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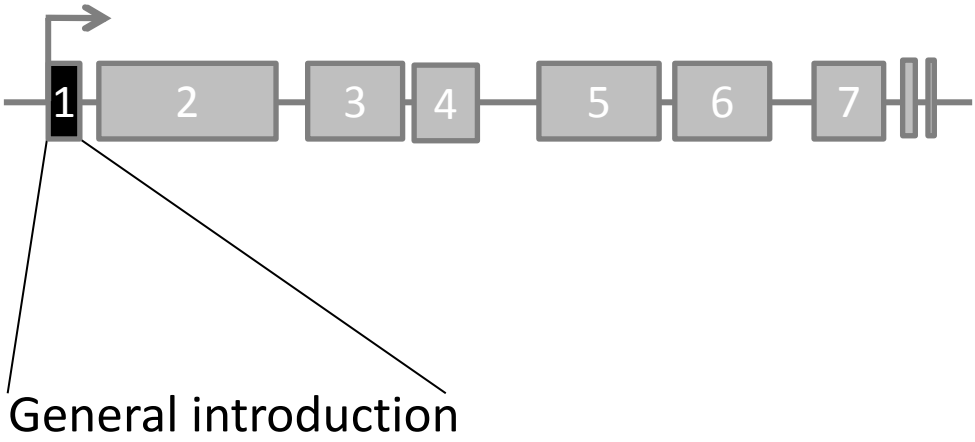
List of abbreviations

AIC	Akaike information criterion
AJCC	American Joint Committee on Cancer
BC	breast cancer
BMI	body mass index
BMP	bone morphogenetic protein
BPE	bovine pituitary extract
BSseq	bisulfite sequencing
ccRCC	clear cell renal cell carcinoma
cDNA	copy deoxyribonucleic acid
chrRCC	chromophobe renal cell carcinoma
CI	confidence interval
CIN	chromosomal instability
COBRA	combined bisulfite restriction analysis
CRC	colorectal cancer
DAC	5-aza-2'-deoxycitidine
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
DNMT	DNA methylatransferase
ECOG	Eastern Cooperative Oncology Group
ECP	endothelial cell proliferation
EGF	epidermal growth factor
EMT	epithelial to mesenchymal transition
FBS	fetal bovine serum
FDA	Food and Drug Administration
FFPE	formalin-fixed paraffin-embedded
HDAC	histone deacytelases
HLA	human leukocyte antigen
HMT	histone methyl transferase
HPH	HIF prolyl hydroxylase
HR	hazard ratio
HRE	hypoxia responsive element
HUVEC	human umbilical vein endothelial cells
IUAC	International Union Against Cancer
K-SFM	Keratinocyte serum free medium
LLN	lower limit normal
LOH	loss of heterozygosity
LRES	long-range epigenetic silencing
MBD	methyl binding domain
MFS	metastasis free survival
MIRA	methylated-CpG island recovery assay
mRCC	metastatic renal cell carcinoma
mRNA	messenger ribonucleic acid
MSI	microsatellite instability
MSKCC	Memorial Sloan-Kettering Cancer Center

MSP	methylation-specific PCR
mTOR	mammalian target of rapamycin
MVD	microvessel density
NCI-EORTC	National Cancer Institute - European Organization for Research and Treatment of Cancer
NGS	next-generation sequencing
NLCS	Netherlands cohort on diet and cancer
OR	odds ratio
OS	overall survival
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pRCC	papillary renal cell carcinoma
PTX	paclitaxel
(q)RT-PCR	(quantitative) reverse transcriptase PCR
RCC	renal cell carcinoma
RCT	randomized clinical trial
REMARK	REporting recommendations for tumor MARKer prognostic studies
RIN	RNA integrity number
RNA	ribonucleic acid
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute medium
RR	risk ratio
TCP	tumor cell proliferation
TGF-β	transforming growth factor-β
TKI	tyrosine kinase inhibitor
TNM	tumor node metastasis
TSA	Trichostatin A
TSG	tumor suppressor gene
TSS	transcription start site
ULN	upper limit normal
VSMC	vascular smooth muscle cells
WHO	World Health Organization

Contents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General introduction

Renal cell cancer in the year 2011

Kidney tumors account for approximately 2% of all adult malignancies and in 2008, worldwide more than 270,000 new cases have been diagnosed and more than 115,000 people died of this disease[1]. Renal cell cancer (RCC) represents the vast majority (80-85%) of all kidney tumors and is histologically classified into clear cell (ccRCC, 60-80%), papillary (pRCC type I and type II, 10-15%), chromophobe (chrRCC, 5-10%), and some less frequent subtypes like collecting duct carcinoma and medullary carcinomas (<1%). In case of localized disease, RCC is curable with surgery, but for those patients with distant metastases the prognosis is poor. Approximately 30% of all RCC patients have metastatic disease at time of diagnosis and in another 30% of patients recurrence develops after complete resection of the primary tumor. Current surgical and pharmaceutical interventions have limited success in treating patients with advanced RCC. From 1992 until 2005, interleukin 2 (IL-2) and interferon α (IFNα) immunotherapy has been the treatment of choice for metastatic RCC (mRCC) but with limited efficacy. Only about 5% to 15% of cases of mRCC respond to immunotherapy and most of these responses are partial or of short duration[2, 3], although for (the highly toxic) IL-2 in some patients a complete remission has been reported.

Since 2005, the high angiogenic potential of RCC has become the first target of choice for treatment options. Tumor angiogenesis is one of the major hallmarks of cancer in general[4] and the high angiogenic potential of (advanced) RCC provided a strong rationale to investigate targeted therapy in this type of tumors. Abnormal functioning of the *von Hippel-Lindau* (*VHL*) gene plays a key role in induced angiogenesis through a hypoxia-driven pathway (accumulation of hypoxia-inducible factor (HIF)) and is the predominant genetic aberration in RCC. The vascular endothelial growth factor (VEGF) and the mammalian target of rapamycin (mTOR) signal transduction pathways have particularly been exploited. Thanks to this improved understanding of the molecular biology of mRCC, its treatment has recently evolved from predominantly cytokine-based to the use of drugs targeting VEGF and its receptor (VEGFR), platelet derived growth factor (PDGF) and its receptor (PDGFR) or mTOR. Since 2005, agents such as the tyrosine kinase inhibitors (TKIs) sorafenib, sunitinib, and pazopanib, the anti-VEGF monoclonal antibody bevacizumab (in combination with IFNα), and the mTOR inhibitors temsirolimus, everolimus dominate the mRCC treatment protocols and more are yet to come. Figure 1 shows the timeline of registration of these agents in the USA and Europe and indicates the targets within the angiogenesis-related pathway.

The significant progress in unraveling the biology of RCC and the treatment opportunities identified through this knowledge for mRCC generate possibilities for a personalized approach and tools to improve management of the disease (see Figure 1) are being developed. Currently, patient performance status, TNM stage and Fuhrman nuclear grade are the most useful predictors of patient outcome[5]. In metastatic RCC a prognostic model was developed by Motzer et al. at the Memorian Sloan Kettering Cancer, which is known as the MSKCC risk system, or informally known as the “Motzer criteria”[6]. This model is widely used as standard prognostic criteria for selecting patients to participate in clinical trials and for recommending treatment options to patients not enrolled in trials. However, this system, only used for RCC patients with metastatic disease, was derived in the cytokine era leading to importance of re-

evaluation of prognostic markers in the current era of 'targeted' therapy. In order to better identify subgroups of patients with different clinical outcome, the increasing knowledge of the biology of cancer and the identification of molecular markers for targeted therapy provide opportunities for medical oncologists to better manage RCC.
Although defective VHL functioning is a key event in the development ccRCCs[7-9], alterations in the structure[9, 10] or regulation of the *VHL* gene[11, 12] do not appear to be directly associated with tumor cell proliferation and patient prognosis[13, 14]. Therefore a need exists for molecular markers predicting prognosis (so-called prognostic markers) and for molecular markers predicting response to therapy (so-called predictive markers). These markers should provide a personal tumor profile to guide personalized therapy of RCC and prevent the serious treatment-related toxicity experienced by a large group of RCC patients as a consequence of 'trial and error' treatment.

Molecular alterations in cancer provide opportunities to develop specific biomarkers. Genetic aberrations have been characterized in RCC and for each subtype one or more important mutations have been described, like *VHL* in ccRCC, the *MET* proto-oncogene and *fumarate hydratase* (*FH*) tumor suppressor gene (TSG) in pRCC, *Birt-Hogg-Dubé* (*BHD*) in chrRCC, and *tuberous sclerosis complex 1* (*TSC1*) and 2 (*TSC2*) TSGs in all three subgroups. So far, additional genomic research in RCC has not yet revealed genetic marks in a significant portion of RCCs with a biomarker potential.
Besides genetic alterations, epigenetic abnormalities (heritable changes in gene expression by processes other than altered DNA sequence) have been recognized at the level of DNA (postreplicative DNA methylation), RNA (RNA interference (RNAi)), and protein (posttranslational histone modifications and polycomb group complexes). DNA methylation is the most widely studied epigenetic modification in humans and of importance in maintaining normal development and biology. The most common sequence context where this epigenetic mark is found is the CpG dinucleotide. Many diseases are associated with the addition of methyl (CH3)-groups to cytosine bases at the wrong time or at the wrong place, especially cancer [15]. Cancer cells are characterized by a massive global loss of DNA methylation[16, 17] and, in contrast, at the same time by specific patterns of hypermethylation at CpG-rich regions (CpG islands) of certain gene promoters.

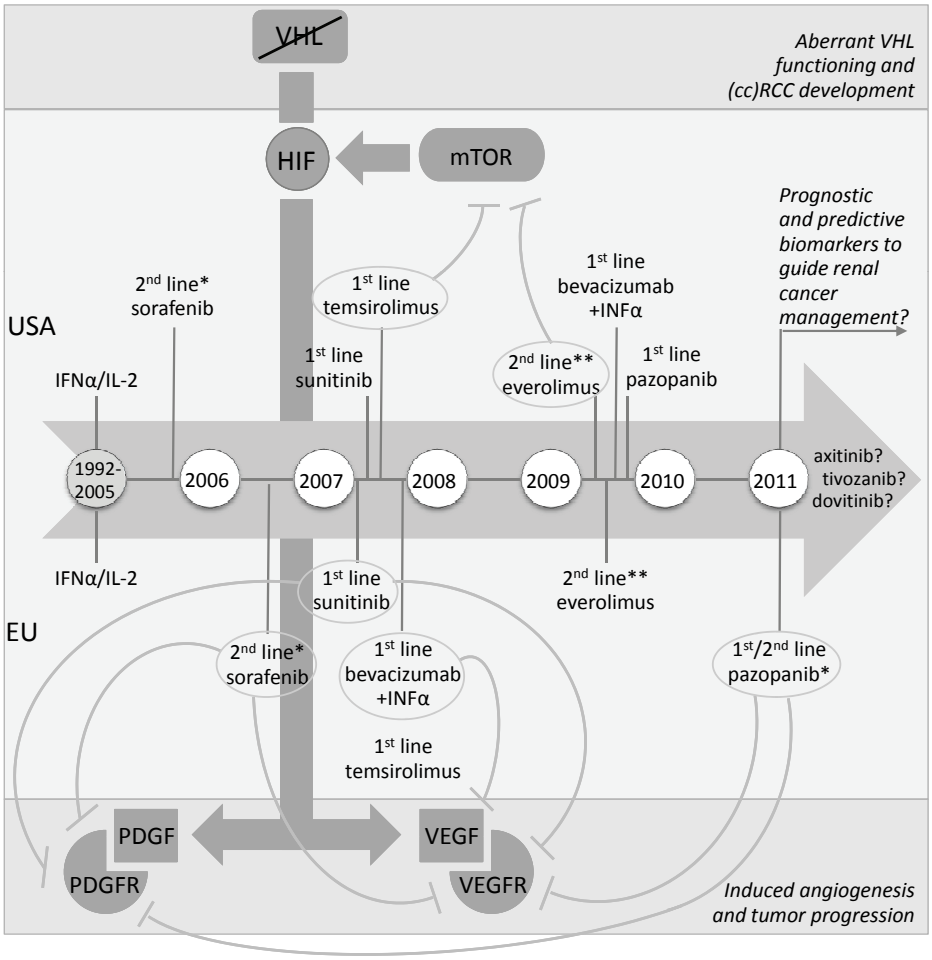

Figure 1. Timeline of targeted therapy registration for mRCC in the USA and Europe
Chronology of registered molecular-targeted agents in metastatic renal cell carcinoma in the U.S. and Europe is depicted in relation to their targets. VHL functioning is abnormal in most ccRCCs and deregulates the HIF-pathway. Targeted agents have been developed against one or more of the players in this pathway. Since December 2005 and July 2006 targeted therapies have been registered for the treatment of (cc)RCC in the USA and Europe, respectively. Sequence of therapy administration and several other agents are still under investigation. From now it is important to personalize these treatments and development of biomarkers is needed to guide renal cancer management. Prognostic biomarkers should indicate which patients benefit from adjuvant therapy, while predictive biomarkers should indicate from which therapy (and in what dose) an individual patient would benefit, with the lowest toxicity. *2nd line therapy after 1st line IFNα, **2nd line therapy after 1st line TKI.

Genomic research in RCC has revealed that, in contrast to other tumor types, candidate TSGs are mutated in less than ten per cent of the RCCs with the exception of *VHL*[18] and the recently identified SWI/SNF chromatin remodeling complex gene *PBRM1*, which showed truncating mutations in 41% of cases[19]. However, a much larger number of candidate TSGs is frequently silenced by cancer-specific promoter hypermethylation[20, 21]. The stability and the frequency of aberrant DNA hypermethylation in cancer, the possibility to determine hypermethylation in formalin fixed paraffin-embedded (FFPE) tissue, and the simple and low cost tests that are available for as-

sessing DNA hypermethylation, make these epigenetic marks promising potential biomarkers[22]. Proof of principle for the clinical value of hypermethylation markers in cancer has been reported for early detection and classification of cancer[23-33], risk assessment and prognosis [31, 34-36] and prediction of therapy response[37-39], with some already having demonstrated their importance in (pre)clinical practice. Increasing interest in epigenetics and especially in DNA hypermethylation has resulted in rapid technological progress to analyze methylation loci in a high-throughput manner. Moreover, current methods enable unbiased profiling of DNA methylation in a genome-wide scale identifying a so-called methylome. Comprehensively exploring the RCC methylome will be a major step towards a better understanding of how the RCC genome works and provides opportunities to identify and develop clinically relevant biomarkers. In order to personalize treatment protocols for RCC patients, prognostic and predictive methylation markers are urgently needed.

Hypothesis, aim and outline of the thesis

As the biology of RCC is incompletely understood and biomarkers for RCC are lacking, we decided to address these issues in this thesis. We **hypothesized** that exploration of the renal cancer methylome will identify methylation marks that are associated with prognosis of RCC patients and can be developed into clinically useful disease markers. Therefore, the **aim of this thesis** was to comprehensively study the renal cancer methylome, specifically clear cell renal cell carcinoma, the most common type of renal cell cancer, to gain insight into RCC biology and to identify novel prognostic biomarkers.

In chapter 2 we review the current knowledge on RCC, including the epidemiology, clinical presentation, risk factors, classification, prognosis and therapy. We highlight the genetic presentation of the common RCC subtypes and comprehensively describe the current role of epigenetics and the reported hypermethylation of putative TSGs involved in RCC development.
Using a well established method to identify hypermethylated TSGs (expression microarrays on pharmacological demethylated cells)[40], we identified tumor specific promoter CpG island hypermethylation of *Gremlin1* (*GREM1*). In chapter 3 we describe its prognostic significance in two large series of ccRCC. Investigating methylation of the *GREM1* promoter in detail, we discovered that the clinical relevance of methylated CpG dinucleotides is strongly dependent on the location in the promoter CpG island. We show that only hypermethylation of so-called 'core-regions' is correlated with prognosis, while hypermethylation at other regions does not have any clinical impact. This led us to evaluate the general importance of the location of CpG dinucleotide hypermethylation in regulating gene expression and associations with clinicopathological characteristics in cancer (chapter 4). We clarify the misconception and oversimplification of the topic of hypermethylation and gene silencing in cancer. Appreciating the significance of location in methylation-based research, we comprehensively explored the RCC methylome by integrating expression arrays following pharmaceutical demethylation and genome-wide methylation specific next-generation sequencing. Chapter 5 covers the gene discovery and the clinical and biological validation of the identified candidate genes identified by this methodology. In chapter 6 we investigate the simi-

larities and differences between several cancer methylomes as assessed by the method used in the previous chapter. We hypothesized that the methylome of RCC is distinct from that of extensively studied epithelial cancers such as colorectal or breast cancer. Finally, the general discussion in chapter 7 summarizes and discusses the major findings of the studies performed and builds upon the putative role of methylation markers in RCC and its treatment.

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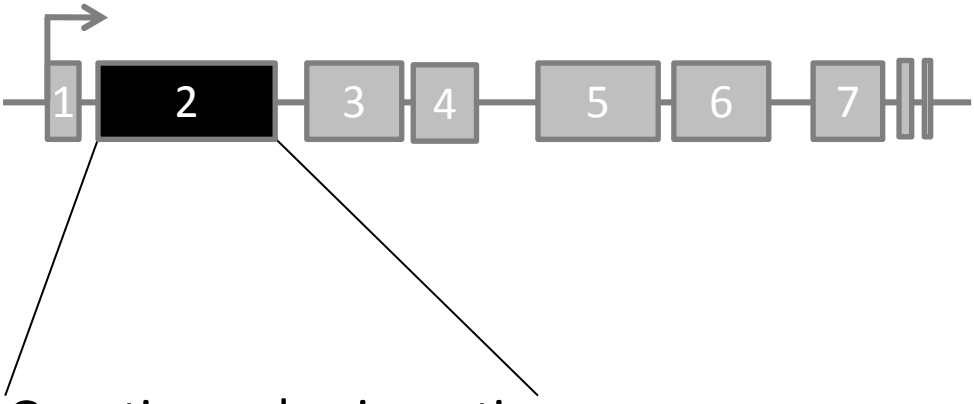

Genetics and epigenetics of renal cell cancer

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Abstract

Renal cell carcinoma (RCC) is not a single disease, but comprises a group of tumors of renal epithelial origin, each with a different histology, displaying a different clinical course and caused by different genetic alterations. Since cure rates are inversely associated with stage and response to the available treatment regimes is limited to a subgroup of patients, diagnostic methods facilitating early detection and new therapeutic modalities are necessary. Increased knowledge of the underlying pathophysiology of RCC has resulted in the identification of genetic alterations involved in renal cell cancer carcinogenesis. Promising agents to target these pathways, especially the angiogenesis pathway, are being developed, some of which are already standard of care. In addition to genetics, knowledge on epigenetics in the process of renal tumorigenesis has been significantly increased in the last decades. Epigenetics will play an increasing role in the development of new therapeutic modalities and may deliver new prognostic and early diagnostic markers.
In this review we discuss the background of RCC and the clinical applications of RCC genetics and epigenetics.

Introduction

Descriptive epidemiology

In 2002, worldwide about 210,000 cases of kidney cancer have been diagnosed and 102,000 persons died because of kidney cancer[1]. In the European Union and in the United States, kidney cancer is the 9th most common cancer. Incidence and mortality rates are approximately twice as high for men as for women[1].
Incidence rates for renal cell cancer have been rising steadily each year in Europe and the United States over the past three decades[2]. This increase cannot be explained completely by the observed decrease in mean tumor size, indicating improved detection[3], since an increasing incidence of late-stage renal cell cancers also has been observed[2, 4]. Rates of renal cancer vary internationally more than tenfold, suggesting a strong role for environmental risk factors. Incidence is generally highest in several Western and Eastern European countries and Scandinavia, as well as in parts of Italy, in North America and in Australia/New Zealand. The lowest rates are reported in Asia and Africa[1].

Risk factors

Several factors have been investigated in association with the risk of renal cell carcinoma (RCC). Some risk factors, such as cigarette smoking[5-8], obesity[5, 6, 8, 9], hypertension and/or use of antihypertensive medication[7, 10, 11] were consistently reported to be positively associated with RCC risk, although risk ratios (RRs, see appendix I for explanation) were only moderately increased.
A recent meta-analysis on cigarette smoking in relation to RCC concluded that inhaled tobacco smoke is clearly implicated in the etiology of RCC. Risk was increased for former (RR: 1.21; 95% CI: 1.07-1.36) and current (RR: 1.45; 1.26-1.66) smokers with a strong dose-dependent increase in risk[12]. Most studies observed increasing risks, not only with the number of cigarettes smoked per day but also with the number of years smoked and decreasing risks with number of years since cessation, as is also shown in a meta-analysis by Hunt et al.[12].
A positive association between body weight and risk of renal cell cancer has been observed in almost all case-control and cohort studies[7]. In a quantitative review, a cumulative risk per 1kg/m2 increase in BMI of 1.07 (95% CI: 1.05-1.09) was found for men and women[13]. The attributable proportion of RCC for excess body weight is estimated to be 25 percent for both men and women in the European Union[14].
Hypertension and antihypertensive medication have been found to be risk factors for RCC in many epidemiological studies[10, 11]. In a meta-analysis[10] based on 13 case-control studies a pooled adjusted odds ratio of 1.75 (95% CI, 1.61-1.90) was calculated for the association between hypertension and RCC. In another meta-analysis, a pooled odds ratio was calculated for use of diuretics and risk of RCC[11]. Based on nine case-control studies, an average odds ratio was calculated of 1.55 (95% CI, 1.42-1.71). It is unclear, whether the increased risk is caused by hypertension itself, or by the use of antihypertensive medication. Some recent studies showed that diuretic medication is no longer a risk factor after controlling for the diagnosis of hypertension[15, 16], suggesting that not medication but hypertension is a risk factor for RCC. It is also conceivable that hyper-

tension acts as biomarker for another underlying disease that might be causally associated with RCC.
Furthermore, dialysis patients with acquired cystic disease of the kidney showed a 30 times greater risk than in the general population for developing RCC[17]. Whether prolonged ingestion of analgesic combinations (phenacetin, aspirin), leading to chronic renal failure, enhances the incidence of RCC remains controversial[18-20].
Several studies have been conducted investigating whether occupational exposures are associated with RCC risk. Exposure to asbestos, polycyclic aromatic hydrocarbons, gasoline have not been shown to be consistently associated with RCC risk[21]. Interestingly, occupational exposure to trichloroethylene has been shown to be associated with specific mutations in the *VHL* gene in RCC[22, 23].
Reports on other factors showed less consistent associations with RCC risk. These factors include alcohol consumption, vegetable and fruit consumption, intake of micronutrients and vitamins[5, 6, 8, 9]. Most studies on alcohol consumption and RCC risk observe null associations[24-30] while some more recent studies suggest an inverse association[31-34], although this effect may be limited to women[35]. In a recent, pooled analysis of 12 prospective cohort studies, intake of alcohol was associated with a decreased risk of renal cell cancer. Compared to non-drinkers, alcohol consumption of $\geq$15 grams/day was associated with a pooled RR of 0.72 (95% CI: 0.60-0.86)[36].
Finally, patients with certain inherited disorders such as von Hippel-Lindau disease, hereditary papillary renal cancer, a hereditary leiomyoma RCC syndrome and Birt-Hogg-Dubé syndrome, show an enhanced risk of RCC[37-39]. These inherited disorders show specific genetic abnormalities (see also the genetic section).

Classification

The majority of kidney cancers (80-85%) are RCCs originating from the renal parenchyma. The remaining 15-20% are mainly transitional cell carcinomas of the renal pelvis. Other less frequent malignant tumors of the kidney are metanephric adenosarcomas, mesenchymal tumors, mixed mesenchymal and epithelial tumors, neuroendocrine tumors, hematopoietic and lymphoid tumors, germ cell tumors and metastatic tumors. Though nephroblastomas (Wilms tumor) are the most common renal tumors of childhood, few cases have been reported in adulthood[40-42].
RCC is almost exclusively a cancer of adults and is rare in the first two decades of life, comprising only 2% of pediatric renal tumors.
RCC is not a single entity (see figure 1), but comprises a group of tumors, which arise from the epithelium of renal tubules. Extensive histological and molecular evaluation has resulted in the development of a consensus classification of different RCC subtypes. In 1996, the Heidelberg classification of RCC was proposed, which intends to integrate the understanding of genetic alterations and readily recognizable histological criteria[43]. In this classification, three major histological subtypes of RCC, clear cell (ccRCC), papillary (pRCC) and chromophobe (chrRCC) RCC have been identified in addition to some less frequent subtypes such as multilocular ccRCC, collecting duct carcinomas, medullary carcinomas and unclassified types. The latest WHO classification of 2002 is more comprehensive and includes, in addition to the previous subtypes, renal

carcinoma associated with neuroblastoma and two recent added subtypes, the Xp11 translocation carcinomas and mucinous tubular and spindle cell carcinoma (Table 1).

