquisition of multiple driver mutations (VHL plus ≥2 BAP1, PBRM1, SETD2, or PTEN) or the parallel BAP1 mutation results in “punctuated” evolution. These tumors are characterized by high wGII but low ITH and belong to group 3. Punctuated evolution, driven mostly by high wGII, leads to rapid dissemination and is also observed among VHL wild-type tumors, which constitute the fourth group [10].

I described a concept of exploiting the aforementioned trajectories for guiding intervention and surveillance. In our article "Targeting the Deterministic Evolutionary Trajectories of Clear Cell Renal Cell Carcinoma" published in the *Cancers* magazine [11],

I proposed seven anti-evolution strategies, and estimated their effectiveness against each evolutionary trajectory. The presented patient stratification is to help predict future steps of malignant progression, thereby informing optimal and personalized clinical decisions. This article sets a new direction for research into targeted anti-evolutionary therapy. In this case, the targeted therapy is for a specific histological type of tumor evolving in a certain direction.

6.2. *Identification of potential molecular targets for targeted, anti-evolutionary cancer therapy against clear cell renal cell carcinoma.*

6.2.1 Introduction.

As previously described, targeting individual components of pathways that drive cancer progression is unlikely to be effective. From the evolutionary perspective, however, it is a reasonable option once these components directly affect the genomic instability. Genomic instability of cancer cells drives genetic diversity required for the natural selection of adaptive traits, but there is a threshold beyond which cells cannot replicate successfully [12]. Hence, it is tempting to alter (increase or decrease) the frequency of mutations within the cancer genome.

Modulation of genomic instability is one of the vital options to overcome the ccRCC's evolution [11]. In particular, I recommended decreasing genomic instability before the loss of 9p or 14q, which represents the acquisition of metastatic competence. This approach is exceptionally attractive in Group 1, characterized by low wGII. In contrast, Groups 3 and 4, due to high wGII and a punctuated evolution pattern, are expected to respond to increasing genomic instability. Modulating genomic instability in Group 2 could be unsuitable because of high wGII and branched mode of evolution.

Kidney cancer is characterized by a moderate level of genomic instability and the absence of mutations in canonical DNA damage response (DDR) genes, such as *RAD9*, *BRCA1*, or *TP53* [13, 14]. As a result, kidney cancer patients are commonly unresponsive to DNA-damaging therapies, such as chemo- or radiotherapy. For that reason, reducing genetic instability could be a more suitable approach. It can be achieved, among others, by the regulation of the transforming growth factor β (TGF-β) axis. TGF-β has been shown to inhibit DNA double-strand breaks (DSB) repair mechanisms to heighten the genetic diversity and adaptability of cancer cells [15]. In ccRCC cell cultures, TGF-β enhances proliferative capacity and promotes metastatic growth [16]. Early phase Ib clinical trial (NCT00356460) investigated the use of a monoclonal antibody against TGF-β fresolimumab in RCC patients and showed preliminary evidence of antitumor activity [17].

On the contrary, particular ccRCC driver genes do influence DNA damage response (DDR) and there is preclinical evidence to support the poly(ADP)-ribose polymerase (PARP) inhibition in VHL- or BAP1-mutated ccRCC [12, 18, 19]. Moreover, cells harboring SETD2 mutation undergo synthetic lethal interaction with WEE1 blockade due to the depletion of nucleotide pools [20]. AZD1775, an experimental inhibitor of WEE1, is currently being evaluated for patients with SETD2-deficient tumors, including RCC (NCT03284385).

Based on the available data and *in silico* analysis, I selected two proteins, TRIP13 and KIF11, which could become targets for this strategy in ccRCC.

TRIP13 is a protein encoded by the *TRIP13* gene. TRIP13 plays a significant role in various cell cycle phases, including meiosis, G2/Prophase, and during the mitotic spindle assembly checkpoint (SAC) activation. TRIP13 is required for the development of higher-order chromosome structures and contributes to synaptonemal complex formation. It also promotes early steps of the DNA double-strand breaks (DSBs) repair process [21].