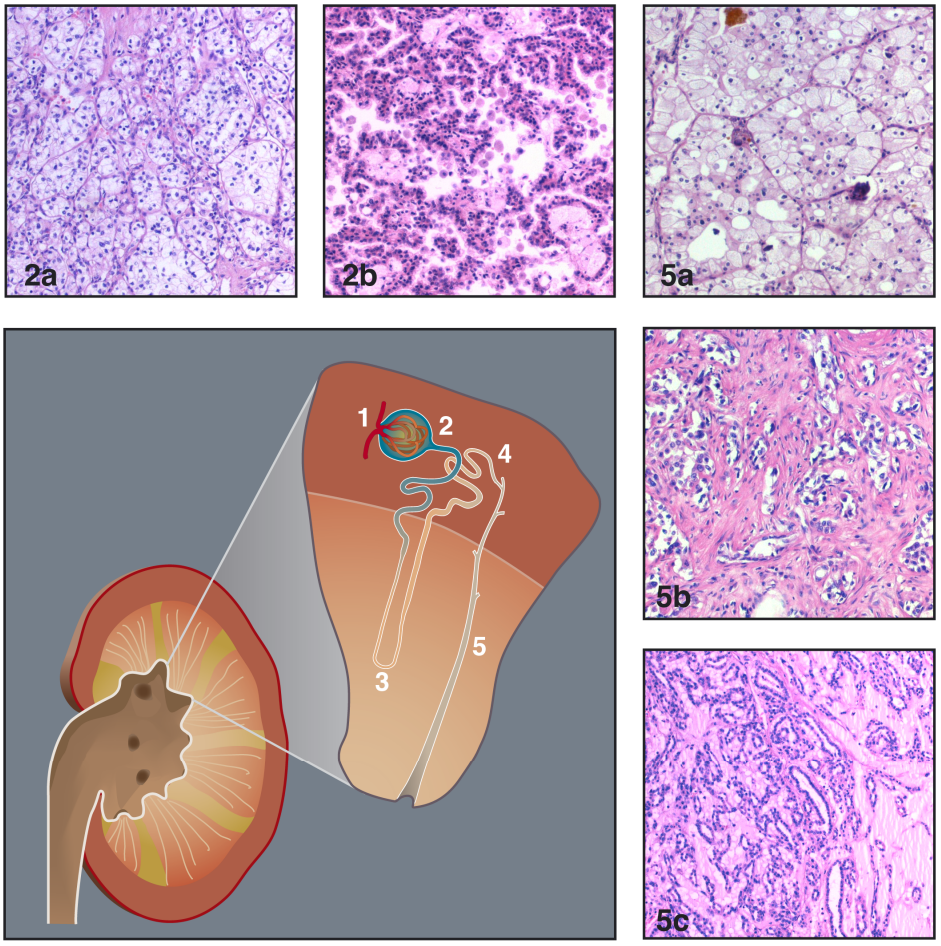

Figure 1. Classification of RCC subtypes: cell of origin
The nephron, consists of the renal corpuscle (1) and renal tubule, which is divided into four histo-physiological zones: proximal convoluted tubule (2), loop of Henle (3), distal convoluted tubule (4) and collecting tubule (5). The different RCC subtypes are thought to originate from different parts of the renal tubular system: mainly the proximal convoluted tubule (2a clear cell RCC and 2b papillary RCC) and the collecting tubule (5a chromophobe RCC, 5b collecting duct carcinoma, and 5c mucinous tubular and spindle cell carcinoma).

Table 1. Histological subtypes of RCC (WHO classification, 2002)

	Frequency (%)	Age	Male/female ratio	Ref.
Clear cell renal cell carcinoma	70	61[a]		43, 251
Papillary renal cell carcinoma	10-15	61[a]	1.8/1 to 3.8/1	43, 44, 57, 252
Chromophobe renal cell carcinoma	5	60[a]	1/1	43, 58
Renal cell carcinoma unclassified	4-5			43, 62
Multilocular clear cell carcinoma	< 5	51[a]	3/1	43, 253
Carcinoma of the collecting ducts of Bellini	< 1	55[a]	2/1	43
Renal medullary carcinoma	< 1	22[a]	2/1	61, 254, 255
Xp11 translocation carcinomas	< 1	15[b]	4/7	63, 64
Carcinoma associated with neuroblastoma	< 1	13.5[b]	1/1	66
Mucinous tubular and spindle cell carcinoma	< 1	53[a]	1/4	65

[a] mean age (years)
[b] median age (years)

Clear cell carcinoma (70% of all RCCs) and pRCC (10-15%) account for the majority of RCCs. They are thought to arise from cells in the proximal convoluted renal tubule[38, 43, 44]. Most cases of ccRCC are unilateral and unifocal. Invasion of perirenal and sinusoidal fat and/or extension into the renal vein occurs in about 45% of ccRCC[45]. ccRCC most commonly metastasize hematogeneously via the vena cava primarily to the lung, although in 7% to 17% lymphatic metastases occur[46, 47]. In 5% of ccRCCs a predominantly cystic growth pattern is seen. These tumors are called multilocular cystic RCCs. This RCC subtype mostly present with low Fuhrman grade and stage at diagnosis and display a favorable outcome, suggesting a tumor with low malignant potential[48-53]. Though no recurrences or metastasis have been reported, additional outcome based research is needed because of the rarity of this subtype and the limited number of patients reported so far in the literature.

pRCC is subclassified in two subtypes depending on the morphology of the tumor cells covering the papillary lesions[54]. Type 1 tumors have papillae covered by a single layer of small cells with scant cytoplasm, whereas in type 2 tumors the papillary cores are covered by pseudostratified epithelium with a larger amount of eosinophilic cytoplasm and often higher nuclear grade. Up to 70% of pRCCs are intrarenal at diagnosis[55, 56]. Type 1 tumors are usually of lower grade and stage than type 2 tumors[56, 57]. Bilateral and multifocal tumors are more common in pRCC than in the other malignant subtypes. The chromophobe variant of RCC accounts for 5 percent of all cases of RCC and is thought to originate from type B intercalated cells of renal collecting ducts[44, 58]. Only 10% of chrRCC show extension through the renal capsule into the surrounding adipose tissue and in only 4% there is involvement of the renal vein[58]. Collecting duct (Bellini duct) carcinomas are thought to arise from the collecting ducts within the renal medulla[44, 59]. These tumors may present at any age, although they tend to occur in younger patients than the former subtypes. These tumors are very aggressive, one third exhibiting metastases at the time of presentation. Approximately two-thirds of patients die of this disease within two years of diagnosis[60]. A variant of the collecting duct carcinoma is the so-called medullary carcinoma[61], which develops preferentially in young patients with sickle trait. These tumors are now widely regarded as a more aggressive variant of collecting duct carcinoma, with a mean survival time after surgery of 15 weeks[60]. The unclassified RCCs are a diagnostic category of renal tumors which do not fit into one of the other subtypes. In surgical series, this group often amounts to 4-5% of all cases[43, 44]. Many of the tumors from this category are high cytomorphologic

grade and show aggressive clinical behavior[62]. Translocation-associated RCC affect predominantly children and young adults and present at advanced stage[63, 64]. Mucinous tubular and spindle cell carcinoma is recently identified as a novel entity of low-grade renal epithelial neoplasm, characterized by an indolent course and, in contrast with the other subtypes, female predominance. Its immunohistochemical and ultrastructural features are consistent with distal nephron origin[65]. Finally, RCC associated with neuroblastoma occurs in long-term survivors of childhood neuroblastoma, by which males and females are equally affected. These RCCs are morphologically heterogeneous and prognosis correlates with tumor grade and stage[66].
Most cases of RCC occur sporadically. An inherited predisposition to renal cancer accounts for only 1-4% of all cases[38]. Inherited renal neoplasms are more likely to be diagnosed at an younger age and are more likely to be multifocal and bilateral.

Prognosis

A gradual improvement in prognosis has been observed over time, with 5-year relative survival rates for RCC as high as 64% in 2002, compared with less than 40% in the early 1960s[67]. The clinical behavior of RCC results from complex interactions between multiple prognostic factors. Currently, patient performance status, tumor stage and grade are the most useful, clinical available predictors of patient outcome[68-70]. The performance status is measured using the Eastern Cooperative Oncology Group (ECOG) assessment scale. This scoring system stratifies patients on the basis of ambulatory status, in order to denote the impact of disease on the overall health of the patient[71].
Currently, the most commonly used staging system is the International Union Against Cancer (IUAC) and the American Joint Committee on Cancer (AJCC) tumor-node-metastases (TNM) staging system. The 2002 modification of this system (sixth edition) has recently been confirmed to have a better prognostic ability than the previous 1997 staging system[72]. Using the 2002 staging system, the 5-year cancer specific survival rates ranged from 97% for pT1A to 20% for pT4. Presence of lymph node metastases predicts a poor prognosis, with cancer-specific survival rates of 5-30% and 0-5% at 5 and 10 years, respectively[73]. Metastases to other organs (lung, brain, bone) are associated with survival rates of 50%, 5-30% and 0-5 % at 1, 5 and 10 years respectively. Grading is based on the Fuhrman system, which consists of four grades, based on the nuclear size, contour and conspicuousness of nucleoli[74].
Kim et al. examined a cohort of 1,046 patients with localized and metastatic RCC and identified hypoalbuminemia, weight loss, anorexia and malaise as independent predictors of poor prognosis[75]. The presence of thrombocytosis (platelet count > 400,000/mm^3) has been shown to be an independent predictor of poor outcome in patients with metastatic RCC treated with nephrectomy and adjuvant therapy[76].
Whether different histologic RCC subtypes have different clinical behavior and outcome remains a topic of discussion. Some reports demonstrated worse outcome for ccRCC compared with pRCC and chrRCC[77, 78]. However, in multivariate analyses, TNM stage, Fuhrman grade and ECOG, but not histology, were retained as independent prognostic variables[69]. Tumor necrosis was found to be a strong independent predictor of poor outcome for clear cell but not for chromophobe or papillary RCC[79, 80]. Micro-

vascular invasion and presence of sarcomatoid features are reported negative prognostic indicators[81, 82].

Since multiple factors affect prognosis, many centers have aimed to integrate independent prognostic indicators into comprehensive outcome models for both nonmetastatic and metastatic RCC to assist clinicians in patient counseling, management decisions and also for clinical trial comparisons[80, 83-94]. The most widely accepted prognostic system is the Memorial Sloan Kettering system for clinical trial assessment and treatment assignment. This model was based on a study of 670 patients with advanced RCC treated in 24 separate clinical trials at Memorial Sloan-Kettering Cancer Center (MSKCC; New York, NY) between 1975 and 1996[92]. Initially, five prognostic variables associated with poor survival: low performance status (Karnofsky < 80%), high serum lactate dehydrogenase (> 1.5 times the upper limit of normal), low hemoglobin (less than the lower limit of normal), high corrected serum calcium (> 10 mg/dL), and absence of prior nephrectomy were identified using uni- and multivariate analysis. The number of risk factors present is used to stratify patients into favorable- (zero risk factors), intermediate- (one or two risk factors), and poor-risk groups (three or more risk factors). In a subsequent analysis of 463 interferon alfa (IFNα) treated patients, the MSKCC model remained the same, except time from diagnosis to treatment with IFNα of less than 1 year was found to be a better predictor of short survival compared with absence or prior nephrectomy[91]. This MSKCC-model is nowadays used in counseling and stratification of patients in phase III trials.

Recent identification and incorporation of molecular markers into current staging systems may result in better prognostic tools. By measuring protein expression or gene copy number, several molecular factors have been identified as independent risk factors for developing RCC, including B7-H1, Smac/DIABLO, survivin, PTEN, p27 and VEGF[95-99]. Other reported prognostic indicators are HIF-1α, CAIX, Ki 67, gelsolin, vimentin, p53 and EphrinA2[100-102]. Until now, there have been no molecular markers for RCC that meet the College of American Pathologists criteria for a marker to be used generally in patient management or even to have been studied sufficiently biologically to provide for any degree of acceptance[103].

Therapy

Current treatments of patients with RCC can be divided in local therapy and systemic therapy.

Local therapy

For patients with localized tumors larger than four centimeters in diameter regardless of histological subtype, radical nephrectomy is currently the therapy of choice[104]. Partial nephrectomy has gained acceptance for treating tumors smaller than four centimeter in diameter or indications such as solitary kidney, bilateral renal tumor localization, renal insufficiency as well as the presence of hypertension, diabetes or hereditary renal cell carcinoma syndromes[104].

In 20-25% of patients, metastases are present at the time of diagnosis of the disease. Based on the results of two randomized studies, showing a survival advantage of 3[105] to 10 months[106] nephrectomy as cytoreductive treatment before embarking on sys-

temic therapy with cytokines has been accepted as standard care. However, since high dose interleukin-2 (IL-2) can be effective in patients with high metastatic burden and a relatively small primary tumor, some reports suggest that nephrectomy should not be offered in this specific group of patients[107]. In patients who will be treated with targeted therapy, the role of cytoreductive surgery is not yet known and needs to be reassessed.
The response rate of RCC to radiotherapy is 50% and it may be useful as palliative therapy in patients who are not eligible for surgery or in case of bone or central nervous system metastases[108].

Systemic therapy
Hormonal and chemotherapy are not standard treatment in RCC because response rates of these agents are low[109]. Drug resistance may be related to the expression of the multidrug resistance transporter in the proximal tubule cells, from which ccRCC and pRCC originate[110, 111]. For more than 20 years, immunotherapy has been the leading treatment for metastatic RCC. Prolonged stabilization of disease in the absence of systemic therapy and rare spontaneous regressions suggest that host immune mechanisms play a role in tumor growth regulation[112]. Therefore, RCC became attractive to test immunologic interventions, e.g. IL-2, IFNα, stem cell transfusions from HLA-compatible siblings following immunoablative chemotherapy[113] and tumor vaccines[114].
Approximately 5 to 15% of cases of metastatic ccRCC respond to IFNα, however most of these responses are partial or of short duration[115, 116]. Treatment with IL-2, the current Food and Drug Administration (FDA)-approved agent for patients with advanced renal carcinoma is associated with a complete response in 10% and partial response in 11% of patients[61]. Given the toxicity, expense and variable response to IL-2 among patients with metastatic RCC, several clinical, histological and molecular response predictors have been evaluated[107, 117]. Cytokine based therapy currently remains an option for patients with metastatic ccRCC and good prognosis according to MSKCC-risk classification[118].
Targeted agents, such as Sunitinib, Sorafenib and Temsirolimus have shown efficacy in phase III trials, and were recently approved by the US FDA for treatment of metastatic RCC and are currently included in clinical practice.
Sunitinib (SU11248) is a multi-targeted tyrosine kinase inhibitor (TKI) inhibiting the receptor tyrosine kinases VEGFR2, PDGFR, FMS-like tyrosine kinase 3 (FLT-3) and c-KIT. Sunitinib was the first therapy to demonstrate superiority over standard cytokine therapy (IFNα) as first line treatment in 750 patients with mainly good and intermediate prognosis according to MSKCC-risk classification[119].
Another oral multi-targeted TKI is Sorafenib (BAY 43-9006) which inhibits the non-receptor serine threonine kinases BRAF and CRAF and the receptor tyrosine kinases VEGFR2, VEGFR3, FLT-3, c-KIT and PDGFR. Sorafenib showed clinical benefit as compared to placebo in patients with intermediate risk or low risk status according to MSKCC-prognostic score120.
Temsirolimus is an inhibitor of the non-receptor tyrosine kinase mTOR which revealed activity against metastatic RCC with MSKCC-poor risk characteristics[121].
An additional drug in the near future may be Bevacizumab, a monoclonal antibody directed against VEGF. Compared to placebo this drug improved progression free sur-

vival[122]. Results of two randomized trials comparing a combination of IFNα with Bevacizumab with IFNα monotherapy or with a placebo are underway.

Genetics of RCC

During the past two decades, genetic and clinical studies have shown that RCCs are not only heterogeneous in their histology and clinical behavior, but also in their genetic alterations. The identification of families with distinct histological subtypes of RCC has permitted a better understanding of the molecular mechanism of carcinogenesis of the different subtypes, and for each subtype one or more important mutations have been described.
Clear cell, papillary, and chromophobe RCC cover the majority of all renal cancers and are mostly investigated, therefore (genetic) knowledge of the other subtypes, with a very low prevalence, is limited. The genetics of the three most common subtypes will be discussed in this review.

Clear cell renal cell carcinoma – von Hippel-Lindau (VHL) tumor suppressor gene

The most and probably best studied adult renal neoplasm is ccRCC, and particularly the inherited form, related to the *von Hippel-Lindau (VHL)* syndrome. RCC in the heritable *VHL*-syndrome is uniformly of the clear cell subtype[123, 124]. In 1988 the *VHL* gene was mapped to the short arm of chromosome 3 by linkage analysis[125] and in 1993 the gene was identified as a result of positional cloning strategies performed in *VHL* kindreds[126]. Heritable ccRCC is caused by a germline mutation of one allele (80% is inherited of one of the parents and 20% is a 'de novo' mutation) and an acquired mutation of the second allele. Germline *VHL* mutations are identified in nearly 100% of *VHL*-families[127]. The *VHL* gene has been found to have characteristics of a tumor suppressor gene (TSG), in which >150 different germline mutations linked to VHL disease have been identified since 1993[123, 124, 128, 129]. *VHL* mutations are extremely heterogeneous and are distributed throughout the coding sequence, except that mutations are rarely observed within the first 50 codons[124, 130].
In the majority (50%-75%)[130-132] of sporadic ccRCCs, *VHL* inactivation of both alleles have been observed[133], whereby loss of heterozygosity (LOH) of a *VHL* allele is detected in almost all (90%) sporadic clear cell tumors[134]. Inactivation can be a result of somatic mutations, deletions, LOH or epigenetic inactivation such as promoter DNA methylation (see also the epigenetic section). Although mutations in the *VHL* gene are mostly restricted to ccRCC[135], van Houwelingen et al. reported mutations in the other sporadic RCC subtypes, although in much smaller proportions[130].
Heritable and sporadic ccRCC often show two inactivated or silenced *VHL* alleles, which indicates that the loss of function of the *VHL* gene is an important event in the pathogenesis of ccRCC[132].

The VHL protein (pVHL) is the product of the *VHL* gene. Cultured cells produce two proteins, a protein consisting of 213 amino acid residues with a molecular weight of 30 kDa ($pVHL_{30}$) and a variant which corresponds to pVHL residues 54-213 of $pVHL_{30}$. The

latter migrates with a molecular weight of 18/19 kDa ($pVHL_{18/19}$) and is most likely generated by internal transcription initiation from the second methionine start site within the *VHL* open reading frame (Met-54)[136-138].

Insights into the function of pVHL have come from studying the effects of reintroducing the wildtype *VHL* gene into cultures of cells which lack functional pVHL[129, 135, 139]. In these experiments, several aspects of cellular behavior, related to tumor suppressor function, like growth inhibiting, cell cycle arrest, and increased susceptibility to apoptosis, were restored upon reintroduction of the wildtype *VHL* gene[129, 135].

Probably the best known alteration in *VHL* defective cells is the failure to (poly)ubiquitinate and degrade hypoxia inducible factor α (HIF-α) in the presence of oxygen. HIF-1α and HIF-2α, which are unstable but continuously transcribed and translated, are key mediators in the complex cellular and systemic oxygen adaptation cascade triggered by hypoxia[140]. Under normoxic conditions, HIF-α subunits become hydroxylated, enabling binding and ubiquitination by pVHL. Under hypoxic conditions, HIF-α subunits remain non-hydroxylated and are able to complex with stable β-subunits. HIFα-β heterodimers bind DNA at hypoxia responsive elements (HREs) and transcriptionally activate genes involved in acute and chronic adaptation to hypoxia, normally inhibited by pVHL[140, 141]. The proteins regulated by hypoxia-inducible genes are involved in angiogenesis (vascular endothelial growth factor (VEGF)), cell growth (transforming growth factor α (TGF-α) and platelet derived growth factor (PDGF)), glucose uptake (glucose transporter GLUT-1), and acid-base balance (carbonic anhydrase IX (CAIX)). Thus, like in hypoxic conditions, absence of (functional) pVHL leads to accumulation of HIF-α subunits, forming heterodimers with HIF-β, resulting in overexpression of the above mentioned proteins, which creates a microenvironment favorable for tumor proliferation[135, 140]. In addition, the above mentioned proteins act on the neighboring vasculature to promote tumor angiogenesis[135].

Both *VHL* gene products, $pVHL_{30}$ and $pVHL_{18/19}$, can inhibit the production of the hypoxia-inducible proteins when reintroduced into renal carcinoma cells that lack the wild-type *VHL* allele. Therefore, mutations 5' of codon 54 may not lead to a functionally inactive protein, however mutations in the first 50 codons are rarely observed[130].

It has been observed early that RCCs are highly angiogenic tumors with aberrant expression of HIF proteins, however, Maxwell et al. were the first to report (1999) the relationship between *VHL* disease and HIF-α in RCC[142]. They reported that cells lacking pVHL are unable to degrade HIF-α under *both* normoxic and hypoxic conditions, thus cells deficient in pVHL behave as being hypoxic (pseudohypoxia), even in normoxic conditions, due to continuous HIF-α activation[131, 135, 142].

Although the HIF-α accumulation is important for pathogenesis or progression, it is not per se sufficient to induce tumorigenesis of ccRCC. In *VHL* disease only a subgroup of *VHL* mutation carriers develops RCC.[135, 143] In addition, in approximately 25%-50% of sporadic ccRCC no alteration in the *VHL* gene was detected[130-132]. This suggests involvement of other genes that may also predispose to tumorigenesis of ccRCC, possibly affecting the same signaling pathway as *VHL*, or other mechanisms to inactivate *VHL*.

Papillary renal cell carcinoma – MET proto-oncogene

The second most prevalent kidney cancer is pRCC, which was first reported by Zbar et al in 1994[144], who observed kidney cancer in a family setting, which differed in several aspects from familial ccRCC. All tumors displayed a papillary histology without evidence of *VHL* mutations. A novel inherited form of kidney cancer was detected, namely hereditary papillary renal cell carcinoma[144, 145]. Genetic studies in kindreds of familial pRCC led to the identification of the *MET* proto-oncogene gene, located on the long arm of chromosome 7[146]. Patients with (germline) mutations in the *MET* gene develop so-called pRCC type 1 tumors. Activating (gain of function) germline mutations in the tyrosine kinase domain of *MET* were found in familial pRCC patients[132, 147]. *MET* encodes a transmembrane receptor tyrosine kinase with hepatocyte growth factor (HGF) as its ligand[128, 145]. Activation of *MET* by HGF activates the tyrosine kinase activity, which initiates several signal transduction cascades, resulting in multiple cellular processes like mitogenesis and migration[145].
Only a small percentage (~5%)[146, 147] of sporadic pRCC cases have *MET* mutations[132, 146, 147]. Cytogenetically, trisomy of chromosome 7 is observed in ~75% of the sporadic papillary cases[135]. Since this chromosome is harboring the *MET* gene, the gene dose is increased. Pathogenesis of pRCC is different in the heritable and sporadic form, and as in ccRCC there may be a role for other genes in the tumorigenesis.

Papillary renal cell carcinoma – fumarate hydratase (FH) tumor suppressor gene

In addition to the *MET* gene, a second gene involved in pRCC has been identified; the *fumarate hydratase* (*FH*) gene, located on chromosome 1q42.3-43. This gene encodes FH, an enzyme part of the tricarboxylic acid (Krebs) cycle, catalyzing the conversion of fumarate to malate. Little is known on the etiology and pathogenesis of these cancers: the gene is thought to act as a tumor suppressor gene[123, 148] as loss of the wildtype allele is found frequently in pRCC. Tomlinson et al. evaluated seven familial pRCCs of which five showed allelic loss, one family had a 2-bp deletion and one family carried a missense mutation[149].
In patients with (germline) *FH* mutations, papillary tumors are of type 2. Tumors of affected families have reduced or undetectable FH activity, and show an angiogenic phenotype[149]. These renal tumors overexpress HIF proteins as well as products of the HIF-regulated target genes (like VEGF and GLUT1). Isaacs et al. reported that excess intracellular fumarate, due to loss of FH, upregulates VEGF and GLUT1 transcripts in a HIF-dependent manner[150]. They further demonstrated a link between fumarate dysregulation and impaired HIF hydroxylation, due to inhibited HIF prolyl hydroxylase (HPH) activity by fumarate. Because VHL recognition of HIF requires hydroxylation by HPH, which is impaired in *FH* mutation-bearing tumors, HIF proteins accumulate and contribute to the development of these renal tumors[150]. Pollard et al. confirmed this pseudohypoxia phenomenon in a *Fh1* “knock-out” mouse model showing induction of hypoxia pathway components, resulting from HIF protein overexpression, as a direct consequence of Fh1 inactivation[151].
It is clear that *FH* germline mutations are associated with the development of pRCC, but the role of *FH* alterations in sporadic kidney cancer remains to be determined since

there is no conclusive evidence that somatic mutations of *FH* have a significant role in sporadic kidney cancers[148].

Chromophobe renal cell carcinoma – Birt-Hogg-Dubé (BHD) tumor suppressor gene

Birt-Hogg-Dubé (BHD) is a hereditary cancer syndrome characterized by kidney tumors in 15-25% of the affected patients[152]. *BHD* syndrome was first characterized in 1977,[153] but renal manifestations were not described as part of *BHD* until Roth et al[154] suggested an association in 1993. In 1999, Toro et al[155] denominated *BHD* as a novel marker of kidney neoplasia. The predominant pathology of *BHD*-related kidney tumors is chromophobe[145].