Study on multiple myeloma cells revealed that overexpression of TRIP13 abrogates SAC. The underlying mechanism includes activation of the PI3K-Akt signaling pathway that induces proteasome-mediated degradation of MAD2, the key component of SAC [22]. Dysfunctional SAC contributes to chromosomal instability (CIN), aneuploidy, and eventually facilitates cancer progression [23-25]. Biallelic loss-of-function mutations in TRIP13 have been shown to predispose to Wilms tumor, a kidney cancer that primarily affects children [26]. The authors of this report indicate a substantial impairment of SAC, which eventually leads to a high rate of chromosome missegregation in these patients. This study supports the existence of a close relationship between TRIP13 and SAC, which when disturbed, increases cancer risk or drives its progression.

In damaged cells, TRIP13 functions to favor non-homologous end joining (NHEJ) over homologous recombination (HR). Both are the major pathways for DNA DSBs repair. While HR results in accurate repair, NHEJ is an intrinsically error-prone pathway and may lead to CIN and eventually carcinogenesis [27].

KIF11, as a motor protein encoded by the *KIF11* gene, assists in spindle dynamics. Among its main functions are chromosome positioning, centrosome separation, and establishing a bipolar spindle during mitosis [28, 29]. KIF11 has been shown to promote CIN and activate many molecular mechanisms involved in cancer progression, including Wnt/β-catenin, PI3K/AKT/mTOR, and MAPK/ERK pathways [30-33].

Targeting KIF11 inhibits invasion, proliferation, and self-renewal in glioblastoma cell lines [34]. A similar effect was observed in breast cancer and prostate cancer cells [30, 35-38]. Filanesib, a potent KIF11 inhibitor, has recently demonstrated clinical efficacy in patients with multiple myeloma [39].

I explored the clinical association of TRIP13 and KIF11 with ccRCC histology and oncologic outcomes using the tissue microarray (TMA) ccRCC cohorts.

6.2.2. Materials and methods

Tissue microarray

Tissue microarray (TMA) slides were obtained from a commercial supplier (US Biomax, Rockville, MD; TMA catalog number HKid-CRC180Sur-01).

The first TMA contained specimens from 92 and the second from 90 patients followed up for 7 years. Each slide contained samples from the tumor and matched normal adjacent tissue (1 core/case).

Retrievable patient data included age, pathology diagnosis, TNM, grade, stage and overall survival. The quality of TMAs was additionally approved by our pathologist. The study followed the principles of the Declaration of Helsinki. The tissues were collected under the highest ethical standards and HIPPA approved protocols with the donor being informed completely and with their consent. Since the tissues were commercially purchased, the study has been exempted from requiring ethical approval.

Immunohistochemistry

The TMA slides were processed at the Department of Clinical Pathology. The primary rabbit polyclonal anti-TRIP13 (HPA005727; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and anti-KIF11 (HPA010568; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) were applied to estimate the expression of TRIP13 and KIF11 proteins, respectively.

The protocol has been standardized using a series of positive and negative control reactions. The positive control reaction was performed on a tissue model selected according to reference sources (The Human Protein Atlas: http://www.proteinatlas.org) and the antibody data-sheet. TRIP13- and KIF11-positive control reactions performed on pancreatic cancer tissue presented cytoplasmic and nuclear expression.

All negative control reactions were performed on additionally analyzed tissue sections by substituting the primary antibody with a solution of 1% BSA (bovine serum albumin) diluted in PBS (phosphate buffered saline). Immunohistochemical staining was performed using primary rabbit polyclonal antibodies (1:200) and visualization system EnVisionFlex+ Anti-Mouse/Rabbit HRP-Labeled Polymer (Dako, Agilent Technologies) on an Autostainer Link48 platform. Finally, tissue sections were dehydrated in ethanol of increasing concentration (from 80% to 98%), then cleared in a series of xylenes (from I to IV) and cover-slipped in a medium (Dako, Agilent Technologies, USA).

IHC analysis and scoring

Initially, two experienced pathologists blinded to the clinical data evaluated the immunostained slides using the light microscope ELIPSE E800 (Nikon Instruments Europe, Amsterdam, Netherlands) at 20× and 40× original objective magnification. IHC revealed cytoplasmic and nuclear proteins expression.