The *BHD* gene, or *FLCN*, is located on the short arm of chromosome 17. In approximately 80% of *BHD* kindreds, a germline mutation is found. The gene contains a hotspot for germline mutations in a mononucleotide tract of eight cytosines at exon 11 and approximately all these mutations result in truncated *BHD* protein, folliculin[145, 152]. Folliculin is suggested to have a tumor suppressor function, since germline mutations combined with somatic mutations or LOH resulted in truncation mutants of the folliculin protein. In addition, low to undetectable levels of *BHD* mRNA were found by Warren et al in kidney tumors from *BHD* patients, suggesting a classical TSG[156].

The identification of candidate genes involved in familial kidney cancers may provide insight into sporadic kidney cancers. Examination of the possible role of the *BHD* gene in sporadic chrRCC confirmed alteration of chromosome 17, but mutations in the *BHD* gene could not be detected[157, 158]. Recently Gad et al. published the (first) report of four different somatic *BHD* mutations in two (out of 46 chrRCC) sporadic chrRCC[159]. Interestingly, no exon 11 hotspot mutations as reported in *BHD* patients have been observed, indicating difference in tumorigenesis in the familial and sporadic form, as in pRCC.

A lack (or low incidence) of somatic mutations in the *BHD* gene suggests that the folliculin pathway may contain other causative genes in sporadic chrRCC, or other mechanisms of inactivation may be involved.

Renal cell carcinoma in tuberous sclerosis – Tuberous sclerosis complex 1 (TSC1) and 2 (TSC2) tumor suppressor genes

Tuberous sclerosis complex (TSC) patients have an increased risk for development of malignant RCC[160, 161]. In contrast to the other genetic syndromes, renal carcinomas in TSC constitute a heterogeneous group, including ccRCC, pRCC, and chrRCC[162].

TSC has been linked to the germline inactivating mutations of either of two tumor suppressor genes *TSC1* (9q34), encoding hamartin or *TSC2* (16p13.3), encoding tuberin. TSC2 forms a complex with TSC1 which functions to integrate growth factor, nutrient, and energy depletion signals with the energy requiring protein translation apparatus through the regulation of the mTOR (mammalian target of rapamycin) protein[160, 161] The TSC1/TSC2 protein complex inhibits mTOR and is involved in signaling pathways that regulate cell growth[162].

Only small numbers of TSC patients with RCC have been studied because of the very low prevalence[163-165]. However, rodent models (the Eker rat/knockout mice) show evidence for a role of the *TSC1* and *TSC2* orthologues in RCC[166, 167].
A report concerning *TSC* mutations in sporadic RCC did not support a role for TSC inactivation in sporadic tumorigenesis[168].
Interestingly, Liu et al. reported that loss of *Tsc-2* tumor suppressor gene, in Eker rats which retain wild-type *Vhl*, up-regulates VEGF via a HIF2α-mediated mechanism. In contrast to human RCC, the *Tsc-2* tumor suppressor gene is the primary target for RCC in rodents. They suggest that loss of *Tsc-2* and *VHL* tumor suppressor gene function have similar consequences in Eker rats and humans respectively. This marks the HIF-regulated pathway to be important for the development of RCC in different species and in tumors with different molecular etiologies[169].

Clinical applications

A growing understanding of the underlying molecular biology of RCC has established new potential therapies. The most significant progress has been made in the area of agents targeting the angiogenesis pathway.
It has been suggested that the resistance of RCC to chemo- and radiotherapy might be partly due to increased levels of the transcription factor nuclear factor κB (NFκB)[170, 171]. Loss of VHL leads, through HIF-α dependent and HIF-α independent pathways, to increased NFκB activity and resistance to apoptosis[172-175]. These findings support the idea that drugs targeting HIF or HIF-responsive gene products should be effective in the treatment of renal carcinomas. A number of drugs have been identified that indirectly down-regulate HIF-α, including drugs that inhibit mTOR[176], HSP90[177] and histone deacetylases[178]. mTOR is a downstream component in the PI3K/Akt pathway and has been identified as an upstream activator of HIF, enhancing translation of the HIF mRNA and preventing degradation[179, 180]. Overexpressed wild-type histone deacetylase 1 downregulates expression of *TP53* and *VHL* tumor suppressor genes and stimulates angiogenesis[178]. HSP90 stabilizes HIF-1α, EGFR and c-*MET*[181].
Other VEGFR targeted tyrosine kinase inhibitors (Pazopanib, AG013736, VEGF-trap) and mTOR inhibitor (RAD001) are still under study in RCC[182-184]. Through combining agents that target different points in the VHL-hypoxia-inducible gene pathway the activity of the targeted approach to RCC therapy may be enhanced[185].
In addition, a number of approaches are under study to block the signal transduction pathway in c-*Met* associated tumors[186, 187]. Small molecules inhibiting c-*MET* (PHA-665752, SU11274) have been shown to inhibit proliferation and growth of c-*MET*-overexpressing cell lines in mouse xenograft models[188, 189].
Further gene expression profiling of tumor specimens will help to identify new candidate genes or patterns of gene expression that can predict response to new and pre-existing therapies, leading to an appropriate patient selection for certain therapies.
Combinations of FDA-approved and other targeted therapies may overcome resistance that develops with single-agent therapy. Also combinations of targeted agents and cytokine therapy are under investigation. Phase I and II trials evaluating the safety and efficacy of these combinations are underway.

Epigenetics of RCC

Recently, it has become clear that gene function can be altered by epigenetic alterations. Epigenetics refers to mechanisms that initiate and maintain heritable (reversible) changes of gene expression and gene function in an inheritable manner without changing the sequence of the genome[190, 191]. Epigenetic regulation of gene expression is organized at the level of DNA (postreplicative DNA methylation), RNA (RNA interference (RNAi)), and protein (posttranslational histone modifications and polycomb group (PcG) protein complexes, the latter mediate long-term transcriptional silencing)[190-193].

DNA methylation is the most widely investigated epigenetic hallmark involving a postreplicative covalent DNA modification, whereby a methyl group is added to the cytosine ring by DNA methyltransferases (DNMTs) to form methyl-cytosine. In mammalian cells, DNA methylation occurs at so-called CpG dinucleotides, cytosines preceding a guanine[194]. CpG dinucleotides are not uniformly distributed throughout the human genome, the prevalence is low, except in CpG-rich regions called CpG islands, which span the 5' end region (promoter, untranslated region, exon 1) of approximately half of all genes, and within repeat sequences and transposable elements[195]. Methylation of cytosines enables the formation of a complex of proteins, including methyl cytosine-binding proteins (MBDs), DNMTs, histone deacetylases (HDACs), and histone methyl transferases (HMTs) (see figure 2) to mediate the transcriptional repressive activities of DNA methylation[191, 194, 196-198].

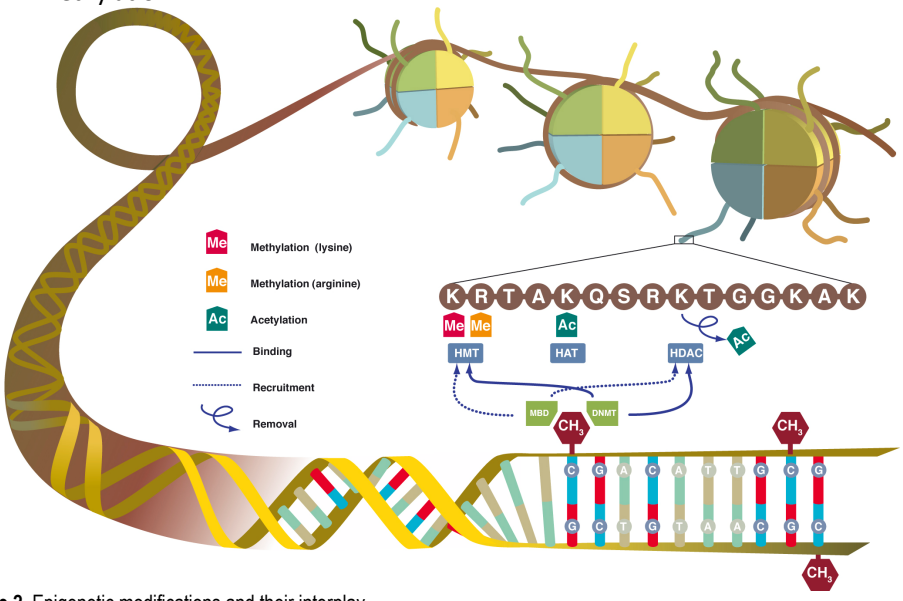

Figure 2. Epigenetic modifications and their interplay
The two main epigenetic hallmarks are postreplicative DNA methylation and posttranslational histone modifications, which both play a role in gene silencing and interact to repress gene transcription. (Additional histone modifications are known but not depicted here; histone methylation and acetylation are the most understood.)
DNA methylation is catalyzed by DNA methyltransferases (DNMTs) and characterized by the addition of a methyl group (CH_3) to a cytosine preceding a guanine (CpG). Histone (de)acetylation results from an imbalance between histone deacetylases (HDACs) and histone acetyltransferases (HATs), whereby deacetylation is associated with gene repression. HDACs and histone methyltransferases (HMTs) are recruited by methyl-binding proteins (MBPs), which bind methylated CpGs, or are directly bound to DNMTs to epigenetically modify histone tail residues and regulate gene expression.

DNA methylation is linked to aberrant patterns of post-translational histone modifications[197, 199], including acetylation, methylation, phosphorylation, ubiquitination, and sumoylation[190, 191, 197]. These modifications occur primarily at specific positions within the N-terminal histone tails[200]. Specific combinations of histone modifications confer to the overall expression status of a certain chromatin region, which is known as the 'histone code' hypothesis[197]. Modifications on histones are dynamic and rapidly changing, depending on the signaling conditions within the cell[200].
The vast majority of all modifications remain poorly understood, although considerable progress in the understanding of histone (de)acetylation and (de)methylation has been made recently. An imbalance between histone deacetylases (HDACs) and histone acetyltransferases (HATs) results in (de)acetylation of histone lysine residues, whereby deacetylation is associated with gene repression. In addition, histone lysine methylation (mono-, di-, and trimethyl lysine) and arginine methylation (mono-, and (a)symmetrical dimethyl arginine) are catalyzed by histone methyltransferases (HMTs). Unlike histone acetylation, histone methylation has long been considered as a permanent modification. However, recent discovery of more than ten (potential) histone demethylases, mediating the reversal of methylation at specific histone residues, has been documented[201, 202].
Both histone modifications contribute to both active and repressive effects on chromatin function, depending on which residue is modified[203]. HDACs and HMTs are both associated with complexes involving DNMTs and MBDs, mediating DNA packaging and gene expression[198]. Depending on the histone residue modified, and the interaction with DNA methylation to form a complex regulatory network, it correlates (among other effects) with chromatin structure/accessibility, transcriptional activity and genome function[192, 200, 204].

The importance of epigenetics in the process of tumorigenesis has been increasingly discovered during the last decades and has been accepted as a true alternative for mutations and deletions associated with a loss of gene function that can provide a selective advantage for neoplastic cells. Epigenetic changes could well be as important as changes in DNA sequence or copy number in altering gene expression, and the interplay between genetics and epigenetics during the progression of cancer is becoming more and more a focus of research interest[205-207].
Regarding RCC, mutations do not cover 100% of the cases and are less often found in sporadic RCC compared to heritable RCC. Also, sporadic cancers are known to arise from multiple (epi)genetic events, and additional aberrations remain to be discovered. Therefore, promoter hypermethylation of genes is thought to be involved in sporadic and/ or hereditary forms of RCC.
Here, we are focusing on the role of promoter methylation of tumor suppressor genes in the three most prevalent histological types of RCC, ccRCC, pRCC and chrRCC.
Recently, many (candidate) tumor suppressor genes silenced by DNA methylation and associated with one or more histological subtypes have been reported for primary RCC cases (Table 2a) and/or analyzed in RCC cell lines (Table 2b). Different techniques to analyze methylation were used to obtain the gene promoter-specific methylation data. An initial approach includes restriction enzyme digestion with Southern hybridization techniques, based on the (in)ability of methylation sensitive restriction enzymes to

digest methylated cytosines. Later, bisulfite treatment of DNA, which converts unmethylated cytosines to uracil, whereas methylated cytosines remain unchanged, allowed combined bisulfite restriction analysis (COBRA), (nested) methylation specific PCR (MSP), and bisulfite sequencing[197]. MSP is a very sensitive approach to detect DNA methylation, enabling detection of one methylated allele in a background of 1,000 unmethylated alleles, while nested MSP allows detection of one methylated allele in 50,000 unmethylated alleles[208].

The genes reported to be associated with RCC (tables 2a and 2b) can be grouped by the biological function/pathways involved in tumorigenesis and/or may be important targets for anti tumor therapy, i.e. interference in cell cycle, DNA-repair mechanisms, extra-cellular matrix molecules with adhesion and/or invasion mechanisms, angiogenesis, apoptosis, metabolism, proliferation, transport, WNT-signaling, or genes with a known role in the oncogenesis of other types of cancer, without a known specific function.

Table 2.a Overview of TSG methylation frequencies in RCC

Function	Gene	Map locus	RCC (%)	ccRCC (%)	pRCC (%)	chrRCC (%)	Norm. (%)	Ref.
Adhesion	*CDH1*	16q22.1	-	55 (14-83)	36 (16-69)	13* (0-20)	8**	212, 217, 220
	CDH13	16q24.2-q24.3	3	-	-	-	-	218
	CTNNB1	3p21	-	0	0	0*	0**	220
	EPB41L3	8p11.3	-	45	-	-	-	219
	JUP	17q	87 (83-91)	-	-	-	10 (9-11)	215
	LSAMP	3q13.2-q21	n.a	26	-	-	-	216
Angiogenesis	*VHL*	3p26-25	5	18 (16-20)	12* (0-50)	0*	0*	196, 211-213, 228
Apoptosis	*APAF1*	12q23	97	-	-	-	15	221
	CASP8	2q33-q34	6 (0-16)	-	-	-	0	218, 221
	DAPK1	9q34.1	35 (24-41)	-	-	-	5	218, 221
	RARB	3p24	-	10 (2-52)	12 (0-20)	6* (0-17)	0*/**	212, 217, 218, 220
	XAF1	17p13.2	14 (10-30)	-	-	-	-	221, 223
Cell cycle	*CDKN2 (P14^{ARF})*	9p21	71	12 (10-23)	18* (8-33)	27* (10-100)	23*/** (0-27)	212, 213, 217, 220
	CDKN2 (P16^{INK4})	9p21	5 (0-7)	10 (0-35)	13* (0-33)	9* (0-50)	0*/**	212,213, 217, 218, 220, 224, 228
	FLCN (BHD)	17p11.2	-	20 (0-33)	36	2* (0-11)	-	158, 159, 214
	RASSF1	3p21.3	37 (20-98)	51 (23-91)	71* (44-100)	27* (0-40)	78*/** (0-100)	212, 217, 220, 213, 226-230, 232
	RASSF5	19q31.1	19	32	-	-	-	216, 218
DNA- repair	*MGMT*	10q26	-	4 (2-6)	6 (0-10)	0*	10* (0-11)	212, 217, 218, 220
Invasion	*COL1A1*	17q21.31-q22	-	65	40*	50*	-	234
	TIMP3	22q12.1-q13.2	78	45 (19-71)	38* (23-50)	44* (0-100)	21*/** (0-24)	212, 213, 217, 220, 233
Krebs cycle	*SDHB*	1p36.1-p35	4	-	-	-	-	218
Metabolism	*ABCB1*	7q21.1	-	87	85	80	97**	220
	ESR1	6q25.1	-	67	77	60	77**	220
	ESR2	14q23.2	-	56	46	50	44**	220
	FHIT	3p14.2	-	52	54	50	69**	220
	GSTP1	11q13	-	8 (6-12)	18 (15-20)	0*	0*/**	212, 217, 220
	MTHFR	1p36.3	-	100	100	100	100**	220
	PTGS2	1q25.2-q25.3	-	96	92	90	100**	220

Proliferation	*APC*	5q21-q22	-	15 (10-29)	26* (20-50)	0*	0*/**	212, 217, 218, 220
	DLC1	8p22-p21.3	35	-	-	-	3	238
	HOXB13	17q21.2	30	-	-	-	0***	237
	IGFBP3	7p14-p12	3	35	40*	50*	0	221, 234
	IGFBP1	7p14-p12	-	35	20	50*	-	234
	MT1G	16q13	20	-	-	-	-	218
	PML	15q22	3	-	-	-	0	221
	SLIT2	4q15.2	25	-	-	-	-	235
	SPINT2	19q13.1	-	30	40	-	-	236
Transport	*RBP1*	3q21-q22	9	-	-	-	-	218
Wnt- signaling	*DKK3*	11p15.2	25	-	-	-	9**	219
	SFRP1	8p12-8p11.1	34 (13-68)	-	-	-	2**	219, 256
	SFRP2	4q31.3	40	-	-	-	26**	219
	SFRP4	7p14-p13	14	-	-	-	6**	219
	SFRP5	10q24.1	41	-	-	-	32**	219
	WIF1	12q14.3	37	-	-	-	26**	219

Gene nomenclature according to HUGO Gene Nomenclature Committee
Frequencies are weigthed averages in case of ≥1 studies, range from frequencies are between brackets.
- not analyzed
* n ≤ 10 (in ≥1 studies)
** normal tissue from tumor-bearing kidney
*** n unknown
RCC: renal cell carcinoma, without dividing in subtypes; ccRCC: clear cell renal cell carcinoma; pRCC: papillary renal cell carcinoma, chrRCC: chromophobe renal cell carcinoma

Table 2.b Overview of TSG methylation frequencies in RCC cell lines vs primary RCC

Gene	Cell line (%)	n	Ref.	RCC (%)	ccRCC (%)	pRCC (%)	chrRCC (%)	Ref.
DLC1	14	7	238	35	-	-	-	238
EPB41L3	47	19	219	-	45	-	-	219
FLCN (BHD)	0	6-9	158, 214	-	20 (0-33)	36	2 (0-11)	158, 159, 214
HOXB13	73	15	237	30	-	-	-	237
JUP[a]	100	3	215	83	-	-	-	215
LSAMP	78	9	216	-	26	-	-	216
RARB	33	6	257	-	18 (8-52)	20	17*	212, 217
RASSF5	67	9	216	19	32	-	-	216, 218
SPINT2[b]	45	11	236	-	30	40	-	236
TIMP3	33	12	223, 233	78	60 (54-71)	46* (45-50)	100*	212, 213, 217, 233
AF1[a]	67	15	223	14 (10-30)	-	-	-	222, 223

Gene nomenclature according to HUGO Gene Nomenclature Committee
Frequencies are weigthed averages in case of ≥1 studies, range from frequencies are between brackets.
- not analyzed
* n ≤ 10 (in ≥1 studies)
[a] indirect measurement of methylation status; (mRNA) expression measurements
[b] indirect measurement of methylation status; upregulation after demethylation treatment
RCC: renal cell carcinoma, without dividing in subtypes; ccRCC: clear cell renal cell carcinoma; pRCC: papillary renal cell carcinoma, chrRCC: chromophobe renal cell carcinoma

Methylation of the VHL promoter

Somatic *VHL* mutations are found in 50-75% of sporadic ccRCC[131-133, 209]. The absence of *VHL* mutations in a proportion of sporadic ccRCCs indicates a role for alternative methods of VHL inactivation[210]. Herman et al. investigated *VHL* promoter CpG island methylation and associated inactivation by RT-PCR and restriction enzyme analysis and Southern hybridization techniques[196]. None of the examined *VHL* hypermethylated tumors expressed the *VHL* gene and treatment of a renal cell culture line with 5-aza-2'-deoxycytidine (5-aza-dc, a demethylating agent) resulted in reexpression of the *VHL* gene. Methylation analysis of the wildtype *VHL* alleles revealed promoter methylation in 19% of the examined RCC tumors. These were the first data suggesting that aberrant DNA methylation of the *VHL* gene, in addition to LOH or gene mutations, is involved in ccRCC carcinogenesis. These data were confirmed by other groups reporting *VHL* methylation in 20%[211], 16%[212], and 17%[213] in ccRCC.
In addition to ccRCC, involvement of *VHL* promoter hypermethylation in other subtypes is mostly unknown[212, 213]. As Van Houwelingen et al. reported *VHL* mutations also in other subtypes of RCC[130], Banks et al. reported methylation of VHL in pRCC analyzed by MSP[211].

Methylation of the FH promoter

One report regarding epigenetic silencing of *FH* in renal cancers has been published, indicating absence of *FH* promoter CpG island methylation as analyzed by bisulfite sequencing, in fifteen pRCCs [212].

Methylation of the BHD promoter

So far, only three groups investigated *BHD* (*FLCN*) promoter methylation and associated silencing in chrRCC[158, 159, 214]. da Silva et al. analyzed promoter methylation in twenty primary RCCs by COBRA and bisulfite sequencing. Promoter hypermethylation was not detected in the primary RCCs[214] and six tested RCC cell lines. In addition, no significant changes in expression after treatment of the demethylating agent 5-aza-dc[214] were observed. In contrast, Khoo et al. observed methylation in a wide spectrum of primary renal tumors, using MSP: 11% of chrRCC (n=9), 36% in pRCC (n=11), and 33% in ccRCC (n=12), while none of the tested kidney cancer cell lines showed methylation[158]. On the other hand, Gad et al. analyzed methylation status of the *BHD* promoter using restriction enzyme analysis on 39 chrRCC and seven ccRCC, but no evidence of promoter methylation was found[159].

Methylation of other tumor suppressor gene promoters

In addition to the genes described above, other TSGs have been investigated (mainly by MSP), which are known to be involved in common cancer types, or which have been identified by microarray studies (see table 2a and 2b for details).
Several adhesion molecules (*CDH1, CDH13, CTNNB1, EPB41L3, JUP,* and *LSAMP*) were analyzed for promoter methylation in RCC, revealing methylation frequencies ranging from 3% (*CDH13*) to 87% (*JUP*)[212, 215-220].
Many genes associated with apoptosis (*APAF1, CASP8, DAPK1, RARB,* and *XAF1*) have been recently investigated for their methylation frequencies in RCC, and approximately 10-30% methylation is observed for the majority of genes, except for *APAF1*, which is methylated in 97% of the tested RCCs[212, 217, 218, 220-223].
The cell cycle genes *CDKN2a* (*$p14^{ARF}$* and *$p16^{INK4A}$)* were methylated in ~10-20% of the RCC cases, while normal renal tissue was mostly unmethylated[212, 213, 217, 218, 220, 224]. The *RAS association domain family 1A (RASSF1)* gene at 3p21.3, thought to be involved in the cell cycle, apoptosis and/or proliferation[225], has been implicated in the pathogenesis of a wide spectrum of tumors[225], including different RCC subtypes. Methylation is observed in ~40-50% (weighted mean) in RCC[212, 213, 217, 220, 226-230], however some studies detect high methylation frequencies in normal renal tissues. These histological normal samples were obtained from tumor-bearing kidneys, therefore a field effect[231] can cause this methylation and/or as reported before *RASSF1* might be an early marker for RCC tumorigenesis[232]. In addition, *RASSF1* methylation was detected in urine obtained from kidney cancer patients[217]. Potential importance of *RASSF1* in early diagnosis is already highlighted in breast/lung cancer patients[225]. For a second *RAS association domain family* member, *5 (RASSF5)*, a methylation frequency of ~20-30% has been observed[216, 218].
The DNA-repair gene *O6-methylguanine-DNA methyltransferase (MGMT)* is methylated in ~5% of the renal tumors[212, 217, 218, 220].
Genes involved in invasion/metastasis, such as *COL1A1* and *TIMP3*, showed methylation in a high percentage (~50%) in all RCC subtypes[212, 213, 217, 220, 233, 234].
For the Krebs cycle gene (*SDHB*) a very low methylation percentage (4%) was observed[218].

The role of the detoxification gene *Glutathione S-Transferase P1 (GSTP1)* is not clear in RCC, but is it is methylated in 10-15% of the tumors[212, 217, 220]. Several other metabolism genes (*ABCB1, ESR1, ESR2, FHIT, MTHFR,* and *PTGS2*) have been analyzed by Costa et al. by MSP, which are all methylated in high percentages (≥50%), however, also the normal renal samples show frequent methylation[220].
A methylation frequency of ~30% is reported for several proliferation genes (see table 2a for details)[212, 217, 218, 220, 221, 234-238]. Also a gene involved in transport of retinol (*RBP1*) was analyzed and a methylation frequency of 9% was observed[218].
Very recently, Urakami et al reported methylation analyses of six WNT- antagonist family genes (*SFRP1, SFRP2, SFRP4, SFRP5, WIF1,* and *DKK3*) in RCC (mostly ccRCC), analyzed by MSP. In this study all *Wnt* antagonist genes had significantly higher methylation frequencies in RCC compared to matched normal renal tissue. Methylation frequencies RCC versus normal were: *SFRP1*: 13% vs. 1,5% *, SFRP2*: 40% vs. 25.5% *, SFRP4*: 13.5% vs. 5.5%*, SFRP5*: 41% vs. 31.5%*, WIF1*: 37% vs. 25.5%*,* and *DKK3*: 25.5% vs. 9% (all $p<0.005$). mRNA expression for most WNT-antagonists was increased in two (out of two) RCC cell lines after treatment with 5-aza-2'deoxycytidine and confirmed to be methylated by bisulfite sequencing[239].

Variables in promoter methylation studies

Comparison of data from different studies on methylated tumor suppressor genes in RCC (see tables 2a and 2b) is complicated by a considerable number of variables. First, instead of considering different subtypes of kidney cancer, RCC is considered as one single entity in some studies. Since RCCs are heterogeneous in histology, clinical behavior, genotype, prognosis and response to therapy, data should be presented for the different subtypes. An interpretation of the diagnostic or therapeutic value of these results is only possible when subtyping is taken into account. Recently, Costa et al. emphasized this importance of subtyping by reporting a gene panel which could discriminate between RCC subtypes by the presence/absence of methylation[220]. In addition to subtyping, consideration of tumor grade/stage is of importance in interpreting (epi)genetic alterations in RCC. The composition of tumor series used to study (epi)genetic alterations in RCC varies between different research groups, therefore it is important to include this variable.
In addition, different methods of measuring promoter methylation can account for part of the differences in results. Since MSP is very sensitive, especially when nested PCR has been performed[208], results might differ from those obtained by restriction enzyme-based methods. Furthermore, the position of measuring promoter methylation in a certain CpG island might differ between studies. Therefore, optimal and consensus primer location and design is critical to achieve reliable results[240].
Finally, when analyzing promoter methylation, the origin of normal renal tissue should be considered[231], and the number of RCC cases investigated should be sufficient to draw valid conclusions.

Clinical applications

DNA methylation markers have a promising future in clinical diagnostics, since DNA methylation changes have been reported to occur early in carcinogenesis[241] and early

detection of disease results in an improved clinical outcome. In case of RCC, clinical signs and symptoms are often not useful in making an early diagnosis. The classic triad of pain, hematuria and a palpable flank mass is encountered in only 10% of patients and is usually associated with advanced disease[242]. Since identification of patients with organ-confined RCC is important for long-term disease-free survival after radical or partial nephrectomy[243], development of novel diagnostic noninvasive approaches for the early detection of kidney cancer is imperative, especially in patients with inherited predisposition. Cancer specific DNA methylation patterns can be found in detached tumor cells in bodily fluids, such as blood and urine and detection of methylated DNA by the very sensitive (nested) MSP is noninvasive and relatively cheap.
Table 3 shows the results of studies of DNA methylation markers in urine and serum from patients with RCC[213, 217, 239].

Table 3. Studies of DNA methylation markers in urine and serum from patients with RCC

Samples	DNA source	Markers	Analytical sensitivity (%)	Clinical Sensitivity (%)	Specificity (%)	Ref.
23 ccRCC,	urine	***APC***	4/5 (80)	10/26 (38)	96	217
1 pRCC,		***CDH1***	6/10 (60)	10/26 (38)	95	
1 chrRCC,		***GSTP1***	1/2 (50)	4/26 (15)	100	
1 collecting duct		***MGMT***	0/1 (0)	2/26 (8)	100	
		P16	4/6 (67)	9/26 (35)	100	
		RARB	4/9 (44)	8/26 (31)	91	
		RASSF1	11/15 (73)	17/26 (65)	89	
	serum	***APC***	1/5 (20)	1/18 (6)	97	
		CDH1	06/10 (60)	6/18 (33)	93	
		GSTP1	0/2 (0)	1/18 (6)	100	
		MGMT	0/1 (1)	0/18 (0)	97	
		P14	1/4 (25)	1/18 (6)	97	
		P16	3/6 (50)	4/18 (22)	100	
		RARB	1/9 (11)	1/18 (6)	100	
		RASSF1	2/15 (13)	2/18 (11)	97	
35 ccRCC,	urine	***APC***	7/7 (100)	9/50 (18)	100	213
6 pRCC,		***P14***	8/8 (100)	9/50 (18)	100	
2 chrRCC		***P16***	4/5 (80)	5/50 (10)	100	
		RASSF1	23/24 (95)	26/50 (52)	100	
		TIMP3	23/26 (88)	30/50 (60)	100	
		VHL	6/6 (101)	6/50 (12)	100	
33 RCC	serum	***DKK3***	6/10 (60)	9/33 (27)	100	239
		SFRP1	5/8 (62)	9/33 (27)	100	
		SFRP2	12/13 (92)	7/33 (21)	100	
		SFRP4	5/15 (33)	8/33 (24)	100	
		SFRP5	9/16 (56)	15/33 (45)	100	
		WIF1	5/13 (38)	9/33 (27)	100	

Gene nomenclature according to HUGO Gene Nomenclature Committee

Analytical sensitivity is defined as the fraction of cases in which methylation of a marker is found in serum or urine for cases with confirmed methylation of the same marker in the associated tumor.
Clinical sensitivity is defined as the fraction of confirmed cases of disease, in which methylation of a marker is found in serum or urine, regardless of whether methylation of that marker is present in the associated tumor.
Specificity is defined as the fraction of controls without the disease that show a lack of detectable methylation in serum or urine.
RCC: renal cell carcinoma, without dividing in subtypes; ccRCC: clear cell renal cell carcinoma; pRCC: papillary renal cell carcinoma, chrRCC: chromophobe renal cell carcinoma

Furthermore, Urakami et al reported a higher frequency of methylation in serum DNA from RCC patients with higher-grade/stage tumors compared to lower-grade/stage tumors, indicating putative progression markers[239]. Other studies have demonstrated independent prognostic values for DNA methylation markers in RCC (*PTEN*[244], *JUP*[215], EPB41L3[219], *APAF1* and *DAPK1*[221]), being predictors of poor prognostic outcome. Inactivation of *RASSF1* is often because of promoter methylation and this gene has the potential to be used as a marker for early detection and tumor surveillance/monitoring of (RCC) cancer patients (along with a panel of other TSGs to obtain a higher sensitivity).

Obviously, methylation markers with a high sensitivity and specificity in urine and/or serum would be a significant improvement, as this would offer a non-invasive method for (early) diagnosis, prognosis, or response on therapy[231].

The quest for DNA methylation markers that are predictive for therapy response is still in its infancy[245-247], and such markers in RCC have not been reported. A better understanding of the molecular pathways involved in renal carcinogenesis and insight into the functional significance of methylation changes, will allow development of new therapeutic approaches by use of demethylating agents. Resistance of several tumor types, including RCC, for certain anti-cancer agents such as chemotherapeutics, might be a result of epigenetic mechanisms that regulate genes involved in resistance, e.g. proapoptotic genes silenced by DNA methylation and/or histone acetylation. Demethylating agents or HDAC inhibitors might reverse these repressive effects[248].

Reu et al. demonstrated augmentation of immune response by DNA methyltransferase 1 inhibitors, through reactivation of apoptosis-associated Interferon response gene *XAF1*[249]. The overcoming of resistance to Interferon-induced apoptosis in RCC cells by demethylating agents may have clinical significance for cytokine therapy response.

Shang et al. reported a synergistic growth suppression of RCC (*in vitro*), caused by the DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine, and a chemotherapeutic agent, Paclitaxel (PTX). They suggest that 5-aza-2′-deoxycytidine could increase the susceptibility of RCC to PTX and that combination chemotherapy with this inhibitor and PTX might be a novel strategy to improve the clinical response rate of RCC[250].

Conclusion

RCC is the most important tumor of the kidney in adults. In etiological, biological and clinical characteristics it represents a heterogeneous entity, which, through lack of clinical symptoms, may be difficult to detect in an early stage. Surgery is the first treatment of choice, and most successful if the tumor is confined to the kidney. Systemic treatment of RCC has made considerable progress in patients with metastatic RCC, e.g. by development of new emerging strategies (targeted agents) in recent years. Further studies are needed to address the questions regarding the efficacy of new targets and the efficacy of combination treatments.

Developing knowledge of genetic and epigenetic changes implicated in tumor development and behavior is becoming increasingly important for advancing the efficacy of disease management.

Defective VHL functioning appears to be the key event, in both hereditary and sporadic cases. However, the variable nature of the resulting neoplasm is most likely strongly determined by the complex interplay of additional downstream modifications, among which the role of epigenetic alteration of gene expression is becoming more and more acknowledged.

Elucidation of epigenetic modifications in RCC holds great promise for early detection, prediction of sensitivity for non-surgical therapy, but also for therapeutical interventions through reversal of epigenetic gene silencing. However, for definitive progress to be made in this field of research, uniformity in study design and consensus in clinicopathological definitions and scientific methods is a prerequisite.

Appendix I:

Odds ratio (OR): The OR is a measure of association between exposure and disease that can be calculated in case-control studies. An OR >1 denotes an increased risk, an OR of < 1 denotes a decreased risk, and an OR of 1 points at no association between the exposure and the disease.

Pooled OR or RR: A pooled OR or RR can be calculated when individual studies are pooled and a OR or RR is calculated using either the outcomes of the data (meta-analysis) or the individual data from the studies.

Relative risk or relative rate (RR): the RR is the ratio of the risk of an event occurring in an exposed group versus a non-exposed group. A RR >1 denotes an increased risk, a RR of < 1 denotes a decreased risk, and a RR of 1 points at no association between the exposure and the disease. A RR can be calculated in cohort studies and randomized controlled trials.

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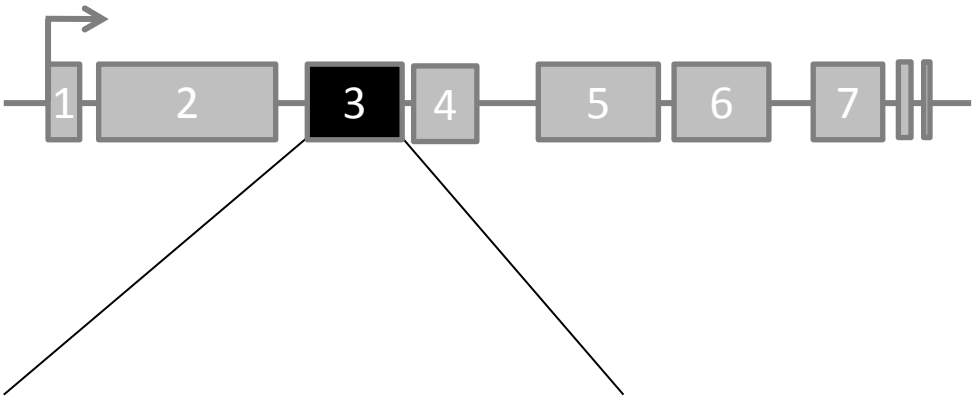

Prognostic significance of *Gremlin1* (*GREM1*) promoter CpG island hypermethylation in clear cell renal cell carcinoma

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Abstract

Gremlin1 (*GREM1*), a bone morphogenetic protein antagonist and putative angiogenesis-modulating gene, is silenced by promoter hypermethylation in human malignancies. Here we study *GREM1* methylation in clear cell renal cell carcinoma (ccRCC) and its impact on tumor characteristics and clinical outcome. Three *GREM1* promoter CpG island regions(i, ii, iii) were analyzed by methylation specific PCR and/or bisulfite sequencing in ccRCC cell lines and ccRCCs from two independent patient series. Results were correlated with clinicopathological- and angiogenic parameters. Bisulfite sequencing of ccRCC cell lines showed *GREM1* methylation, associated with absence of *GREM1* mRNA. *GREM1* methylation prevalence in ccRCCs varied between regions: 55%, 24% and 20% for regions-i, -ii and -iii respectively. *GREM1*-region-iii methylation was associated with increased tumor size(p=0.02), stage(p=0.013), grade(p=0.04), tumor-(p=0.001) and endothelial cell(p=0.0001) proliferation and decreased mean vessel density(p=0.001) in a hospital-based ccRCC series(n=150). In univariate analysis, *GREM1*-region-iii methylated ccRCCs had a significant worse survival when compared to unmethylated ccRCCs (hazard ratio (HR)=2.35, 95% CI:1.29-4.28), but not in multivariate analysis (HR=0.88, 95% CI:0.45-1.74). In a population-based validation series(n=185), *GREM1*-region-iii methylation was associated with increased Fuhrman grade(p=0.03) and decreased overall survival(p=0.001) in univariate and multivariate analysis (HR=2.32, 95% CI:1.52-3.53 and HR=2.27, 95% CI:1.44-3.59, respectively). The strong correlation between *GREM1*-region-iii promoter methylation and increased malignancy and its correlation with active angiogenesis indicates a role for *GREM1* in ccRCC carcinogenesis and tumor angiogenesis.

Introduction

Clear cell renal cell carcinoma (ccRCC) accounts for ~75% of all cases of renal cell cancer[1], and is characterized by increased vascularization and an unclear clinical prognosis. Currently, patient performance status, tumor size, nodal and distant metastasis (TNM)–stage and Fuhrman nuclear grade are the most useful predictors of patient outcome[2]. However, interest in additional prognostic molecular markers is growing. Inactivation of the von *Hippel-Lindau* (*VHL*) gene has been shown to be a common and early event in the carcinogenesis of ccRCC[3-5]. 50 to 70% of ccRCC tumors harbor a *VHL* mutation[5, 6] and in 5 to 20% of ccRCCs *VHL* is silenced by promoter CpG island hypermethylation[7, 8]. Although defective VHL functioning is a key event in the development in both sporadic and hereditary ccRCCs, alterations in the structure or regulation of the *VHL* gene do not appear to be directly associated with tumor cell proliferation and patient prognosis[9, 10], suggesting a complex interplay of additional genetic and epigenetic changes which may accumulate during RCC development.

One intriguing candidate gene in this development may be the highly conserved *Gremlin1* (*GREM1*) which we have identified to be expressed upon 5'aza-2-deoxycytidine (DAC) treatment in four ccRCC cell lines in a epigenome-wide screen. *GREM1* is a secreted glycoprotein that binds and antagonizes bone morphogenetic proteins (BMPs) -2, -4, and -7, thereby preventing the ability of these ligands to interact with their receptors resulting in inhibition of downstream transforming growth factor-β (TGF-β) signaling[11-13]. BMPs, the largest subfamily of the TGF-β superfamily, are pleiotropic growth factors serving multiple functions in many cell and tissue types including angiogenesis, proliferation, apoptosis, differentiation, chemotaxis, and extracellular matrix production during development as well as in adult life[14]. BMPs and BMP-antagonists such as Gremlin1, have been demonstrated in regulating renal development[15-18] and in the pathogenesis of nephropathy[19-21]. BMP-independent activities of *GREM1* in cancer[12, 22] and angiogenesis[23] have also been demonstrated. However, the role of *GREM1* in renal cancer pathogenesis and the mechanisms by which *GREM1* gene expression is regulated remains incompletely understood.

GREM1 has been identified as one of the targets of Polycomb Repressive Complex 2 (PCR2) subunit protein SUZ12[24], which marks repressive chromatin during early stages of embryonic stem cell differentiation. Compared to unmarked stem cell genes, Polycomb group (PcG) targets have up to 12-fold increased susceptibility to develop cancer-specific promoter CpG island hypermethylation[25], suggesting that epigenetic mechanisms may play a key role in regulating *GREM1* expression. Further evidence that this mechanism is important in cancer development has recently been provided by the observations that *GREM1* promoter CpG island methylation is prevalent in other tumor types such as gastric-[26], bladder- and prostate cancer[27].

The aim of this study was to investigate *GREM1* promoter CpG island methylation and its association with clinicopathological- and angiogenesis parameters in ccRCC.

Materials and Methods

Cell lines, study populations, and clinical specimens

Four ccRCC cell lines (SKRC1, SKRC10, SKRC52, SKRC59), kindly provided by Dr. E. Oosterwijk, Nijmegen Center for Molecular Life Sciences (NCMLS), Nijmegen, The Netherlands, were cultured in RPMI 1640 media (Invitrogen, Breda, the Netherlands) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Etten-Leur, the Netherlands).

Formalin-fixed and paraffin embedded tumor samples (n=150) from patients with sporadic ccRCC, treated with radical or partial nephrectomy without any neo-adjuvant therapy, were collected retrospectively and further referred to as hospital-based series. Of 50 ccRCC patients also histological normal renal tissue was available. In addition, histological normal renal tissue samples (formalin-fixed, paraffin embedded) of 61 non-RCC patients was collected. All samples were derived from the archives of the Department of Histopathology, University Hospital of Leuven and Department of Pathology, Maastricht University Medical Center. Hematoxylin eosin-stained slides were revised by an experienced genitourinary pathologist to confirm nuclear Fuhrman grading. Tissues were sectioned into 20 µm slides and genomic DNA was isolated and purified using the Puregene DNA purification kit according to the manufacturer's instructions (BIOzym, Landgraaf, The Netherlands). Data of postoperative follow-up (median follow-up 64 months, range 1–153 months) at regular intervals by means of physical examination, chest X-ray, abdominal computed tomography or ultrasound, and when indicated a bone scan, was available (for patient characteristics see table 1).

A second independent, population-based validation series of formalin-fixed, paraffin embedded ccRCC samples (n=185) was obtained from the Netherlands Cohort Study on diet and cancer (NLCS)[28] and collected from 51 pathology laboratories throughout the Netherlands with a median follow-up period of 79 months, range 0–218 months. This series will be further referred to as population-based series. Tissue collection and DNA isolation have been described in detail elsewhere[29]. Hematoxylin eosin-stained slides were revised by an experienced genitourinary pathologist. Information on tumor size and stage was available from the pathological reports and cancer registries. Follow-up was accomplished by record linkage to the municipal population registry and the causes of death registry from Statistics Netherlands[10] (for patient characteristics see table 2). This study was approved by the Medical Ethical Committee of the Maastricht University Medical Center.

Table 1. *GREM1* promoter CpG island methylation associated with clinical, histopathological, and angiogenesis-related patient characteristics (hospital-based series)

		All ccRCC	Methylated *GREM1*-i	Methylated *GREM1*-ii	Methylated *GREM1*-iii
		n=150 (100%)	n=77 (55%)	n=33 (24%)	n=28 (20%)
Sex					
Male (*n*)		99 (66%)	50 (65%)	22 (67%)	22 (79%)
Female (*n*)		51 (34%)	27 (35%)	11 (33%)	6 (21%)
P (U versus M)			0.38	0.95	0.11
Age (mean)		60.1 (± 12.1 SD)	60.3 (± 12.6 SD)	62.3 (±11.6 SD)	60.3 (±10.4 SD)
P (U versus M)			0.80	0.33	0.96
Tumor size in cm (mean)		6.36 (± 3.45 SD)	6.91 (± 3.55 SD)	7.25 (± 3.54 SD)	7.72 (± 3.17 SD)
P (U versus M)			0.01	0.08	0.02
Fuhrman grade	1	15 (10%)	5 (6%)	2 (6%)	1 (4%)
(*n*, %)	2	72 (48%)	36 (47%)	15 (46%)	11 (39%)
	3	46 (31%)	26 (34%)	10 (30%)	9 (32%)
	4	17 (11%)	10 (13%)	6 (18%)	7 (25%)
P (U versus M)			0.54	0.61	0.04
Tumor stage	1	8 (5%)	3 (4%)	0 (0%)	0 (0%)
(*n*, %)	2	89 (60%)	42 (54%)	17 (53%)	12 (43%)
	3	30 (20%)	16 (21%)	9 (28%)	7 (25%)
	4	22 (15%)	16 (21%)	6 (19%)	9 (32%)
P (U versus M)			0.23	0.32	0.013
ECP % (mean)		1.4 (±2.17SD)	1.8 (±2.72 SD)	2.5 (±3.37 SD)	3.0 (±2.97 SD)
P (U versus M)			0.097	0.004	0.0001
TCP % (mean)		6.7 (±9.25 SD)	8.2 (±11.40 SD)	9.0 (± 12.70 SD)	12.0 (± 11.90 SD)
P (U versus M)			0.13	0.16	0.001
MVD per mm^2 (mean)		197.5 (± 91.0 SD)	177.4 (± 81.70 SD)	174.8 (± 89.20 SD)	152.5 (± 92.2 SD)
P (U versus M)			0.01	0.09	0.001

Tumor stage based on International Union against Cancer (UICC) TNM classification of malignant tumors, fourth, fully revised edition, 1987
P: p-value for comparison of methylated and unmethylated cases
ECP: endothelial cell proliferation
TCP: tumor cell proliferation
MVD: micro vessel density
OS: overall survival
MFS: metastasis-free survival

Table 2. *GREM1* promoter CpG island methylation associated with clinical, histopathological, and angiogenesis-related patient characteristics (population-based series)

		All ccRCC	Methylated *GREM1*-iii
		n=185 (100%)	n=68 (40%)
Sex			
Male (*n*)		108 (59%)	44 (65%)
Female (*n*)		74 (41%)	24 (35%)
P (U versus M)			0.32
Age (mean)		67.4 (± 4.7 SD)	67.2 (± 5.2 SD)
P (U versus M)			0.49
Tumor size in cm (mean)		7.9 (± 3.9 SD)	7.5 (± 3.17 SD)
P (U versus M)			0.16
Fuhrman grade	1	42 (23%)	10 (15%)
(*n*, %)	2	66 (36%)	22 (32%)
	3	47 (26%)	23 (34%)
	4	27 (15%)	13 (19%)
P (U versus M)			0.03
Tumor stage	1	4 (2%)	0 (0%)
(*n*, %)	2	81 (45%)	27 (40%)
	3	63 (35%)	23 (34%)
	4	32 (18%)	18 (26%)
P (U versus M)			0.08

Drug treatment and gene expression analysis

ccRCC cell lines SKRC1, SKRC10, SKRC52, and SKRC59 were treated with 5'aza-2-deoxycytidine (DAC) or Trichostatin A (TSA), which inhibit DNA methylation and histone deacetylases, respectively. ~10% confluent RCC cells were cultured in RPMI 1640 media containing 10% FBS with 5μM (DAC) (DAC; Sigma, St. Louis, MO; stock solution: 1 mmol/L in PBS) for 96 hours, replacing media and DAC every 24 hours. Cell treatment with 300nM TSA (Sigma; stock solution: 5mM dissolved in ethanol) was performed for 18 hours, starting at ~30% cell confluency. Mock treated cells were grown in parallel with the DAC treatment by addition of equal volumes of PBS without drugs.

RNA of mock-, DAC-, and TSA-treated cells was isolated using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturers' instructions. For real time RT-PCR, 1 μg of total RNA was reverse transcribed by using Ready-To-Go™ You-Prime First-Strand Beads (Amersham Biosciences/GE Health care, Piscataway, NJ) with addition of random hexamers (0.2μg per reaction). RNA of a subset of the hospital-based series primary ccRCCs and normal renal tissue from non-cancerous patients was isolated from frozen tissue using the GenElute™ Total RNA kit (Sigma) according to the manufacturers' instructions. For real time RT-PCR, 1 μg of total RNA was reverse transcribed by using the iScript™ cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands). (Primer sequences and PCR conditions are provided in table 3).

GREM1 methylation-specific PCR and bisulfite sequencing

The highly conserved human *GREM1* gene (accession number NM_013372) is located on chromosome *15q13-q15*. For CpG island prediction, EMBOSS (http://bioinfo.hku.hk/EMBOSS) software using the following criteria (Obs/Exp: 0.6, MinPC: 50, length: 200) was used. This resulted in the identification of three closely located CpG islands from -650 bp to +1671 bp relative to the transcription start site (TSS) (figure 1.A), which will be further considered as one island. To cover a large part of the promoter region for *GREM1* methylation analysis, three different nested methylation-specific PCR (MSP) primer sets (*GREM1*-region i, -region ii, and -region iii) were designed (see figure 1.A). *GREM1* promoter CpG island methylation was determined by chemical modification of genomic DNA with sodium bisulfite and subsequent MSP as described in detail elsewhere[30-32]. In brief, 500 ng of DNA was modified by sodium bisulfite using the EZ DNA methylation kit (Zymo Research, Orange, California) according to the manufacturer's instruction. To facilitate MSP analysis on DNA retrieved from formalin-fixed paraffin embedded tissue, DNA was first amplified with flanking PCR primers that amplify bisulfite-modified DNA but do not preferentially amplify methylated or unmethylated DNA. The resulting fragment was used as a template for the MSP reaction. *GREM1*-region i, -region ii, and -region iii methylation was analyzed on the hospital-based series of ccRCCs. On the population-based series, *GREM1*-region iii MSP was performed. All PCRs were performed with controls for unmethylated alleles (DNA from normal lymphocytes or DNA from human umbilical vein endothelial cells (HUVEC), methylated alleles [normal lymphocyte DNA treated in vitro with SssI methyltransferase (New England Biolabs, Leusden, the Netherlands)], and a control without DNA. Primer sequences and PCR conditions are provided in table 3. Ten µl of each MSP reaction was directly loaded onto 2% agarose gels containing GelStar Nucleic Acid Gel Stain (Cambrex, New Jersey, USA), and visualized under UV illumination. The methylation status of the hospital-based ccRCC series was successfully obtained in 93-95% (138-141 of 149, one sample failed to provide a sufficient amount of DNA), dependent on the primer set used, and in 91% (168 of 185) of the population-based series. To assess reproducibility, MSP reactions have been performed in duplicate or triplicate starting from DNA amplification with flanking PCR primers. The reproducibility was 82%, 95%, and 97% for primer set i, ii, and iii, respectively in the hospital-based series and 84% in the population-based series. Non-concordant MSP results were analyzed a third time and a two out of three concordance was used as end result.
For bisulfite sequencing, PCR products were cloned using the TOPO TA cloning kit (Invitrogen, Breda, the Netherlands), single colonies were sequenced using M13 primer, BigDye Terminator v1.1 Cycle Sequencing Kit in accordance to the manufacturer's instruction. Primer sequences and PCR conditions are provided in table 3.