The cytoplasmic staining intensity of cells and percentage of cells at each staining intensity level were determined for each fixed core in the TMA. Staining intensity was graded as 0 (negative), 1+ (weak), 2+ (moderate), and 3+ (strong). The H-score was assigned using the following formula: [1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)], obtaining a value from 0 to 300.

Statistical analysis

All the statistical analyses were performed using Statistica version 10 (StatSoft) and Microsoft Excel 2019. The comparative studies were analyzed statistically using the nonparametric chi-square test. The p value < 0.05 was considered statistically significant.

6.2.3. Results

TRIP13 expression

IHC was performed on 87 pairs of ccRCC and corresponding normal tissues. Five cores of corresponding tissues were lost during the IHC staining procedure.

Cytoplasmic TRIP13 staining was observed in 77 (88.51%) of 87 ccRCC tissues and the median expression was 100 (interquartile range 0-215). Among adjacent controls, 70 (85.37%) of 82 cores were positive and the median expression was 115 (interquartile range 70-200). Cytoplasmic expressions of TRIP13 in ccRCC tissues were lower than those in adjacent controls ($P < 0.05$). I dichotomized the cytoplasmic expressions of TRIP13 to low expression and high expression using the tool *Cutoff Finder*. The cutoff has been set at 105 [40]. Mean TRIP13 expression in TRIP13-rich tumors was significantly higher comparing to adjacent normal tissues ($P < 0.05$). Similarly, adjacent normal tissues were characterized by elevated TRIP13 expression when compared to TRIP13-depleted tumors ($P < 0.05$).

Univariate analysis revealed that patients with high cytoplasmic TRIP13 protein expression had significantly shorter overall survival (OS) comparing to those with low expression ($P < 0.05$, HR = 2.88 [1.35-6.15]). TRIP13 expression did not significantly correlate with stage nor tumor grade ($P > 0.05$).

KIF11 expression

The study included 88 pairs of ccRCC and corresponding non-cancerous tissue. During the IHC staining procedure, 5 cores of corresponding tissue and 2 cores of ccRCC were lost.

64 of 88 ccRCC tissues (72.7%) presented KIF11 cytoplasmic expression with the median value of 20 (interquartile range 0–200). Among controls, cytoplasmic expression was identified in all cases, with a median value of 230 (interquartile range 45–290). Cytoplasmic KIF11 expression in ccRCC tissues was lower compared to control tissues ($p < 0.05$). Cytoplasmic KIF11 expression positively correlated with tumor grade

(p = 0.0013) and mortality (HR 2.17; 95% Cl: 0.99–4.73; p = 0.047). Cox Proportional Hazard was statistically significant only for T1 tumors. HR estimates of 0.19 (95% Cl: 0.04–0.96; p = 0.045) for low KIF cytoplasmic expression and 0.42 (95% Cl: 0.15–1.16; p = 0.049) for high KIF cytoplasmic expression were calculated. KIF11 cytoplasmic expression did not correlate with tumor stage.

6.2.4. Discussion

High levels of two proteins, TRIP13 and KIF11, in the cells of patients with ccRCC are linked to worse survival outcomes. These proteins have the ability to cause serious changes to chromosomes, which suggests they may play a big role in the progression and spread of ccRCC. Therefore, targeting these proteins in addition to other treatments that aim to disrupt cancer evolution may be a promising approach for treating ccRCC.

Evolution-targeted therapy in ccRCC is a novel concept that relies on patient stratification according to the deterministic evolutionary trajectory of the tumor [11].

Currently there are 7 well described evolutionary trajectories in ccRCC according to the tumor's genomic characteristics, evolution mode, and clinical course [10]. While the evolutionary trajectory could be used as a biomarker for guiding the intervention, modulation of TRIP13 or KIF11 expression could further curb ccRCC's evolution.

The cBioportal for Cancer Genomics, a database of genomic data, shows that TRIP13 and KIF11 overexpression in ccRCC is mostly caused by changes to the epigenome, which can be reversed. These changes play a major role in the development of renal cancer. The remaining causes of overexpression include genetic amplifications and mutations [41]. Therefore, if we are able to target these epigenetic changes, it could lead to the reversal of the cancer. Researchers are currently looking into therapies that specifically target these changes. One promising approach is to use these therapies in combination with other treatments such as antiangiogenic or immune checkpoint

inhibitors, as this can help overcome resistance to using a single therapy alone [42, 43-45].