Assessment of angiogenesis and tumor parameters

Quantification of micro vessel density (MVD), endothelial cell proliferation (ECP), and tumor cell proliferation (TCP) in the hospital-based series has been described in detail elsewhere[33].
Briefly, CD34/Ki67 double immunohistochemical staining[34] was used to analyze MVD, ECP and TCP. One tissue slide was analyzed per tumor. For assessment of the vascular

density within a tumor tissue slide, 1 hotspot (most vascularized microscopic field) was selected and 4 areas were chosen randomly. Vessel counts were performed at 200x magnification using an optical grid. The presence of a vascular lumen was not necessary to identify a micro vessel. MVD was expressed as vessels per mm^2. Next, a total number of at least 500 intratumoral endothelial cells and 500 tumor cells were evaluated in consecutive fields at a 400x magnification and the fractions of proliferating endothelial cells (ECP%) and tumor cells (TCP%) were assessed. ECP% and TCP% were calculated according to the following formulas: ECP% = (the number of endothelial cells with Ki67-stained nuclei/total number of endothelial cells evaluated) x 100; TCP% = (the number of tumor cells with Ki67-stained nuclei/total number of tumor cells evaluated) x 100.

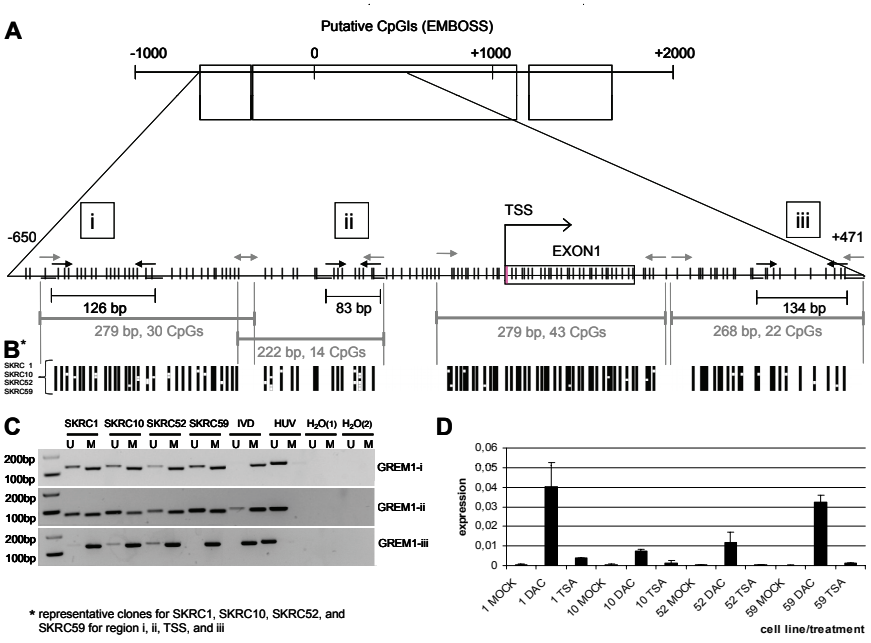

Figure 1. *GREM1* promoter CpG island, methylation analyses, and mRNA expression in ccRCC cell lines
GREM1 putative promoter CpG island relative to the transcription start site (TSS), including the locations of BSseq primer sets (i, ii, TSS, and iii; gray arrows: sequence primer locations) and MSP primer sets i, ii, and iii (underlined: flank PCR primer locations, black arrows: methylation specific PCR primer location, respectively) (A) with representive BSseq (B), MSP (C), and mRNA expression (D) results of ccRCC cell lines SKRC1, SKRC10, SKRC52, SKRC59. mRNA expression data resulted after three independent experiments.

Open/closed squares indicate unmethylated/methylated CpGs. U: unmethylated product; M: methylated product; IVD: methylation positive control; HUV: methylation negative control; H2O(1): Flank PCR control without DNA; H2O(2): MSP control without DNA. DAC: 5'aza-2-deoxycytidine, TSA: Trichostatin A.

Table 3. PCR primers and conditions for *GREM1*

method		Forward 5'>3'	Reverse 5'>3'	Tm (°C)	cycles (n)	length (bp)
RT		CAGCCTA-CACGGTGGGAGC	CTGCTCTGAGTCATTGTGCTGG	60	40	128
MSP i	FI	TGGYGA-TAGGTTTTTGTTGG	CCCCTCTAAAAAAAACRAAAC-TA	56	35	156
	U	GTTTTTGTTGGTGG TTAGTGAGTTT	AACAAAACTACAAAA-TACTCTAATCAAACA	62	30	134
	M	TGTTGGCGGTTAGC GAGTTC	GAAACTACGAAA-TACTCTAATCGAACG	62	30	126
MSP ii	FI	TTTTGGGGTGATTTT TTTTAGT	CACCCCTCCTACTTCCCC	56	35	96
	U	GTGATTTTTTTTAGT TGGTTTGGGT	ACCCCTCCTACTTCCCCACA	64	35	88
	M	ATTTTTTTTAGTCGG TTCGGGC	CCCTCCTACTTCCCCGCG	64	35	83
MSP iii	FI	GGGGTTTTTTTTGTT GAGGT	CTACTACCACCAACAC-CAAAAAC	56	35	161
	U	TTTTTTTGTTGAGGT TGTGGATATT	CAAAAACATATT-CAAAAACCTCCA	65	25	140
	M	TTTGTTGAGGTCGC GGATATC	AAAACGTATTCGAAAACCTCCG	65	25	134
BSseq i		TGGYGA-TAGGTTTTTGTTGG	CCCAAATCTAAACTTTCCCTATC	60	35	279
BSseq ii		GATAGG-GAAAGTTTA-GATTTGGG	CACCCCTCCTACTTCCCC	64	35	222
BSseq iii		AGGGTYGTAAAT-TAATTTAGGATT	CTACTACCACCAACAC-CAAAAAC	60	40	268
BSseq TSS		GGYGTTTGGTTAAT GGAGAGG	CRCCCACTCACATCCCTACC	62	35	279

RT: real-time reverse transcriptase PCR; MSP i, ii, iii: methylation specific PCR *GREM1*-region i, *GREM1*-region ii, *GREM1*-region iii, respectively; M: methylation sensitive primers; U: non-methylation sensitive primers; BSseq i, ii, iii, TSS: bisulfite sequencing *GREM1*-region i, ii, iii, and around transcription start site.

Statistical analysis

Cause-specific survival was defined as the time from cancer diagnosis until renal cancer-related death or until the end of follow-up. Differences in clinicopathological and angiogenesis characteristics between ccRCCs with and without *GREM1* promoter CpG island methylation were evaluated with the Student's t-tests and Pearson's χ^2 tests. Kaplan-Meier analyses and log-rank tests were used to estimate the overall influence of *GREM1* promoter CpG island methylation on cause-specific survival. Hazard ratios (HR) and corresponding 95% confidence interval (CI) were assessed by use of Cox proportional hazard models. Factors were considered possible confounders if they were known prognostic factors for renal cancer and influenced the crude HR. Possible confounders that were included in the model for both series were sex, age at diagnosis, cancer stage, tumor size and nuclear grade. The proportional hazard assumption was tested using the Schoenfeld residuals. Analyses were performed by use of the statistical package STATA 9.0. All reported P values are two-sided and P values ≤0.05 were considered statistically significant.

Results

Promoter CpG island methylation and down regulation of GREM1 mRNA expression in ccRCC cell lines

GREM1 promoter CpG island methylation and mRNA expression were analyzed by MSP and real time RT-PCR in the ccRCC cell lines SKRC1, SKRC10, SKRC52, SKRC59. All four cell lines were heavily methylated at region i, region ii, and region iii of the CpG island (figure 1.C). MSP data were confirmed by bisulfite sequencing of region i (279 bp, 30 CpGs), region ii (222 bp, 14 CpGs) and region iii (268 bp, 22 CpGs). Methylation at the transcription start site (TSS) (279 bp, 43 CpGs) was assessed by bisulfite sequencing (figure 1.B). As shown in figure 1.D, little or no mRNA expression is observed in SKRC1, SKRC10, SKRC52, and SKRC59. mRNA expression could be induced in all four cell lines by DAC but not, or to a much lesser extent, by TSA.

GREM1 promoter CpG island hypermethylation in ccRCCs varies at different regions in the promoter CpG island

To analyze *GREM1* promoter CpG island methylation in primary ccRCCs, a hospital-based series of ccRCCs was analyzed for methylation at three regions in the promoter. 63% (93/147, 3 samples did not yield a MSP result in region i, region ii and region iii) of the ccRCCs showed methylation of one or more of the three analyzed regions. A decrease of methylation prevalence was seen more downstream in the analyzed area, with 55% (77/141) methylation for region i (-648 - -462), 24% (33/139) for region ii (-236 - -140), and 20% (28/138) for region iii (+311 - +472) (table 1). Methylation in matching histological normal renal tissues was observed in 28% (14/50), 16% (8/50), and 10% (5/50) for region i, ii, and iii, respectively (data not shown). In histological normal renal tissues from non-cancer patients, 0-16% methylation was observed in the sub regions (region i: 7/61, region ii: 10/61, region iii: 0/61), data not shown.

GREM1 promoter CpG island methylation at region iii is associated with increased tumor size, tumor grade and -stage, angiogenesis and poor prognosis

Next, we studied the association of *GREM1* promoter CpG island methylation and patient characteristics and clinical outcome. In table 1, patient and tumor characteristics for the 150 ccRCC cases included in the *GREM1* promoter hypermethylation analyses are presented; methylated cases were stratified by methylation site. No differences among methylated and unmethylated cases were observed in the distribution of age and sex (p-values > 0.05, see table 1). Consistent with the number of methylated cases, associations between *GREM1* promoter methylation and clinical-, histopathological- and angiogenesis related patient characteristics are dependent on the region of methylation. *GREM1*-region i methylation was associated with tumor size (p=0.01) and MVD (p=0.01). The median overall survival (OS) and metastasis free survival (MFS) for unmethylated versus methylated cases at region i is 60.5 (1-143) versus 64.5 (range 2-153) and 58.5 (1-143) versus 41 months (0-143), respectively. The Kaplan-Meier curve for *GREM1*-region i methylation (Figure 2.A) shows a borderline statistically significant difference in OS for methylated and unmethylated cases (log-rank, p=0.05 log-rank

MFS, p=0.73, data not shown). Age and sex adjusted Cox proportional hazard analyses show a statistically significant association between *GREM1*-region i methylation and OS (HR_i= 1.84; 95% CI, 1.02-3.32, but not for MFS (HR_i= 1.08; 95% CI, 0.76-1.54). Multivariate analyses however, show no association between *GREM1*-region i and OS or MFS (HR_i= 0.66; 95% CI, 0.33-1.33, and HR_i= 0.82; 95% CI, 0.55-1.21, respectively)
GREM1-region ii methylation was only significantly associated with ECP (p=0.004). The median OS and MFS for unmethylated versus methylated cases at region ii is 60.5 (1-153) versus 76 (range 1-153) and 56 (1-153) versus 60 months (0-153), respectively. Figure 2.B shows the Kaplan-Meier curve for *GREM1*-region ii; no difference was observed between methylated and unmethylated cases (log-rank p=0.84, log-rank MFS, p=0.15, data not shown). Methylation of *GREM1*-region ii was not significantly associated with OS or MFS in the age and sex adjusted Cox regression analyses ($HR(OS)_{ii}$= 0.93; 95% CI, 0.48-1.80, and $HR(MFS)_{ii}$=0.74; 95% CI, 0.49-1.12) nor in the multivariate analyses ($HR(OS)_{ii}$= 0.55; 95% CI, 0.26-1.18, and $HR(MFS)_{ii}$=0.56; 95% CI, 0.36-0.87).
In contrast, ccRCCs methylated at region iii are statistically significant larger in tumor size (p=0.02), and have a statistically significant higher tumor grade (p=0.04), tumor stage (p=0.013), ECP ratio (p=0.0001), and TCP ratio (p=0.001), as compared to tumors unmethylated at region iii, and methylated cases have a lower MVD (per mm^2, p=0.001). The median OS and MFS for unmethylated versus methylated cases at region iii is 65.5 (1-153) versus 56 (range 2-138) and 60 (1-153) versus 23 months (0-138), respectively. Figure 2.C shows the Kaplan-Meier curve for cause-specific survival and *GREM1*-region iii methylation, and methylated cases had a statistically significant worse survival as compared to unmethylated cases (log-rank p=0.002, log-rank MFS p=0.05, data not shown). Age and sex adjusted Cox proportional hazard analyses also show a significant association between *GREM1*-region iii methylation and OS (HR= 2.35; 95% CI 1.29-4.28); the association with MFS was borderline significant (HR= 1.54; 95% CI 0.99-2.39). However, in multivariate analyses adjusted for age, sex, grade, stage, and tumor size, *GREM1*-region iii methylation does not seem to be an independent prognostic factor: HR(OS)=0.88, 95% CI: 0.45-1.74, and HR(MFS)=1.04, 95% CI: 0.65-1.68.

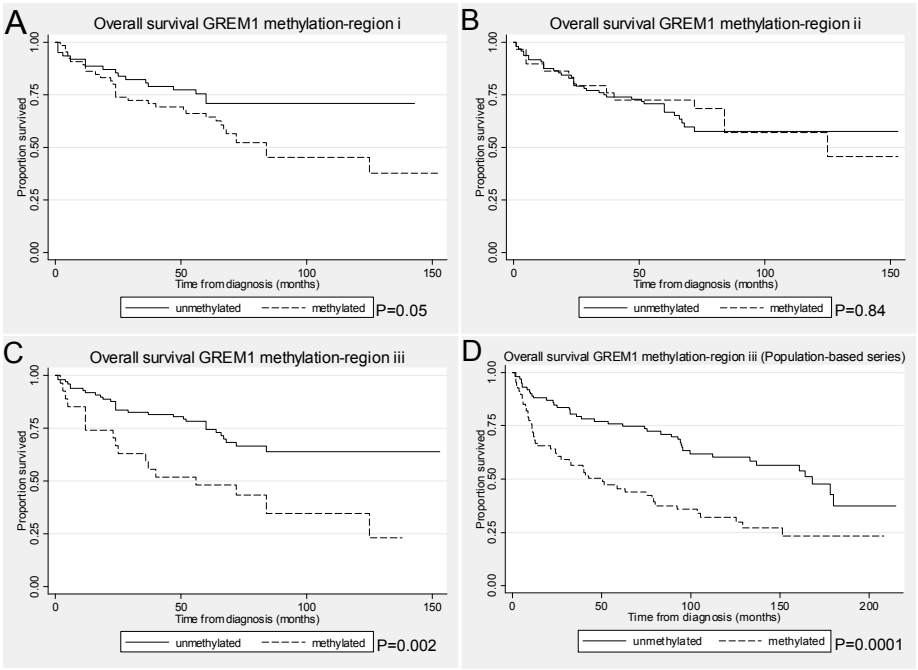

Figure 2. Overall cause-specific survival curves for ccRCC patients according to *GREM1* promoter CpG island methylation at region i (A), ii (B), and iii (C, D), with A, B, and C for patients of the hospital-based series and D for patients of the population-based series.

Since *GREM1* promoter CpG island methylation at region iii was most cancer-specific and because of the result obtained for this region in the hospital-based series, we next aimed to validate the results obtained for *GREM1*-region iii, in an independent population-based series of ccRCC (table 2). Age and sex were equally distributed among the methylated and unmethylated cases in this series, (P-values >0.05, see table 2). 40% (68 of 168) of the ccRCCs with *GREM1*-region iii MSP results were methylated and methylated tumors more often had a higher tumor grade (p=0.03) as compared to unmethylated tumors. A similar trend was seen for tumor stage (p=0.08). The median OS for unmethylated versus methylated cases at region iii is 96 (1-218) versus 42 (range 1-212), respectively. Figure 2.D shows the Kaplan-Meier curve for cause-specific survival and *GREM1*-region iii methylation, and methylated cases had a statistically significant worse survival as compared to unmethylated cases (log-rank test p=0.0001). Cox regression analyses also showed a statistically significant association between methylation and OS with an age and sex adjusted HR of 2.32 (95% CI: 1.52-3.53) and a multivariate HR of 2.27 (95% CI: 1.44-3.59, adjusted for age and sex, grade, stage and size).

Discussion

Epigenetic silencing of *GREM1* by promoter CpG island methylation is present in ccRCC cell lines, and can be reversed by inhibiting DNA methyltransferases, indicating that DNA methylation is involved in *GREM1* silencing. We have also shown an association between *GREM1* promoter CpG island hypermethylation and increased malignancy/angiogenesis in primary ccRCCs, while methylation in histological normal renal tissue is an infrequent event.

Interestingly, *GREM1* promoter CpG island methylation in primary ccRCCs varies within the CpG island, with frequencies of 55%, 24%, and 20% for region i, region ii, and region iii, respectively. We showed that within one CpG island, CpG methylation at loci only a few hundred base pairs apart can differ strongly. As previously shown for *hMLH1* in colorectal cancer, methylation associated transcriptional silencing can occur in a region-specific manner[35], indicating that also clinical effects may be methylation-region specific. This observation emphasizes that the location of CpG dinucleotides to be analyzed and with this, MSP primer location and -design, can influence the observed methylation prevalence and clinical association[36].

Analyses of clinicopathological characteristics revealed that significant differences between *GREM1* methylated and unmethylated ccRCCs are also dependent on the region analyzed. Only for the most downstream region (iii), CpG methylation was statistically significant associated with increased malignancy, i.e. larger tumors, higher tumor grade and -stage, and a worse prognosis. These data suggest that the effect of *GREM1* promoter CpG island methylation on ccRCC biology depends on the region methylated within the CpG island. Whether *GREM1* promoter CpG island methylation initiates at region i and than spreads downstream towards the TSS and region iii or starts in region iii and than retroverts towards region i can not be deduced from these data.

The second series is derived from the population-based prospective Netherlands Cohort Study, and therefore appropriate for validation of the results. This series confirmed the associations between region iii methylation and tumor grade and prognosis which were found in the hospital-based series. In addition, this validation points to *GREM1* as a gene independently influencing the progression of ccRCCs when methylated at region iii (figure 3 lower panel), which was not found in the hospital-based series to the same extent (figure 3 upper panel). Possible differences between the two ccRCC series were not apparent, but could perhaps account for this observation. Nevertheless, both series do indicate a significant effect of *GREM1*-region iii methylation on increased malignant behavior and a worse prognosis of ccRCC.

Our data also show that *GREM1*-region iii methylation is significantly associated with increased ECP, TCP and lower MVD, suggesting that *GREM1* silencing promotes active angiogenesis, pointing to *GREM1* as an anti-angiogenesis factor. Similar observations showing that BMP2 and BMP4 exert angiogenic activity, which may increase with inactivation of the BMP-antagonist *GREM1* have been reported[37-39]. However, a recent study by Stabile et al. indicated a BMP-independent, pro-angiogenic function of *GREM1* by showing *GREM1* expression in human lung tumor endothelium and binding of *GREM1* to the endothelial cell surface thereby activating intracellular signaling and cell motility[23]. An angiogenic role of *GREM1* may be tissue specific and/or dose-

dependent. ECP also may be indirectly influenced by *GREM1* through its strong correlation with TCP (Rho=0.82, p<0.001). Moreover, during tumor progression, cumulative genetic/epigenetic events can activate additional angiogenic growth factors, resulting in an increased tumor driven angiogenesis. Additionally, *GREM1* promoter CpG island methylation may affect maturation of intratumoral blood vessels in ccRCC, as Maciel et al. reported induction of vascular smooth muscle cell (VSMC) proliferation and migration by Gremlin[40]. This is supported by the fact that ccRCC with high ECP, representing high grade ccRCC, are shown to be characterized by a less stabilized, less mature vasculature[33]. Therefore, *GREM1* inactivation may impede tumor vessel maturation, as observed in high grade tumors, in which less differentiated vessels provide an easier access for cancer cells to the circulation.

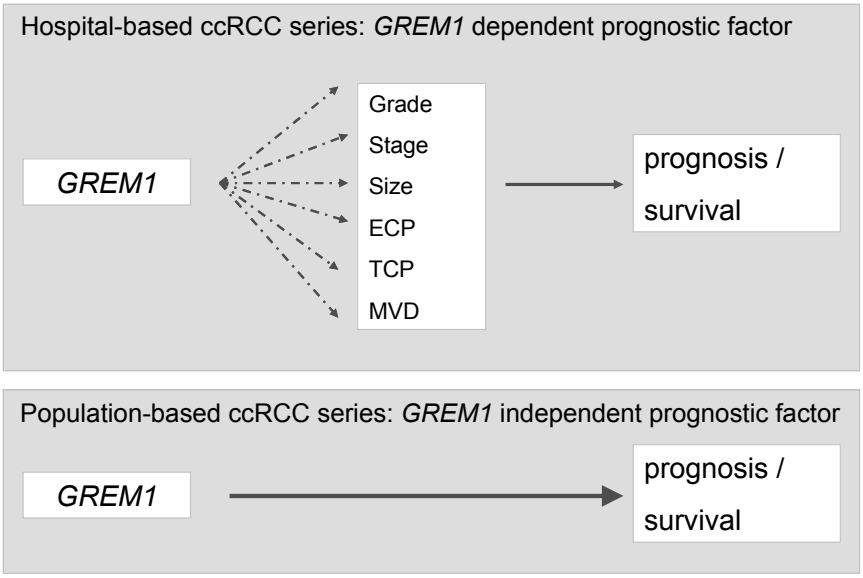

Figure 3. Prognostic significance of *GREM1* promoter CpG island methylation in hospital-based and population-based ccRCC patient series

Except for sclerostin domain-containing-1 (SOSTDC1), another BMP antagonist which suppresses proliferation of RCC cells and is down regulated in RCC[41], no other BMP antagonists have previously been implicated in RCC. *GREM1* has been shown to be down regulated in several tumor cell lines i.e. neuroblastoma, fibrosarcoma, colon adenocarcinoma, breast carcinoma, suggesting a tumor suppressor function[22, 42]. However, the role of *GREM1* in renal carcinogenesis has not yet been elucidated. Silencing of *GREM1* by promoter CpG island methylation may play a role in carcinogenesis both by affecting cell cycle and by upregulation of BMP signaling. Earlier studies have linked BMPs and BMP receptors (BMP-RI and BMP-RII) to cancer[43-45]. Biological responses to BMP are cell type-specific and divergent effects with both oncogenic and tumor suppressor activities have been described[44]. Despite the progress achieved in delineating the functional significance of BMP pathways in carcinogenesis in general during the last decade[46-50], little is known about the importance of BMP signaling in RCC.

To learn more about the role of *GREM1* and the consequence of silencing *GREM1* in ccRCC cancer, the relationship between promoter CpG island methylation and gene and protein expression needs to be explored. In this study *GREM1* promoter CpG island methylation and mRNA expression did not correlate (see supplemental figure 1). Several explanations can explain these results. First, overall the basal expression level of *GREM1* in normal kidney is very low, an observation which has been reported for other (candidate) tumor suppressor genes/biomarkers as well (*TP53*[51], *VIM*[52]), indicating that the regulation of expression of important cancer-associated genes is complex. Our data are in agreement with the literature consensus on *GREM1* expression levels in normal adult renal tissue, which seems to be very low or undetectable[53-56], see also supplemental figure 1. An explanation for this observation could be that *GREM1* is a gene involved in embryogenesis. Many embryologically expressed genes regulate morphogenesis and then become quiescent, which could explain the absence of *GREM1* expression in adult normal renal tissue[57]. It is increasingly being appreciated that some developmental genes are reactivated in the adult in diseased tissues. Gremlin expression in the diabetic glomerulus[53, 54] serves to illustrate the complexities and opportunities posed by this emerging paradigm[57]. These literature observations as well as our own data indicate that analyzing epigenetic silencing of *GREM1* in cancer tissue as compared to normal epithelial tissue is complex.

An additional complicating factor is the observation that the Gremlin protein belongs to the Dan family of secreted glycosylated proteins[13, 58] and that expression is predominantly observed in the stromal counterpart of other cancers types (skin, lung, esophagus, pancreas, breast, bladder, colon)[23, 56]. This complicates studying the correlation between epithelial promoter methylation and epithelial RNA and protein expression. At the same time, Sun et al. reported tissue-specific intracellular interaction between Gremlin and BMP4[59], which indicates again the (micro)environment dependency of the expression and actions of Gremlin1.

In conclusion, our results indicate that promoter CpG island methylation of *GREM1* has an impact on the biology and outcome of this type of cancer. Notably, *GREM1*-region iii promoter CpG island hypermethylation appears to be independently associated with aggressive tumor behavior and increased angiogenesis and therefore could be a candidate tumor suppressor gene and interesting prognostic biomarker in ccRCC. Probably, this prognostic effect is mediated through an increase TCP and ECP. However, the exact mechanism of *GREM1* inactivation and its relation to tumor progression as well as its role in angiogenesis need to be further clarified. In addition, further studies are needed to explore whether *GREM1* promoter CpG island methylation can also be detected in urine or serum samples for use as a possible noninvasive marker for aggressive ccRCC.

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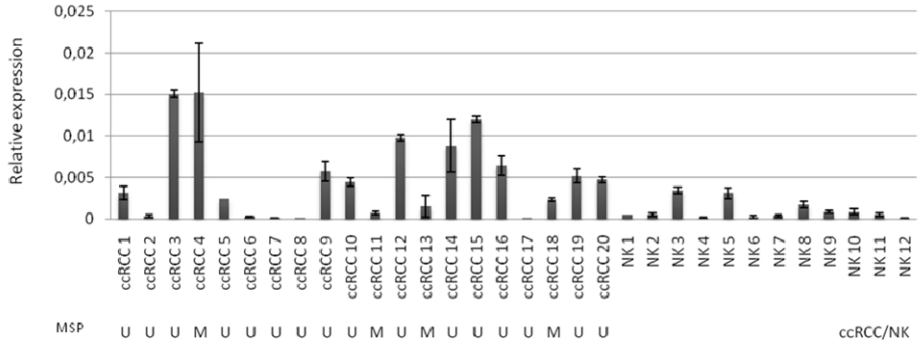
Supplemental figure 1



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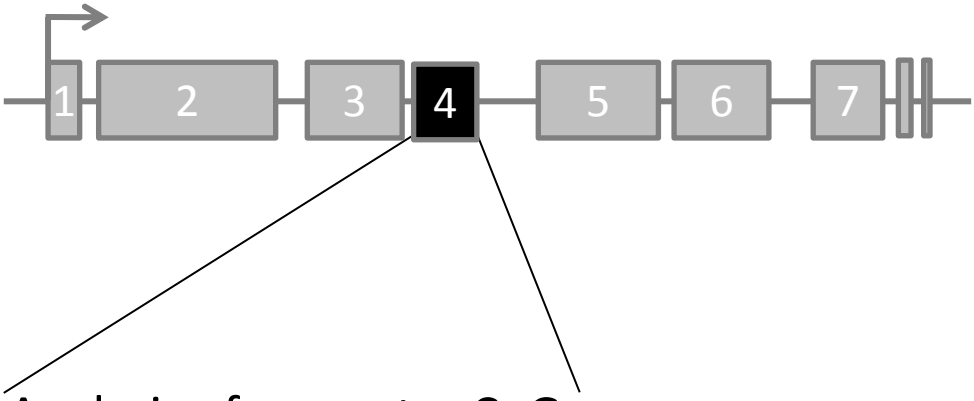

Analysis of promoter CpG island hypermethylation in cancer: location, location, location!

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Abstract

The genetic and epigenetic alterations that underlie cancer pathogenesis are rapidly being identified. This provides novel insights in tumor biology as well as in potential cancer biomarkers. The somatic mutations in cancer genes that have been implemented in clinical practice are well defined and very specific. For epigenetic alterations, and more specifically aberrant methylation of promoter CpG islands, evidence is emerging that these markers could be used for the early detection of cancer as well as prediction of prognosis and response to therapy. However, the exact location of biologically and clinically relevant hypermethylation has not been identified for the majority of methylation markers. The most widely used approaches to analyze DNA methylation are based on primer- and probe-based assays that provide information for a limited number of CpG dinucleotides and thus for only part of the information available in a given CpG island. Validation of the current data and implementation of hypermethylation markers in clinical practice require a more comprehensive and critical evaluation of DNA methylation and limitations of the techniques currently used in methylation marker research. Here, we discuss the emerging evidence on the importance of the location of CpG dinucleotide hypermethylation in relation to gene expression and associations with clinicopathologic characteristics in cancer.

Introduction

DNA methylation is involved in regulating gene expression in normal physiology (e.g., by managing imprinting, X-chromosome activation, and tissue-specific gene expression) and disease (e.g., neurodevelopmental and degenerative disorders, autoimmune diseases, and cancer)[1]. DNA hypermethylation-induced silencing of tumor suppressor and DNA repair genes is a frequent phenomenon affecting the hallmarks of cancer[2, 3]. Aberrant DNA methylation often occurs around the transcription start site (TSS) within a CpG island, and as was recently shown, even outside of the traditionally defined islands[4, 5]. These hypermethylation markers are promising tools to detect cancer cells in tissue and body fluids[6, 7] with the use of simple PCR technology[8-10]. Proof of principle for the clinical value of methylation markers has been reported for early detection and classification of cancer[11-21], risk assessment and prognosis[18, 22-24], and prediction of therapy response[25-27], with some already having shown their importance in (pre)clinical practice. Thus, the promise of methylation changes to become a powerful diagnostic and predictive tool[6] is becoming a reality.

Nevertheless, the clinical value of biomarkers depends on the accuracy and prognostic or predictive value of the marker. The CpG islands of a variety of cancer-associated genes have been evaluated for methylation, and positive, negative, and null associations with gene expression and clinical characteristics are reported. Here, we discuss emerging evidence on the importance of the location of aberrant CpG dinucleotide methylation in relation to gene expression and its clinical value in cancer.

Location of biologically relevant methylation in promoter CpG islands

The dogma that promoter CpG island methylation generally induces gene silencing is currently being specified. Specific regions within the promoter CpG islands, designated as core regions crucial for regulating gene expression, are rapidly being identified. As illustrated in Figure 1, these regions are often situated around the TSS within a CpG island but can also be observed more upstream or downstream of the TSS.

One of the first studies to show that hypermethylation of a specific locus is critical for transcriptional repression was conducted in the human bladder cancer cell line T24. Treatment with the demethylating agent 5-aza-2'-deoxycytidine resulted in different expression levels of cyclin-dependent kinase inhibitor 2A (*CDKN2A*) in the acquired subclones. No direct correlation between the degree of methylation and gene expression was observed. However, demethylation of a specific region upstream of exon 1 did correlate with reexpression, whereas CpGs in the vicinity of this region showed methylation in all subclones[28]. Similarly, expression of human telomerase reverse transcriptase (*hTERT*) was reported in a variety of cancer cell lines despite dense promoter hypermethylation at the region initially analyzed (upstream of TSS)[29]. A more detailed analysis of the promoter CpG island region around the TSS revealed that silencing of *hTERT* expression was associated with dense methylation at, or in close proximity to, the TSS and is independent of methylation more upstream of TSS[29]. Similar observations have been reported for the TGF-β signaling target *RUNX3*[30] and the T-cell differentiation protein *MAL*[31] in gastric cancer cell lines and primary gastric cancers.

Core regions have also been observed outside the direct TSS region. A small region proximal to the *MLH1* TSS has been identified to regulate expression by methylation in

24 colorectal cancer cell lines[32], whereas hypermethylation upstream of this region did not influence *MLH1* expression[32] and was later suggested to be age-related[33]. The same correlation was observed in 64 primary colorectal cancers[34] as well as in 123 patients with colorectal cancer in an independent study[35]. Similarly, mapping of *WIF-1* promoter CpG island hypermethylation reveals regional methylation just proximal to the TSS that correlates with transcriptional silencing, whereas other more upstream regions do not[36].

CpG island methylation analyses of *SOCS1* in hepatocellular carcinoma cell lines revealed one unmethylated cell line without *SOCS1* expression. More detailed analyses by bisulfite sequencing of a larger region revealed regional and clustered hypermethylation more downstream of the initially analyzed region, indicating a silencing effect by methylation in this critical 3′-TSS region[37].

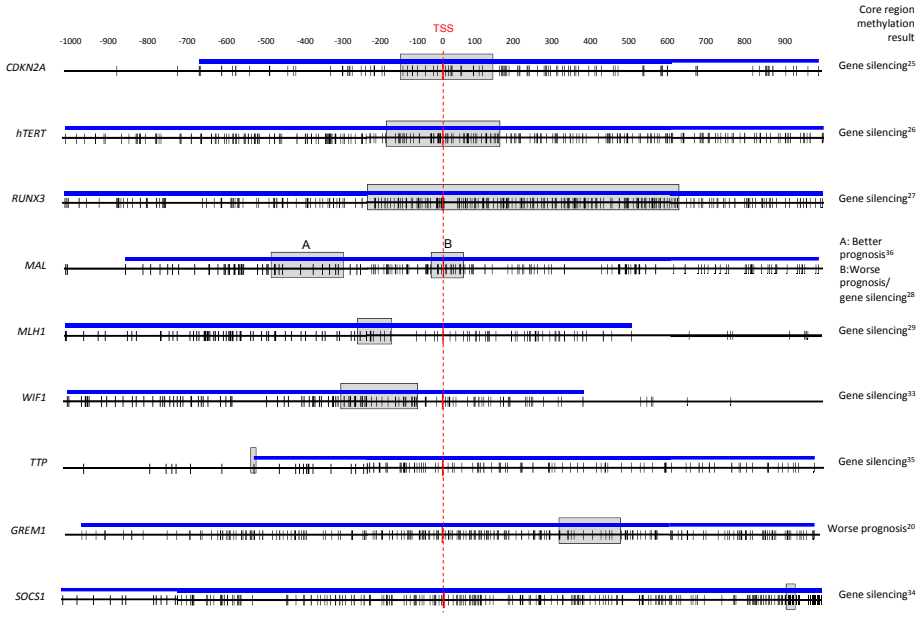
Interestingly, a recent study showed transcriptional silencing of *TTP* in liver cancer by hypermethylation of a specific single CpG site. One specific CpG dinucleotide, located at the 5′-boundary of the CpG island, was exclusively hypermethylated in transcriptionally silenced cell lines[38]. This observation narrows down the core region for hypermethylation-induced silencing of *TTP* to just one CpG dinucleotide.

These studies show that transcriptional silencing does not require hypermethylation of the entire CpG island, but that methylation of a few gene-specific core CpG dinucleotides, most likely associated with transcription, may be sufficient. It is important to realize that data obtained solely in cell lines can be biased, as they exhibit significantly more CpG island methylation than the primary tumors they represent[39] and thus proof of principle in primary tumors is required. Identifying the core regions regulating gene expression is essential for evaluation of the clinical value of DNA hypermethylation (Figure 1). For example, two regions within the *MAL* promoter were analyzed for methylation in gastric cancer samples. Hypermethylation of both regions occurred in 71% and 80%, respectively; however, only methylation at the region closest to the TSS was correlated with a better disease-free survival[31]. In addition, increased expression of *MAL* in serous ovarian cancer patients with a poor prognosis is associated with decreased methylation of a specific region of the *MAL* promoter[40].

We recently described a region in the promoter CpG island of *GREM1* that was specifically associated with poor prognosis in clear cell renal cell carcinoma. Three regions were analyzed for hypermethylation, but only one was correlated with poor survival[23]. This indicates that location of hypermethylation is also important for marker discovery.

These studies clearly indicate that the biological and clinical consequences of promoter CpG island hypermethylation are strongly dependent on silencing of expression-regulating core regions in the CpG island. Although clinically relevant hypermethylation of a specific locus is not always perfectly associated with gene expression and might serve as a surrogate marker for functional hypermethylation of another locus, we expect that the best validated markers will be those for which good correlations between DNA methylation and gene expression exist.

Hypermethylation outside core regions is frequently observed in cancer cells but sometimes also in normal cells[41-44] and is correlated with aging and chronic inflammation[41-43, 45, 46]. This is hypothesized to progress toward the core region, initiating gene silencing[47]. For example, a demarcation has been observed between *RASSF1A* hypermethylation in exon 1 and in its immediate upstream promoter region.



Legend to Figure 1.
Location of biologically relevant methylation in promoter CpG islands. Promoter regions from −1,000 to +1,000 bp are depicted relative to the TSS (at 0 in red) with CpG islands in blue. Vertical lines represent CpG sites, and gray boxes show the relevant regions (core regions) for expression or progression. All genes are presented in a forward fashion and are grouped by core region position relative to the TSS: top genes are around TSS, middle genes are pre-TSS, and bottom genes are post-TSS. Top, core region of cell-cycle regulating gene *CDKN2A* was identified from −121 to +123 relative to the TSS. The catalytic subunit of telomerase, *hTERT*, showed a core region from −150 to +150, relative to TSS. The important region in the human runt–related transcription factor 1, *RUNX1*, was reported from −194 to +451 relative to the TSS. Methylation at *MAL* promoter from −92 to −7 relative to the first ATG correlated with expression and survival (B). Another region was found at −452 to −266 relative to the TSS, which showed a correlation with worse prognosis (A). Middle, the region −248 to −178 relative to the TSS of the mismatch repair gene *MLH1* was identified as the core region. For the Wnt-pathway antagonist Wnt inhibitory factor-1 (*WIF-1*), the core region is reported to be proximal to the TSS from −295 to −95. *TTP*, a negative post-transcriptional regulator of c-Myc, uniquely showed one CpG at −500 bp at the 5'-boundary of the CpG island, as the core dinucleotide. Bottom, for the bone morphogenetic protein (BMP) pathway antagonist *GREM1*, the region +311 to +471 relative to the TSS showed clinical correlations when methylated. The core region of a negative regulator of the Janus-activated kinase (JAK)/STAT pathway *SOCS1* was identified at +901 to +924 (relative to new TSS). Note: differences in location as compared with Yoshikawa and colleagues (ref. 37) are due to a repositioning of the predicted TSS after publishing (680 bp more upstream according to the previous predicted TSS position). Original reported core region at +221 to +244.

In normal breast tissue, exon 1 is methylated without affecting gene expression, whereas in breast cancer samples, hypermethylation is observed in both exon 1 and its immediate upstream promoter region that is associated with *RASSF1A* silencing. A progressive spreading from exon 1 upstream is proposed, which can occur early in breast tumorigenesis[48]. Additional evidence for spreading of hypermethylation in the promoter CpG island region has been observed for *CDKN2B* in leukemia[49], *CDKN2A*[50], *MGMT*[51], and *NDRG4*[19] in colorectal cancer, and for *RUNX* [30] in gastric cancer.
Spreading of DNA methylation is often consistent with increasing density of methylation, but whether density itself or spreading toward (expression regulating) specific regions is correlated with gene silencing is currently not clear.

Location of DNA methylation initiation

It might be speculated that the core region for which hypermethylation is associated with gene silencing and clinical consequences has specific (sequence) characteristics. To study this hypothesis, Feltus and colleagues applied DNA pattern recognition techniques in a DNA cytosine-5-methyltransferase 1 (DNMT1) overexpressing human cell culture model and showed that methylation-prone and methylation-resistant CpG islands can be distinguished by an underlying sequence signature based on 13 DNA motifs[52, 53]. These motifs were proposed to represent protein-binding sites involved in the susceptibility to or prevention of DNA methylation. Although the methylation-prone motifs do not obviously resemble a transcription factor consensus sequence or protein-binding site, transcription factors PML-RAR[54] and c-Myc[55] have shown the ability to initiate DNA hypermethylation by the recruitment of DNA methyltransferase enzymes (DNMT) to specific loci. The opposite is observed for the presence of Alu elements[52] and Sp1-binding sites[56, 57], as well as binding of the insulator protein CTCF[58], which are all associated with resistance to DNA hypermethylation. Subsequent studies have shown that genes with a methylation-prone sequence motif and genes characterized by Polycomb group (PcG) protein occupancy in embryonic stem cells are strongly related[59]. PcG proteins have been shown to mark target genes in the progenitor or stem cell state by targeting H3K27 histone methylation. Several observations indicate that there could be a functional link between PcG protein binding and CpG island hypermethylation. First, the reported percentage of PcG-binding sites that correspond to CpG islands ranges from 50% to 88%[60, 61]. Second, direct interactions have been described between PcG proteins and DNMTs[62, 63]. Third, PcG target genes are up to 12 times more likely to have cancer-specific promoter hypermethylation than non-PcG targets[64-66]. These observations make it tempting to speculate that PcG proteins recruit DNMTs to their target genes and thereby induce aberrant transcriptional silencing of promoter CpG islands by DNA hypermethylation.

Location of methylation outside of classical promoter CpG Islands

DNA methylation studies in cancer initially focused on gene promoter CpG island hypermethylation. However, recent research revealed novel insights on the location of DNA hypermethylation. Hypermethylation of intra- and intergenic CpG dinucleotides might contribute to regulating gene expression by functioning as alternative promoters[5]. For example, in-depth investigation of the human *SHANK3* locus (~60 kb) showed hypermethylation-regulated intragenic promoter activity, expressing alternative transcripts in a tissue- (brain) and cell-type (primary cortical astrocyte)–specific manner[5]. In addition, other gene-regulating regions such as enhancers, which are *cis*-regulatory DNA sequences that increase transcription independent of their orientation and distance relative to the TSS, can be regulated by hypermethylation[67, 68]. For example, hypermethylation-dependent enhancer-like activity, located at a CpG island in *EGFR2* intron 1, is suggested to regulate transcription[69].

Evidence is accumulating that CpG island hypermethylation in bidirectional promoters is correlated with silencing of both genes, thereby possibly accelerating tumorigenesis, for example, in the gene pairs *WNT9A/CD558500*, *CTDSPL/BC040563*,

KCNK15/BF195580, and *MLH1/EPM2AIP1*[70, 71]. Even in promoters without a classical CpG island (low CpG density), hypermethylation still can regulate expression, as has been shown for *Maspin* in breast cancer[72].

Although the impact of DNA hypermethylation has been studied mainly in CpG islands located at TSSs[73], Irizarry and colleagues recently introduced the term "CpG island shores"[4], regions with a relatively low CpG density located within 2 kb of traditional CpG islands. Aberrant methylation in these shores was reported to segregate tissue subtypes and cancerous tissue from matched normal tissues[4]. These observations change the current focus from exclusively CpG islands in promoter regions to much larger regions of interest, which potentially possess regulatory regions previously characterized.

The biological relevance of hypermethylation throughout the gene locus by means of long-range interactions with the promoter region has recently been shown by Tiwari and colleagues[74]. They reported DNA methylation at 6 of 7 CpG islands, including the island spanning the TSS, throughout the *GATA4* gene. Chromatin looping can enable long-range interactions of these islands around a single gene. This can cluster aberrant methylation of CpG islands and other epigenetic markers, thereby facilitating and enhancing transcriptional repression[74]. These findings demand mapping of DNA hypermethylation of genes in higher-order chromatin structures as there might be an additional role for chromatin looping in mediating gene expression.

Frigola and colleagues showed for the first time that clusters of genes could be coordinately repressed by epigenetic mechanisms, a concept termed long-range epigenetic silencing (LRES)[75]. They identified an epigenetically repressed 4-Mb spanning region of chromosome *2q14.2.* Genes located in this cytogenetic region are affected by hypermethylation of clusters of neighboring CpG islands and coordinately inactivated by chromatin remodeling. Similar LRES mechanisms have been observed by others in chromosomal regions *3q22*[76] and *5q35.2*[77]. Recently, 47 LRES regions were identified in prostate cancer, typically spanning about 2 Mb and harboring approximately 12 genes[78]. Global gene silencing by LRES is comparable with genetic deletions by LOH, as large regions become simultaneously inactivated. Therefore, LRES provides an efficient silencing mechanism in cancer development.

Furthermore, nucleosome organization, location, and dynamics are critical for gene regulation. Lin and colleagues studied *MLH1* silencing by hypermethylation and nucleosomal occupancy in cancer[70]. They showed nucleosome depletion just upstream of each start site on the active *MLH1* promoter in normal cells, whereas 3 nucleosomes were present on the hypermethylated, inactive promoter. Moreover, gene reactivation induced by the demethylating agent 5-aza-2′-deoxycytidine involved promoter nucleosome removal, suggesting that epigenetic silencing may involve the (reversible) movement of nucleosomes into previously vacant positions[70]. Changes in nucleosomal occupancy not only occur at TSS regions but also at enhancers acting at variable distances from the start site[79].

Conclusions and perspectives: reflect on location

The above-described location-related complexities of gene expression regulation by aberrant DNA methylation can all, separately or combined, result in unexpected or misinterpreted information on the associations among DNA hypermethylation, gene expression, and clinical parameters. Promoter CpG islands of genes have often been reported as "unmethylated" or "hypermethylated," based on the data of only a small number of CpG dinucleotides independent of location or the assays that have been used. Because it now has become clear that the location of core regions and the density of methylation required for gene silencing can vary per gene, a broader view than just the classical dogma of promoter CpG island methylation and gene silencing is needed to interpret data on DNA hypermethylation, gene expression, and clinicopathologic associations. Unexpected results do not *per se* contradict this dogma regarding the complexity and the number of parameters involved in epigenetic silencing. In addition, all the above-mentioned phenomena might be tissue, cell-type, cancer type, genomic region, or gene specific, thereby complicating data analysis, interpretation, and validation of results and conception of the literature.

These considerations underscore the importance of detailed analysis of CpG dinucleotide analysis and careful data analysis, with regard to diverse techniques and/or primer and probe design. Results of analyses at the same region are dependent on the detection method, that is, primer (design), reagents, detectors, equipment, and protocols, which all influence sensitivity and specificity. Frequently used technologies are restriction enzyme- and/or bisulfite-based analyses, the results of which are highly dependent on primer- and/or probe/microarray design-like methylated-specific PCR (MSP)[80], methylated DNA immunoprecipitation (MeDIP)[81], methylated-CpG island recovery assay (MIRA)[82], and Illumina Infinium methylation assay[83]. Limitations of these techniques can introduce bias; for example, MSP only assesses 2 to 4 CpG dinucleotides per oligo and thus needs to perfectly cover the core region of interest. Methylation-sensitive restriction enzyme digestion can introduce recognition site bias and is prone to false-positive results because of incomplete digestion. Techniques using DNA hybridization to microarrays introduce ascertainment bias (for an extensive overview of the resolution and limitations of the most widely used techniques to analyze DNA methylation, see ref.[84]). The recently developed novel technologies that enable (semi)epigenome-wide analyses such as bisulfite deep sequencing[85] or methyl-binding protein domain (MBD)-sequencing[86] are promising in this respect. However the technical limitations (such as sensitivity/specificity and resolution, but also bisulfite conversion, CpG coverage, number of methylated CpG sites, choice of region analyses, etc.) have to be considered, especially when reporting methylation data. Furthermore, hydroxymethylcytosine (hmC) has been discovered recently, but its role is as yet unknown. It is hypothesized that the presence of hmC in DNA can inhibit methyl-binding proteins, enzymatic functions, and gene expression. Enzymatic- or bisulfite-based approaches cannot discriminate between hydroxy- and 5-methylcytosine because of structural similarity[87]. Therefore, the possible presence of hmC should be considered in future methylation assay design.

The future discovery of clinically relevant hypermethylation markers would preferably be genome-wide and location- and CpG density–independent. In contrast, subsequent

sequence-specific methylation analyses would need to be core-region specific. Careful and thorough experiment and assay design will lead to the development of sensitive and specific hypermethylation markers that can be used for early detection of cancer and prediction of prognosis and response to anticancer therapy. These methods enable independent validation by studying the same core regions, accurate identification of the biologically relevant location of hypermethylation, and translation of data into an accurate biomarker assay.

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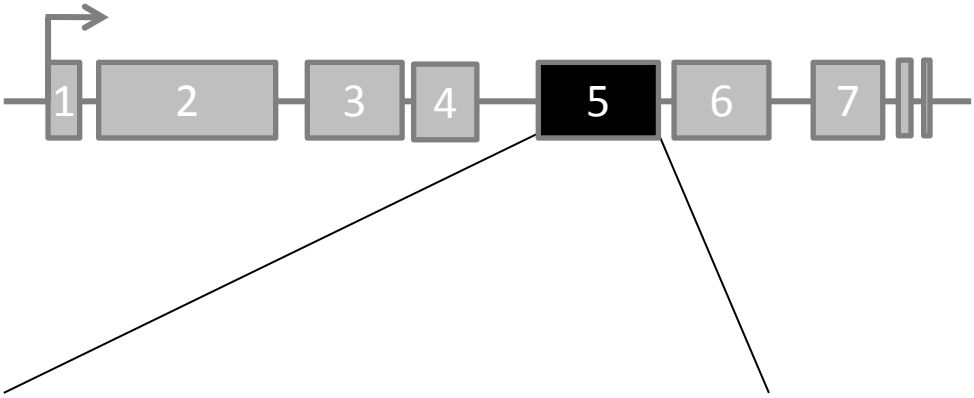

A four gene promoter CpG island methylation marker panel predicts survival of clear cell renal cell cancer patients

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Submitted

Abstract

Clear cell renal cell carcinoma (ccRCC) is the most common adult renal cancer. Although the molecular characteristics of ccRCC are currently being studied, the biologically and clinically relevant ccRCC methylome remains to be elucidated. To explore the RCC hypermethylome we employed massive sequencing of methyl-binding protein enriched DNA and validated the biological relevance of the identified hypermethylated sites by pharmacological inhibition of DNA methylation. We identified four candidate tumor suppressor genes (*GREM1*, *LAD1*, *NEFH*, and *NEURL*) of which promoter CpG island hypermethylation was strongly predictive for ccRCC survival in two independent series (n=150 and n=185) of ccRCC primary samples. The four markers combined are strongly associated with risk for cancer-related death in the test series (HR 3.64, 95% CI 1.02-13.01) as well as, independently of other clinicopathological characteristics, in the validation series (HR 7.54, 95% CI 2.68-21.19) using Cox proportional hazard models. According to Harrell's C statistics and the Akaike Information Criterion (AIC) the four marker panel provides the best predictive capacity with the best fit of the model.
These results provide novel insights into the ccRCC hypermethylome and identify a strong methylation marker panel to potentially guide personalized ccRCC patient management. Preliminary to implementation of this marker panel into clinical practice, enrollment of these patients in a Phase-III trial to study adjuvant treatment efficacy is essential.