Epigenetic therapeutics are classified into agents that have a targeted effect, such as anti-miRNA oligonucleotides, and agents that have a more broad effect and lead to large-scale changes in gene expression, such as histone deacetylase inhibitors (HDACi) [41]. The principal problem with the first group of agents is their difficult delivery to cancer cells [46]. The second group of agents, however, can cause negative side effects because they turn on genes that are typically turned off. This can affect many processes in the body and may cause unintended consequences [47]. Therefore, it is currently difficult to target epigenetic changes in RCC in a safe and precise manner. As a result, it is yet to be determined if the use of epigenetics in RCC will be successful in a clinical setting.

I analyzed data from the TCGA database to see if the levels of TRIP13 and KIF11 mRNA were related to the progression of ccRCC in patients. The data consisted of information from 528 patients diagnosed with ccRCC, with a median follow-up of 3.28 years [48]. Using the FPKM values for TRIP13 and KIF11, patients were divided into two groups based on the expression levels. The group with higher expression levels had worse survival outcomes, with 5-year survival rates of 39% and 44% for patients with high TRIP13 and KIF11 expression respectively, compared to 70% and 69% for those with low expression. These results support our findings that high expression of TRIP13 and KIF11 mRNA is linked to worse outcomes in ccRCC patients. Our study cohort comprised mainly low-grade and low-stage cases. Therefore, further research incorporating advanced, unresectable tumors is needed to translate our results toward a future potential clinical intervention.

6.2.5. Conclusions.

I developed a concept of exploiting cancers' evolutionary trajectories for guiding intervention and surveillance. This means that by understanding the way cancer cells evolve, we can better predict how the disease will progress and make more informed treatment decisions.

I proposed seven anti-evolution strategies to disrupt the way in which ccRCC cells evolve in order to slow or stop the progression of the disease. Moreover, I estimated the effectiveness of these strategies against each evolutionary trajectory.

Thus, I set a new direction for research into targeted anti-evolutionary therapy. By focusing on specific histological type of cancer and the direction in which they evolve, we can develop more personalized and effective treatments. This approach has the potential to improve outcomes for all cancer patients.

I found out that individuals with high amounts of TRIP13 protein in their ccRCC cells tend to have worse prognosis in comparison to those with lower levels.

Similarly, high levels of KIF11 in ccRCC cells have been correlated with poor clinical outcomes.

Given that TRIP13 and KIF11 have been established as factors that contribute to CIN, the results of my research prompt a hypothesis that this mechanism may also play a role in the development of ccRCC.

Our studies focused on patients with early-stage and less aggressive forms of ccRCC. More research is needed to confirm these findings in patients with more advanced, inoperable tumors in order to see if these results can be applied in a clinical setting.

The presented results could potentially serve as a starting point for translational research, where the modulation of TRIP13 and KIF11 expressions would provide new therapeutic strategies for ccRCC.

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I declare that my substantive contribution to creation of this work is:

- conceiving of the presented idea
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- interpretation of the results
- taking the lead in writing the manuscript

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I declare that my substantive contribution to creation of this work is:

- supervising data quality
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I consent to the submission of the indicated work by Adam Kowalewski as part of his doctoral dissertation in the form of a thematically coherent collection of articles published in peer-reviewed scientific journals.

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I declare that my substantive contribution to creation of this work is:

- supervising data quality
- providing critical feedback
- overall directing and planning

I consent to the submission of the indicated work by Adam Kowalewski as part of his doctoral dissertation in the form of a thematically coherent collection of articles published in peer-reviewed scientific journals.

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I declare that my substantive contribution to creation of this work is:

- supervising data quality
- performing part of the analytic calculations
- providing critical feedback

I consent to the submission of the indicated work by Adam Kowalewski as part of his doctoral dissertation in the form of a thematically coherent collection of articles published in peer-reviewed scientific journals.