Introduction

Exploring cancer (epi)genomes provides insight in tumorigenesis and this knowledge might be used to develop biomarkers for the early detection of cancer, for the prediction of prognosis and response to therapy as well as for developing novel therapies. Unlike well-characterized cancers such as colorectal and breast cancer, the biology of renal cancer has not yet been extensively studied. The most common adult renal neoplasm is clear cell renal cell carcinoma (ccRCC), histologically characterized by lipid-rich clear cells and high vessel density. Currently, the patient performance status, TNM stage and Fuhrman nuclear grade are the best predictors of patient outcome[1] but more specific parameters are needed. Although loss of function of the *von Hippel-Lindau* (*VHL*) gene is a key event in the development of ccRCCs[2-4], alterations in the structure[4, 5] or regulation of the expression of the *VHL* gene[6, 7] do not appear to be directly associated with tumor cell proliferation and patient prognosis[8, 9]. Except for *VHL* and the recently identified *PBRM1* gene[10], no genes have been identified that are consistently mutated in a substantial proportion of ccRCCs. Known cancer genes that are frequently mutated in other cancers (*RAS* genes, *TP53*, *CDKN2A*, *BRAF*, *PTEN*, etc) do not play an important role in ccRCC[11]. Clinical management would profit from markers that can predict patient outcome and/or guide treatment decisions.

Promoter CpG island hypermethylation and subsequent gene silencing are involved in RCC oncogenesis more frequently than genetic alterations[11, 12]. This affects numerous cellular processes, including cell cycle checkpoints, apoptosis, signal transduction, cell adhesion, and angiogenesis[13].

Rapid developments in approaches to analyze the methylome, based on expression microarrays, have facilitated the identification of cancer related genes inactivated by DNA hypermethylation. However, probe- and protocol design in array-based strategies introduce detection bias and influence the sensitivity and specificity of detection. There is emerging evidence that CpG dinucleotide methylation, regulating gene expression, is highly location specific[14]. As current probe-design is location- rather than function oriented, this may hinder identification of relevant methylated genes by microarray-based discovery. Next-generation sequencing (NGS) technology is a more direct method and allows genome-wide sequencing of enriched DNA fractions, which overcomes these limitations[15].

We comprehensively explored the clear cell renal cell cancer hypermethylome, by integrating methyl-binding domain (MBD) affinity purification based massive parallel sequencing of ccRCC cell lines with global transcript expression array data obtained by pharmacological inhibition of DNA methylation. This allows us to identify those promoter hypermethylated genes that are functionally relevant, i.e. silenced by promoter hypermethylation. A panel of four genes was identified, of which methylation impacts on tumor cell proliferation and invasion and which strongly predicts prognosis of ccRCC patients in a retrospective series and prospective validation series of ccRCC.

Materials and Methods

Study populations and clinical specimens

DNA from formalin-fixed and paraffin embedded primary tumor samples from two independent well characterized ccRCC patient series was used for this study. The first population is a hospital-based series (n=150) derived from the archives of the Department of Histopathology, University Hospital of Leuven and Department of Pathology, Maastricht University Medical Center. The second population (n=185) was obtained from the Netherlands Cohort Study on diet and cancer (NLCS)[16], a prospective, population-based cohort study in which tumor tissue was collected from 51 pathology laboratories throughout the Netherlands. In addition, histologically normal renal tissue samples (formalin-fixed, paraffin embedded) of 20 non-RCC patients were collected from the archives of the Department of Pathology, Maastricht University Medical Center.
Tissue collection, DNA isolation and patient characteristics (see also Table 3 and Table 4) have been described in detail elsewhere[17, 18]. This study was approved by the Medical Ethical Committee of the Maastricht University Medical Center.

Cell culture, drug treatment and microarray analyses

Four ccRCC cell lines (SKRC1, SKRC10, SKRC52, SKRC59), kindly provided by Dr. E. Oosterwijk, Nijmegen Center for Molecular Life Sciences (NCMLS), Nijmegen, The Netherlands, were cultured in RPMI 1640 media (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The human kidney 2 cell line (HK-2) was purchased from ATCC and cultured in Keratinocyte Serum Free Medium (K-SFM, GIBCO) supplemented with bovine pituitary extract (BPE) and human recombinant epidermal growth factor (EGF) as recommended.
SKRC1, SKRC10, SKRC52, and SKRC59 cells were treated with 5'aza-2-deoxycytidine (DAC) or Trichostatin A (TSA), which inhibit DNA methylation and histone deacetylation, respectively, as recently described[19, 20]. Approximately 10% confluent RCC cells were cultured in RPMI 1640 media containing 10% FBS with 5µM (DAC) (Sigma; stock solution: 1mM in PBS) for 96 hours, replacing media and DAC every 24 hours. Cell treatment with 300nM TSA (Sigma; stock solution: 5mM dissolved in ethanol) was performed for 18 hours, starting at approximately 30% cell confluency. Mock treated cells were grown in parallel with the DAC treatment by adding equal volumes of PBS without drugs.
Microarray expression analyses were performed on mock-, DAC-, or TSA-treated cells (SKRC1, SKRC10, SKRC52, SKRC59), as described by Schuebel et al.[19]. In brief, total RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturers' instructions and quantified using the NanoDrop ND-100 followed by quality assessment with the 2100 Bioanalyzer (RIN=10 (n=10), 9.9 (n=1) or 8.9 (n=1), Agilent Technologies). Sample amplification and labeling procedures were carried out using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) according to the manufacturers' instructions. The labeled cRNA was purified using the RNeasy mini kit (Qiagen) and quantified. Cy3 or Cy5 labeled samples were mixed with control targets (Agilent Technologies), assembled on Oligo Microarray, hybridized, and processed

according to the Agilent microarray protocol. Scanning was performed with the Agilent G2565BA microarray scanner using settings recommended by Agilent Technologies.
Array data were analyzed using R (version 2.10.0) and BioConductor, using the limma package (version 3.2.1)[21]. Median Cy5 and Cy3 signals read from the raw data and M-values, i.e. log2(Cy5/Cy3), were loess normalized. Basal expression was roughly estimated using the single channel Cy3 values, containing the mock treated samples. Based on the threshold used for basal expression, the specificity could be further increased, with obvious implications on sensitivity.

MBD-affinity NGS

SKRC1, SKRC10, SKRC52, SKRC59 and HK-2 cells were cultured as described above and DNA was isolated using the Puregene DNA purification kit (BIOzym, Landgraaf, the Netherlands) according to the manufacturer's instructions. Its genomic DNA was fragmented by use of a COVARIS S2 system with AFA fiber micro tubes to obtain fragments with an average length of 200 bp. Methylated DNA was captured by the equally efficient (more than 94%) pull-down using the MethylCollector kit (Active Motif) or MethylCap with High-Salt elution (Diagenode), both based on autologous methyl-binding domains, according to the manufacturer's protocols. Subsequently, fragments were sequenced using the Illumina Genome Analyzer II. The concentration of the fragmented and captured DNA was determined on a Fluostar Optima plate reader with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen P7589) 480/520nm. For all samples together, the paired-end 40bp sequence reads were mapped using BOWTIE[22] on the human reference genome (NCBI build 37.3). Coverage values were summarized using the map of the human methylome, which consists of putatively independently methylated regions (methylation cores) throughout the genome (manuscript in preparation). For each sample, and each methylation core, the maximum read coverage was used for further analysis. Subsequently, we focused on the broad promoter region for each gene (-2000 to +500bp). A Poisson background model was used to identify significantly methylated regions (alpha = 0.01), with lambda estimated as the sum of the coverage values over all promoter methylation cores divided by the number of promoter methylation cores. This approach takes into account coverage differences between samples, although generally low coverage will result in low sensitivity. For absence of methylation in HK-2 we allowed a maximum coverage value of 2, corresponding with $P \leq 0.067$.

DNA methylation and gene expression analyses

Gene promoter CpG island methylation was determined by chemical modification of genomic DNA with sodium bisulfite and subsequent methylation-specific PCR (MSP) as described in detail elsewhere[23-25]. In brief, 500 ng of DNA was modified by sodium bisulfite using the EZ DNA methylation kit (Zymo Research, Orange, California) according to the manufacturers' instructions. To facilitate MSP analysis on DNA retrieved from formalin-fixed paraffin embedded tissue, DNA was first amplified with flanking PCR primers that amplify bisulfite-modified DNA but do not preferentially amplify methylated or unmethylated DNA. The resulting fragment was used as a template for the MSP reaction. All PCRs were performed with controls for unmethylated alleles (DNA from normal lymphocytes or DNA from human umbilical vein endothelial cells (HU-

VEC), methylated alleles [normal lymphocyte DNA treated in vitro with SssI methyltransferase (New England Biolabs)], and a control without DNA. Primer sequences and PCR conditions are available upon request. Ten µl of each MSP reaction was directly loaded onto 2% agarose gels containing GelStar Nucleic Acid Gel Stain (Cambrex, New Jersey, USA), and visualized under UV illumination.
RNA of mock-, DAC-, and TSA-treated cells was isolated using the RNeasy mini kit (Qiagen) according to the manufacturers' instructions and treated with RNase-free DNase (Qiagen). Synthesis of complementary DNA (cDNA) was performed using the Iscript cDNA synthesis kit (Bio-Rad, Veenendaal, The Netherlands). Quantitative reverse transcription PCR was performed as described previously[26] using SYBR Green PCR master mix (Bio-Rad, Veenendaal, The Netherlands). Cyclophillin A was used as a reference gene for normalization. Primer sequences and PCR conditions are available upon request.

Plasmid constructs and cell transfections

The *GREM1* expression construct was made by cloning the full-length human coding region amplified from normal kidney tissue into the pcDNA3 vector. The NEURL-pIRESneo3 construct has been described before[19]. Plasmid constructs were verified by sequencing.
SKRC1 and SKRC59 cells were transfected with GREM1-pcDNA3 or empty vector (pcDNA3) using Lipofectamine2000 (Invitrogen), or with NEURL-pIRESneo3 (linearized by PvuI) or empty vector (pIRESneo3) (linearized by PvuI) using Nucleofector Kit V (Amaxa Biosystems, Gaithersburg, MD) according to the manufacturer's protocols. Following a 24 hours recovery period, transfected SKRC1, and -59 cells were selected in G418 containing medium (500µg/mL for SKRC1, 750µg/mL for SKRC59). Expression was confirmed by qRT-PCR as described above.

In vitro colony formation, cell proliferation, migration, and invasion assays

SKRC1 and -59 cells were transfected in six-well plates (3 x 10^5 cells per well) with empty pcDNA3 / GREM1-pcDNA3 or empty pIRESneo3 (linearized by PvuI) / NEURL-pIRESneo3 (linearized by PvuI) and selected for 10 or 14 days in G418 containing media as described above. Surviving colonies were stained with Giemsa's azur eosin methylene blue solution (Merck, Darmstadt, Germany) and counted. Cells were transfected in triplicate and colony formation was assessed in three independent experiments.
G418-selected SKRC1 and -59 cells were seeded onto 96-well plates (2500 or 3000 cells per well), and cell numbers were counted 24, 48, 72, and 96 hours later (six wells per time point). In addition, after 96 hours of incubation, the cultures were pulse labeled for 6 hours with [methyl-^{3}H]thymidine (0.3 µCi per well; Amersham Life Science, Roosendaal, the Netherlands). Cells were harvested using a cell harvester, and [^{3}H]thymidine activity was measured by liquid scintillation counting. Three independent experiments were performed.
Cell migration and invasion assays were performed using matrigel-coated (invasion assay) or uncoated (migration assay) 24-well transwell plates (8-µm pore size) (BD Biosciences, Franklin Lakes, NJ), according to the manufacturers' instructions. Briefly, 6 x 10^4 SKRC1 cells or 1.2 x 10^5 SKRC59 cells in RPMI 1640 medium containing 1% FCS

were seeded into the upper chamber of each well, and RPMI 1640 containing 10% FCS was placed in the lower chamber. After 24 hours of incubation and removal of cells on the upper surface of the membrane, cells on the lower surface were fixed with methanol, hematoxylin/eosine stained and counted with the use of a light microscope. Transwell assays were performed in three independent experiments (two replicate wells per experiment).

Data analysis

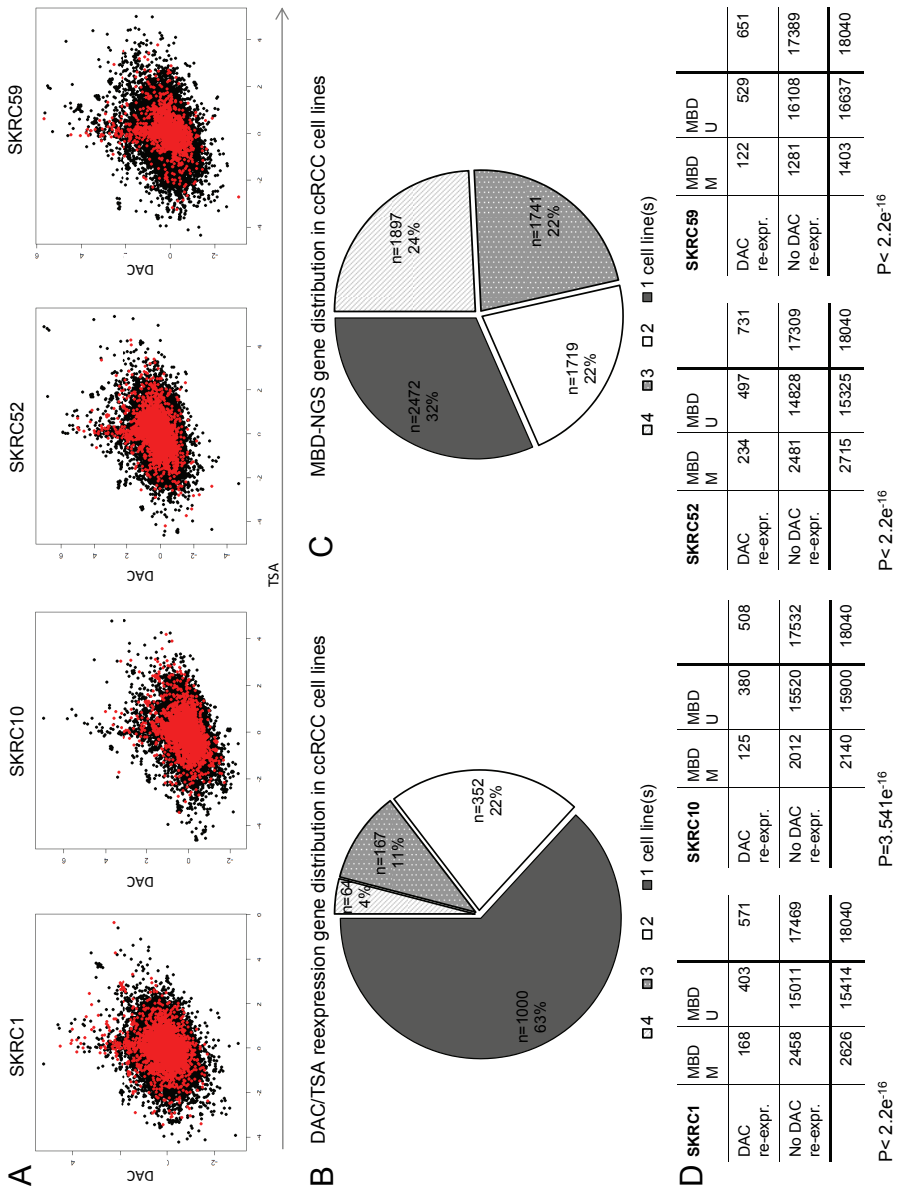
Cause-specific survival was defined as the time from cancer diagnosis until renal cancer-related death or end of follow-up. Differences in clinicopathological and angiogenesis characteristics between ccRCCs with and without candidate gene promoter CpG island hypermethylation were evaluated with the Student's t and Pearson's χ^2 tests, where appropriate. Kaplan-Meier analyses and log-rank tests were used to estimate the overall influence of candidate gene promoter CpG island hypermethylation on cause-specific survival. Hazard ratios (HR) and corresponding 95% confidence interval (CI) were assessed using Cox proportional hazard models. Known prognostic factors for renal cancer were considered possible confounders if they influenced the crude HR by more than 10%. Possible confounders that were included in the model for both series were sex, age at diagnosis, cancer stage, tumor size and nuclear grade. The proportional hazard assumption was tested using the Schoenfeld residuals and the log(-log) hazards plots. Harrell's C statistic and Akaike Information Criterion (AIC) were used to assess the predictive capacity and fit of the models. As a model with a 100% sensitivity and specificity would yield a Harrell's C statistic of 1.00, the model with the highest Harrell's C statistic was regarded as the best model. If two models had similar Harrell's C statistics, the model with the lowest AIC was chosen as the best model. All analyses were performed with the statistical package STATA 11.0.

The Student *t*-test or the Mann-Whitney rank sum test (in case of normality violation) was used to analyze differences in gene expression, colony formation, [^{3}H]thymidine incorporation, migration and invasion. Cell growth curves were analyzed through Kruskal-Wallis one-way analysis of variance. Functional data analyses were performed using PASW statistics 18 (SPSS Inc.). All reported P values are two-sided and P values ≤ 0.05 were considered statistically significant.

Results

Exploring the ccRCC hypermethylome identifies promoter CpG island hypermethylated candidate tumor suppressor genes

In order to identify tumor specific promoter hypermethylation in RCC we combined the novel MBD-affinity based NGS method[27] with up-regulation after demethylation by DAC. Changes in gene expression following global demethylation or histone deacetylase inhibition of four ccRCC cell lines are shown in Figure 1.A. The characteristic spikes identify a zone in which gene expression did not increase by TSA (TSA-negative <1.4 fold), and was not found in wild-type untreated cells, but increased >1.4-fold with DAC treatment (DAC-positive)[19, 20]. In total, 1583 unique genes within the spikes were identified with between 508 and 731 unique genes per cell line. The number of cell lines expressing these genes is shown in Figure 1.B.
Differentially methylated DNA sequences were identified by massive sequencing of MBD binding enriched sheared DNA as described earlier[27]. MBD-affinity NGS of the normal renal epithelial cell line HK-2 was performed to identify tumor specific hypermethylation. In total, 7829 unique genes indicated promoter methylation, with 2802 to 5695 unique genes per cell line. The distribution of differentially methylated genes among the cell lines is depicted in Figure 1.C. Figure 1.D shows the similarity and difference in number of genes between the two approaches per cell line, taking into account the lower genome coverage of the expression arrays. Interestingly, using our stringent restrictions, just 5.8 to 8.6% of the methylated genes were re-expressed after demethylation per cell line, defining the candidates for functional DNA methylation. Genes were selected for functional hypermethylation, defined as MBD-binding and re-expression after demethylation by DAC in 4/4 or 3/4 ccRCC cell lines, but unmethylated in the normal kidney cell line HK-2. This identified candidate genes with a CpG-island in their promoter region (exon1) within the TSA-negative, DAC-positive zone. To increase the possibility of the identification of (renal cell) cancer specific genes, we specifically focused on genes involved in DNA repair, angiogenesis and hypoxia, carbohydrate metabolism, the TGF-β or Notch pathway based on GO-terms, and genes known to be mutated in renal cancer[10, 11] or frequently mutated in breast and colorectal cancer[28]. This resulted in 43 candidate genes that have not been reported previously to show promoter hypermethylation in RCC, except for *CST6*[29] and for *GREM1*[18, 29] (see Figure 2 and for a complete list see Table 1).



SKRC1	MBD M	MBD U	
DAC re-expr.	168	403	571
No DAC re-expr.	2458	15011	17469
	2626	15414	18040

P< 2.2e-16

SKRC10	MBD M	MBD U	
DAC re-expr.	125	380	508
No DAC re-expr.	2012	15520	17532
	2140	15900	18040

P=3.541e-16

SKRC52	MBD M	MBD U	
DAC re-expr.	234	497	731
No DAC re-expr.	2481	14828	17309
	2715	15325	18040

P< 2.2e-16

SKRC59	MBD M	MBD U	
DAC re-expr.	122	529	651
No DAC re-expr.	1281	16108	17389
	1403	16637	18040

P< 2.2e-16

Figure 1. Characteristics of gene discovery approaches in ccRCC cell lines
Gene expression changes for SKRC1, SKRC10, SKRC52 and SKRC59 cells treated with TSA (x-axis) or DAC (y-axis) are plotted by log-fold change, and each dot represents an individual gene (probe) on the array. Overlapping genes with MBD-protein(s) binding are indicated in red (without overlap are in black) (A). Distribution and shared candidate hypermethylated genes in the ccRCC cell lines is shown after DAC/TSA treatment (B) or MBD-affinity based NGS (C).Overlaps in gene expression changes or MBD-protein(s) binding among two, three, or four cell lines are indicated. Integration of the two approaches to induce the likelihood to identify biological relevant candidate genes is depicted per cell line (D). P-values indicate a significant overlap between the two approaches in all cell lines.

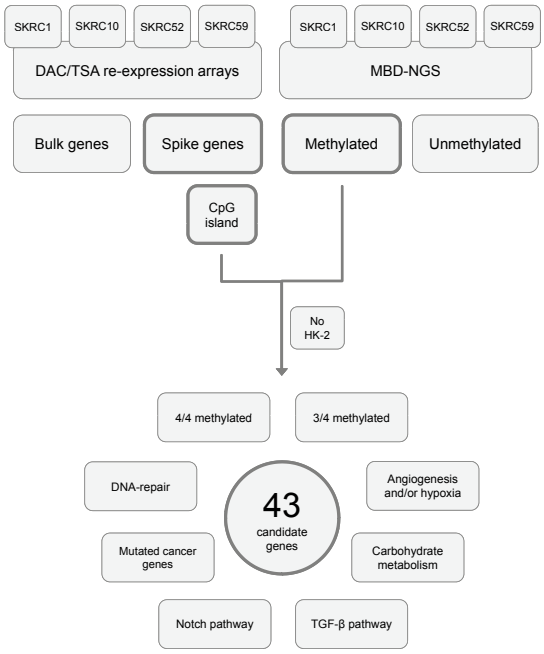


Figure.2. Pipeline to select candidate methylation markers in ccRCC cell lines
Schematic view of developed pipeline applied for the quick identification of biological relevant candidate genes. Promoter CpG island including genes, identified in ccRCC cell lines as specifically up-regulated after DAC treatment and enriched by MBD-protein(s), but not in HK-2 cells, were considered as candidate promoter hypermethylated genes. Those genes that met the downstream indicated requirements were selected for further analyses.

Validation of methylation and expression status in candidate genes

In order to validate the approach used, we examined the methylation and expression status of the candidate genes in ccRCC cells, by MSP and qRT-PCR, respectively. As recently illustrated, the location of methylation is of utmost importance[14]. Therefore, we designed MSP primers for all 43 candidate genes at promoter regions which show binding of MBD protein(s) in RCC cell lines, but not in normal kidney epithelium in the MBD affinity-NGS approach. In total, 172 (43 genes times four cell lines) reactions were performed by MSP with methylation in 138 MSP reactions compared to 30 reactions without methylation (see Table 2, four reactions failed due to technical limitations). Validation of the expression microarray data resulted in a sensitivity and specificity of 80% (111 out of 138 and 24 out of 30, respectively, Table 2). Validation of the MBD-NGS data resulted in a sensitivity of 90% (124 out of 138) and a specificity of 63% (19 out of 30, Table 2). Similarly, gene up-regulation after demethylation by qRT-PCR was validated in 74% using a subset (n=22) of genes.

Table 1. Candidate gene selection

Gene	Acc. No	Map locus	Meth. cell lines	% Meth. pilot RCC	% Meth. pilot NK	% Meth. 1st series	% Meth. 2nd series
CSPG4	NM_001897	15q24.2	4/4	25	0	21	
GREM1	NM_013372	15q13.3	4/4	30	0	20	40
KRT7	NM_005556	12q12-q13	4/4	30	0	19	
LAD1	NM_005558	1q25.1-q32.3	4/4	30	5	27	37
NEFH	NM_021076	22q12.2	4/4	45	0	37	52
NEURL	NM_004210	10q25.1	4/4	60	5	44	48
QPCT	NM_012413	2p22.2	4/4	30	5	45	
RGMA	NM_001166283	15q26.1	4/4	20	0	16	
SORL1	NM_003105	11q23.2-q24.2	3/4	29	10	58	
CD109	NM_133493	6q13	1/4	30	0	0	
CHRDL2	NM_015424	11q14	3/4	29	6	5	
FST	NM_006350	5q11.2	2/4	20	6	5	
PLXDC1	NM_020405	17q21.1	4/4	26	5	2	
RASGEF1A	NM_145313	10q11.21	3/4	20	0	0	
ACOT4	NM_152331	14q24.3	4/4	83	80		
ALDH2	NM_000690	12q24.2	2/4	0	0		
ANK1	NM_020476	8p11.1	4/4	59	32		
APBB1IP	NM_019043	10p12.1	3/4	0	0		
BMP2	NM_001200	20p12	4/4	83	90		
BMPER	NM_133468	7p14.3	4/4	57	86		
CIDEA	NM_001279	18p11.21	3/4	50	17		
COL1A2	NM_000089	7q22.1	-	41	45		
CST6	NM_001323	11q13	4/4	0	0		
CYB5R2	NM_016229	11p15.4	4/4	10	21		
CYP24A1	NM_000782	20q13	4/4	5	0		
DLL1	NM_005618	6q27	4/4	56	76		
FLJ40869	NM_182625	2p24.2	1/4	5	0		
GDF6	NM_001001557	8q22.1	1/4	0	0		
GPR30	NM_001505	7p22.3	3/4	0	0		
GPR68	NM_001177676	14q31	4/4	0	0		
HEYL	NM_014571	1p34.3	3/4	50	40		
IGFBP4	NM_001552	17q12-q21.1	4/4	0	0		
MAPK13	NM_002754	6p21.31	4/4	50	55		
ME3	NM_006680	11cen-q22.3	2/4	15	0		
MMP2	NM_004530	16q13-q21	3/4	60	17		
MYO5C	NM_018728	15q21	2/4	35	30		
PGF	NM_002632	14q24.3	2/4	5	0		
PON3	NM_000940	7q21.3	4/4	95	90		
PYCARD	NM_013258	16p11.2	4/4	0	0		
SECTM1	NM_003004	17q25	4/4	15	0		
SPON2	NM_012445	4p16.3	4/4	55	95		
TACSTD2	NM_002353	1p32	4/4	81	94		
ZNF569	NM_152484	19q13.12	4/4	0	0		

Promoters of 43 selected candidate genes were analyzed for their methylation status in ccRCC cell lines and a pilot of primary ccRCCs and normal kidney samples. 14 genes were further selected for analyses in the hospital-based series and four of them additionally in the population-based series

Table 2. Validation expression microarrays and MBD-NGS by MSP

		ARRAY spike		
		Yes	no	
MSP	M	111	27	138
	U	6	24	30
		117	51	168

		MBD-NGS M		
		yes	no	
MSP	M	124	14	138
	U	11	19	30
		135	33	168

MSP M: methylated by methylation-specific PCR
MSP U: unmethylated by methylation-specific PCR
MBD-NGS M: indicated for methylation by MBD-NGS

Promoter hypermethylation in primary RCC

To determine whether promoter CpG island hypermethylation of candidate genes was also present in primary RCC, we initially performed MSP for the 43 candidate genes on 20 primary RCCs and 20 normal kidney samples in which the methylation frequency ranged from 0-95% (Table 1). To increase the likelihood of relevant gene identification with proper prognostic significance, a high prevalence of tumor-specific methylation is required. Therefore, genes with a promoter methylation frequency of ≤ 20% in RCC (n=15) or >10% in normal kidney (n=14, despite correction for kidney tissue specific methylation using HK-2 MBD-NGS data) were not further analyzed. The remaining 14 genes were tested for promoter methylation in the hospital-based series (n=150) (Table 1). Of these, five were eventually excluded as none or only few additional methylated cases were detected in the first half of the series. Thus, the remaining nine genes were analyzed in all 150 ccRCC cases of the hospital-based series and showed hypermethylation frequencies ranging from 16%-58% (Table 1).

Promoter CpG island hypermethylation of GREM1, LAD1, NEFH and NEURL has prognostic value in primary ccRCC

The association between the promoter hypermethylation status of the ten candidate genes and tumor/patient characteristics and clinical outcome in the hospital-based series (n=150) is shown in Table 3, supplemental Table 1, supplemental Figure 1 A-E and supplemental Figure 2 A,C,E,G. Promoter hypermethylation of *GREM1*, *LAD1, NEFH* and *NEURL* showed significant associations with advanced disease and/or poor prognosis (Table 3). In addition to *GREM1* (as we published earlier, [18]), also promoter hypermethylation of *LAD1, NEFH* and *NEURL* showed an association with poor survival, although for *NEFH* this was not statistically significant (log-rank p-value *LAD1* 0.013, *NEURL* 0.002 and *NEFH* 0.173, supplemental Figure 2C,E,G). In addition, promoter hypermethylation of *LAD1* and *NEURL* was associated with tumor size, -grade, and -stage, tumor cell proliferation (TCP), endothelial cell proliferation (ECP) and micro vessel density (MVD), although for *NEURL* less striking than for *GREM1* (Table 3).
As shown in Figure 3, the association between poor survival and promoter hypermethylation of *GREM1*, *LAD1*, *NEFH*, and *NEURL* was also seen in the age- and sex-adjusted Cox proportional hazard analyses (HR_{GREM1}: 2.3 (95%-CI 1.29-4.28), HR_{LAD1}: 2.3 (95%-CI 1.23-4.22), HR_{NEFH}: 1.5 (95%-CI 0.79-2.79), HR_{NEURL}: 2.5 (95%-CI 1.36-4.47)). Two- and three- marker panels combining *GREM1*, *LAD1*, *NEFH*, and *NEURL* hypermethylation status showed the best predictive capacity according to the Harrell's C statistic for the two marker combination of *GREM1* and *LAD1* (age/sex adjusted HR both genes methylated 3.54 (95%-CI 1.58-7.95), Harrell's C: 0.6415, Figure 3), and for the three marker combination of *GREM1, LAD1* and *NEURL* (age/sex adjusted HR three genes methylated 3.61 (95%-CI 1.36-9.57), Harrell's C: 0.6436, Figure 3). The model combining all four markers showed a slightly elevated age/sex adjusted HR of 3.64 (95%-CI 1.02-13.02, Figure 3) when compared to the best-three marker panel, but the Harrel's C statistic does not indicate a higher predictive capacity of this model (Harrell's C: 0.6212) although the fit of the four marker model is better according to the AIC as compared to the three marker model (AIC: 321 for the four marker panel as compared to 346 for the three marker panel). Figure 4 shows the overall cause-specific survival of

ccRCC patients of the best predictive capacity according to the Harrell's C statistic for the two (A) and three (B) marker panels as compared to the four marker (C) panel. Addition of a methylation marker previously not associated with prognosis such as *VHL*[9] did not increase the prognostic value of the 2, 3 and 4 marker panel.
In multivariate analyses adjusted for age, sex, grade, stage and tumor size, however, the association between promoter hypermethylation of *GREM1*, *LAD1*, *NEFH* and/or *NEURL* was lost suggesting that these markers might not be independent prognostic factors in this hospital-based population (Table 3).

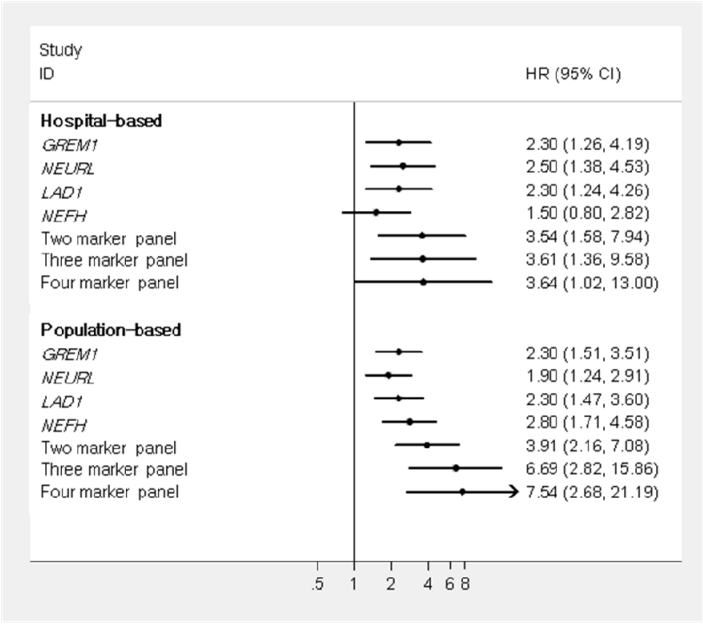


Figure 3. Hazard ratios for RCC-related death for single markers and most prognostic marker panels in hospital- and population-based ccRCC series
Forest plot depicting the increasing risk on RCC-related death by HRs (corrected for sex and age) for *GREM1, LAD1, NEFH* and *NEURL* and the two- and three best predictive marker panels according to Harrels C statistic as well as the four marker panel. Two- and three marker combinations hospital-based series: *GREM1* and *LAD1,* and *GREM1, LAD1,* and *NEURL,* respectively, and population-based series: *GREM1* and *LAD1,* and, *LAD1, NEFH* and *NEURL,* respectively.

Table 3. *GREM1, LAD1, NEFH* and *NEURL* promoter CpG island methylation associated with patient and tumor characteristics, including gene panels (hospital-based series)

		All ccRCC	Methylated *GREM1*	Methylated *LAD1*	Methylated *NEFH*	Methylated *NEURL*	Methylated *GREM1&LAD1*	Methylated *GREM1&NEURL&LAD1*	Methylated *GREM1&LAD1&NEURL &NEFH*
		n=150 (100%)	n=28 (20%)	n=36 (27%)	n=48 (37%)	n=62 (44%)	n= 14 (11%)	n= 10 (8%)	n=7 (6%)
Sex	Male (*n*)	99 (66%)	22 (79%)	22 (61%)	36 (75%)	45 (79%)	10 (71%)	9 (90%)	6 (86%)
	Female (*n*)	51 (34%)	6 (21%)	14 (39%)	12 (25%)	17 (21%)	4 (29%)	1 (10%)	1 (14%)
	P (U versus M)		0.11	0.63	0.14	0.27	0.91	0.53	0.21
Age (mean)		60.1 (± 12.1 SD)	60.3 (±10.4 SD)	61.3 (±13.1 SD)	63.4 (±10.4 SD)	61.6 (±12.3 SD)	60.7 (± 12.8 SD)	61.9 (± 14.1 SD)	63.3 (±11.2 SD)
	P (U versus M)		0.96	0.44	0.01	0.20	0.79	0.34	0.29
Tumor size in cm (mean)		6.36 (± 3.45 SD)	7.72 (± 3.17 SD)	8.03 (± 3.86 SD)	6.88 (± 3.00 SD)	6.96 (± 3.56 SD)	8.11 (± 3.44 SD)	8.72 (± 3.80 SD)	7.92 (± 3.72 SD)
	P (U versus M)		0.02	0.0007	0.30	0.04	0.002	0.002	0.007
Fuhrman grade (n, %)	1	15 (10%)	1 (4%)	1 (3%)	4 (9%)	5 (8%)	0 (0%)	0 (0%)	0 (0%)
	2	72 (48%)	11 (39%)	10 (28%)	17 (35%)	25 (40%)	4 (29%)	3 (30%)	2 (28.5%)
	3	46 (31%)	9 (32%)	14 (39%)	17 (35%)	22 (36%)	4 (28%)	3 (30%)	2 (28.5%)
	4	17 (11%)	7 (25%)	11 (30%)	10 (21%)	10 (16%)	6 (43%)	4 (40%)	3 (43%)
	P (U versus M)		0.04	<0.001	0.10	0.19	0.002	0.021	0.04
Tumor stage (n, %)	1	8 (5%)	0 (0%)	0 (0%)	1 (2%)	2 (3%)	0 (0%)	0 (0%)	0 (0%)
	2	89 (60%)	12 (43%)	16 (45%)	26 (54%)	31 (50%)	5 (36%)	3 (30%)	3 (43%)
	3	30 (20%)	7 (25%)	12 (33%)	10 (21%)	17 (28%)	4 (28%)	4 (40%)	2 (28.5%)
	4	22 (15%)	9 (32%)	8 (22%)	11 (23%)	12 (19%)	5 (36%)	3 (30%)	2 (28.5%)
	P (U versus M)		0.013	0.016	0.118	0.063	0.025	0.007	0.075
ECP % (mean)		1.4 (±2.17SD)	3.0 (±2.97 SD)	2.5 (±3.35 SD)	1.9 (±2.93 SD)	2.1 (±2.92 SD)	4.0 (± 4.21 SD)	4.5 (± 4.8 SD)	4.4 (±5.84 SD)
	P (U versus M)		0.0001	0.0011	0.1295	0.0039	<0.0001	<0.0001	0.0001
TCP % (mean)		6.7 (±9.25 SD)	12.0 (± 11.90 SD)	10.6 (± 12.91 SD)	7.8 (± 10.99 SD)	9.9 (± 12.43 SD)	15.8 (± 13.6 SD)	16.4 (± 15.1)	14.3 (± 17.88 SD)
	P (U versus M)		0.001	0.002	0.372	0.0006	0.0001	<0.0001	0.0008
MVD per mm^2 (mean)		197.5 (± 91.0 SD)	152.5 (± 92.2 SD)	152.8 (± 81.9 SD)	178.1 (± 83.6 SD)	180.0 (± 84.9 SD)	114.0 (± 51.5 SD)	93.3 (± 26.4)	96.1 (± 28.5 SD)
	P (U versus M)		0.001	0.0008	0.132	0.053	0.0003	<0.0001	0.0001
OS in months (mean)		63.5 (± 36.8 SD)	53.8 (± 41.1 SD)	52.6 (± 32.9 SD)	51.9 (± 29.4 SD)	57.4 (± 39.2 SD)	51.3 (± 43.4 SD)	49.8 (± 41.0 SD)	45.2 (± 35.1 SD)
	P (U versus M)		0.11	0.05	0.02	0.08	0.16	0.13	0.20
MFS in months (mean)		51.8 (± 41.0 SD)	35.8 (± 41.8 SD)	35.0 (± 33.3 SD)	38.5 (± 32.3 SD)	40.9 (± 39.9 SD)	32.1 (± 39.3 SD)	25.3 (± 29.0 SD)	31.1 (± 32.9 SD)
	P (U versus M)		0.02	0.007	0.01	0.004	0.018	0.007	0.019

Table 3. continued

	All ccRCC n=150 (100%)	Methylated *GREM1* n=28 (20%)	Methylated *LAD1* n=36 (27%)	Methylated *NEFH* n=48 (37%)	Methylated *NEURL* n=62 (44%)	Methylated *GREM1&LAD1* n= 14 (11%)	Methylated *GREM1&NEURL&LAD1* n= 10 (8%)	Methylated *GREM1&LAD1&NEURL &NEFH* n=7 (6%)
HR (sex/age)		2.3 (CI 1.3-4.3)	2.3 (CI 1.2-4.2)	1.5 (CI 0.8-2.8)	2.5 (CI 1.4-4.6)	3.5 (CI 1.6-7.9)	3.6 (CI 1.4-9.6)	3.6 (CI 1.0-13.0)
HR (multi)		0.9 (CI 0.5-1.7)	0.8 (CI 0.4-1.6)	1.1 (CI 0.6-2.2)	1.4 (CI 0.7-2.7)	0.85 (CI 0.3-2.4)	0.68 (CI 0.2-2.4)	0.3 (CI 0.1-1.9)

Tumor stage based on International Union against Cancer (UICC) TNM classification of malignant tumors, fourth, fully revised edition, 1987
P: p-value for comparison of methylated and unmethylated cases
ECP: endothelial cell proliferation
TCP: tumor cell proliferation
MVD: micro vessel density
OS: overall survival
MFS: metastasis-free survival
SD: standard deviation
HR (sex/age): hazard ratio (RCC related death) corrected for sex and age
HR (multi): hazard ration (RCC related death) corrected for age, sex, tumor size, stage, and grade)
CI: 95% confidence interval

Table 4. *GREM1, LAD1, NEURL and NEFH* promoter CpG island methylation associated with patient and tumor characteristics, including gene panels (population-based series)

		All ccRCC	Methylated *GREM1*	Methylated *LAD1*	Methylated *NEFH*	Methylated *NEURL*	Methylated *GREM1&LAD1*	Methylated *NEURL & LAD1 & NEFH*	Methylated *GREM1&LAD1&NEURL &NEFH*
		n=185 (100%)	n=68 (40%)	n=60 (35%)	n=74 (52%)	n=88 (47%)	n=31 (20%)	n=26 (20%)	n=22 (18%)
Sex	Male (*n*)	108 (59%)	44 (65%)	42 (70%)	50 (68%)	46 (52%)	22 (71%)	17 (65%)	15 (68%)
	Female (*n*)	74 (41%)	24 (35%)	18 (30%)	24 (32%)	42 (48%)	9 (29%)	9 (35%)	7 (32%)
	P (U versus M)		0.32	0.07	0.17	0.05	0.06	0.11	0.16
Age (mean)		67.4 (± 4.7 SD)	67.2 (±5.2 SD)	68.6 (±4.7 SD)	68 (±4.4 SD)	67.0 (±4.6 SD)	67.4 (± 5.3 SD)	67.8 (± 5.1 SD)	67.2 (±4.5 SD)
	P (U versus M)		0.49	0.02	0.78	0.58	0.29	0.33	0.83
Tumor size in cm (mean)		7.9 (± 3.9 SD)	7.5 (± 3.17 SD)	7.9 (± 3.40 SD)	7.3 (± 3.01 SD)	6.9 (± 3.30 SD)	7.4 (± 2.8 SD)	8.4 (± 3.3 SD)	8.1 (± 2.78 SD)
	P (U versus M)		0.16	0.01	0.87	0.08	0.01	0.02	0.08
Fuhrman grade (n, %)	1	42 (23%)	10 (15%)	9 (15%)	11 (15%)	30 (34%)	3 (10%)	2 (8%)	2 (9%)
	2	66 (36%)	22 (32%)	15 (25%)	20 (27%)	35 (40%)	5 (16%)	3 (11%)	2 (9%)
	3	47 (26%)	23 (34%)	23 (38%)	29 (39%)	17 (19%)	15 (48%)	12 (46%)	11 (50%)
	4	27 (15%)	13 (19%)	13 (22%)	14 (19%)	6 (7%)	8 (26%)	9 (35%)	7 (32%)
	P (U versus M)		0.03	0.03	0.01	<0.001	0.007	0.003	0.007
Tumor stage (n, %)	1	4 (2%)	0 (0%)	0 (0%)	0 (0%)	3 (3%)	0 (0%)	0 (0%)	0 (0%)
	2	81 (45%)	27 (40%)	19 (33%)	26 (36%)	47 (54%)	11 (35%)	6 (23%)	5 (23%)
	3	63 (35%)	23 (34%)	26 (45%)	27 (37%)	26 (30%)	10 (32%)	10 (38%)	7 (32%)
	4	32 (18%)	18 (26%)	13 (22%)	20 (27%)	11 (13%)	10 (32%)	10 (38%)	10 (45%)
	P (U versus M)		0.08	0.035	0.009	0.008	0.006	0.001	0.001
OS in months (mean)		82.9 (± 64.1 SD)	63.8 (± 60.7 SD)	51.3 (± 50.4 SD)	60.5 (± 57.3 SD)	64.8 (± 58.6 SD)	52.6 (± 52.2 SD)	46.2 (± 56.1 SD)	47.1 (± 56.3 SD)
	P (U versus M)		0.001	<0.0001	<0.0001	0.001	<0.0001	<0.0001	<0.0001
HR (sex/age)			2.3 (CI 1.5-3.5)	2.3 (CI 1.5-3.6)	2.8 (CI 1.7-4.6)	1.9 (CI 1.2-2.9)	3.9 (CI 2.2-7.1)	6.7 (CI 2.8-15.9)	7.5 (CI 2.7-21.2)
HR (multi)			2.3 (CI 1.4-3.6)	1.8 (CI 1.1-3.1)	2.6 (CI 1.5-4.6)	1.5 (CI 1.0-2.4)	3.4 (CI 1.7-6.6)	5.7 (CI 2.0-15.7)	6.6 (CI 2.1-20.5)

Tumor stage based on International Union against Cancer (UICC) TNM classification of malignant tumors, fourth, fully revised edition, 1987
P: p-value for comparison of methylated and unmethylated cases
OS: overall survival
MFS: metastasis-free survival
SD: standard deviation
HR (sex/age): hazard ratio (RCC related death) corrected for sex and age
HR (multi): hazard ration (RCC related death) corrected for age, sex, tumor size, stage, and grade)

GREM1, LAD1, NEFH and NEURL are strong independent predictors of ccRCC survival

For the four candidate genes identified in the hospital-based series we studied the methylation status in an independent population-based series of ccRCCs derived from the prospective Netherlands Cohort Study on Diet and Cancer. Table 4 and supplemental Figure 2 B,D,F,H show that, as reported previously for *GREM1*, promoter hypermethylation of the single genes *LAD1*, *NEURL* and *NEFH* is associated with increased malignancy and poor prognosis (age/sex adjusted HR_{GREM1}: 2.3 (95%-CI 1.52-3.53), HR_{LAD1}: 2.3 (95%-CI 1.48-3.63), HR_{NEFH}: 2.8 (95%-CI 1.72-4.61), HR_{NEURL}: 1.9 (95%-CI 1.24-2.90)) independent of other prognostic factors (multivariate HR_{GREM1}: 2.3 (95%-CI 1.45-3.61), HR_{LAD1}: 1.8 (95%-CI 1.10-3.06), HR_{NEFH}: 2.62 (95%-CI 1.50-4.59), HR_{NEURL}: 1.5 (95%-CI 0.96-2.42)), although for *NEURL* only borderline statistical significance was reached. The best two-marker panel according to Harrell's C Statistic, was the panel of *GREM1* and *LAD1* (age/sex adjusted HR both genes methylated 3.91 (95%-CI 2.16-7.08), Harrell's C: 0.6837, Figure 3). In contrast to the hospital-based series, the best three marker panel however appeared to be the combination of *LAD1, NEFH* and *NEURL* (age/sex adjusted HR three genes methylated 6.69 (95%-CI 2.82-15.85), Harrell's C: 0.7108, Figure 3), although the second best predictive model, *GREM1, LAD1* and *NEFH* (age/sex adjusted HR three genes methylated 5.76 (95%-CI 2.61-12.74)) only had a slightly lower Harrell's C of 0.7073 but also a lower AIC of 551 as compared to 585 for the *NEURL, LAD1* and *NEFH* combination. Nevertheless, the four marker panel appeared to be superior in predicting prognosis in ccRCC patients (Figure 3, Harrell's C: 0.7129, AIC: 540, log-rank p <0.0001, age/sex adjusted HR four genes methylated 7.54 (95%-CI 2.68-21.17)). Again, adding *VHL* to the best marker panels did not significantly improve the prediction of prognosis (data not show). Figure 4 shows the overall cause-specific survival of ccRCC patients of the best predictive capacity according to the Harrell's C statistic for the two (D) and three (E) marker panels as compared to the four marker (F) panel.

In contrast with the hospital-based population, the association with poor prognosis in the population-based series remained significant when adjusting for other prognostic factors (multivariate HR best two marker panel 3.36 (95%-CI 1.71-6.60); multivariate HR best three marker panel 5.65 (95%-CI 2.03-15.72) and multivariate HR four marker panel 6.56 (95%-CI 2.09-20.52).

GREM1 and NEURL exhibit tumor suppressor activity in vitro

To examine whether the identified candidates *GREM1* and/or *NEURL* exhibit tumor suppressor activity in renal cancer cells, we characterized SKRC1 and SKRC59 cells after induced expression (Figure 5 and 6). *GREM1* transfectants showed increased expression of *GREM1* mRNA compared with control cells transfected with empty vector (SKRC1: p=0.009 SKRC59: p=0.004, Figure 5.A,G). In both cell lines, no statistically significant effect was observed on the growth of G418-selected colonies or cell proliferation (Figure 5. B,C,D, and H,I,J) in *GREM1* transfected cells as compared to control transfectants. However, transfection of *GREM1* reduced migration (SKRC1: p=0.086,

SKRC59: p=0.128) and invasion (SKRC1: p=0.005, SKRC59: p=0.028) in both cell lines, although not statistically significant for migration (Figure 5. E,F and K L).
NEURL transfectants showed increased expression of *NEURL* mRNA compared with control cells transfected with empty vector (SKRC1: p=0.009 SKRC59: p=0.001, Figure 6.A,G). A statistically significantly reduced growth of G418-selected colonies (SKRC1: p=0.003 SKRC59: p=0.010, Figure 6.B,H) and cell proliferation as assessed by cell number (SKRC1: p=<0.0001 SKRC59: p<0.0001, Figure 6.C,I) and [^{3}H]-thymidine incorporation (SKRC1: p<0.0001 SKRC59: p=0.021, Figure 6.D,J) was observed in *NEURL* transfected cells of both cell lines as compared to control transfectants. Transfection of *NEURL* showed no statistically significant effect on migration and invasion in both cell lines (Figure 6.E,F and K,L).

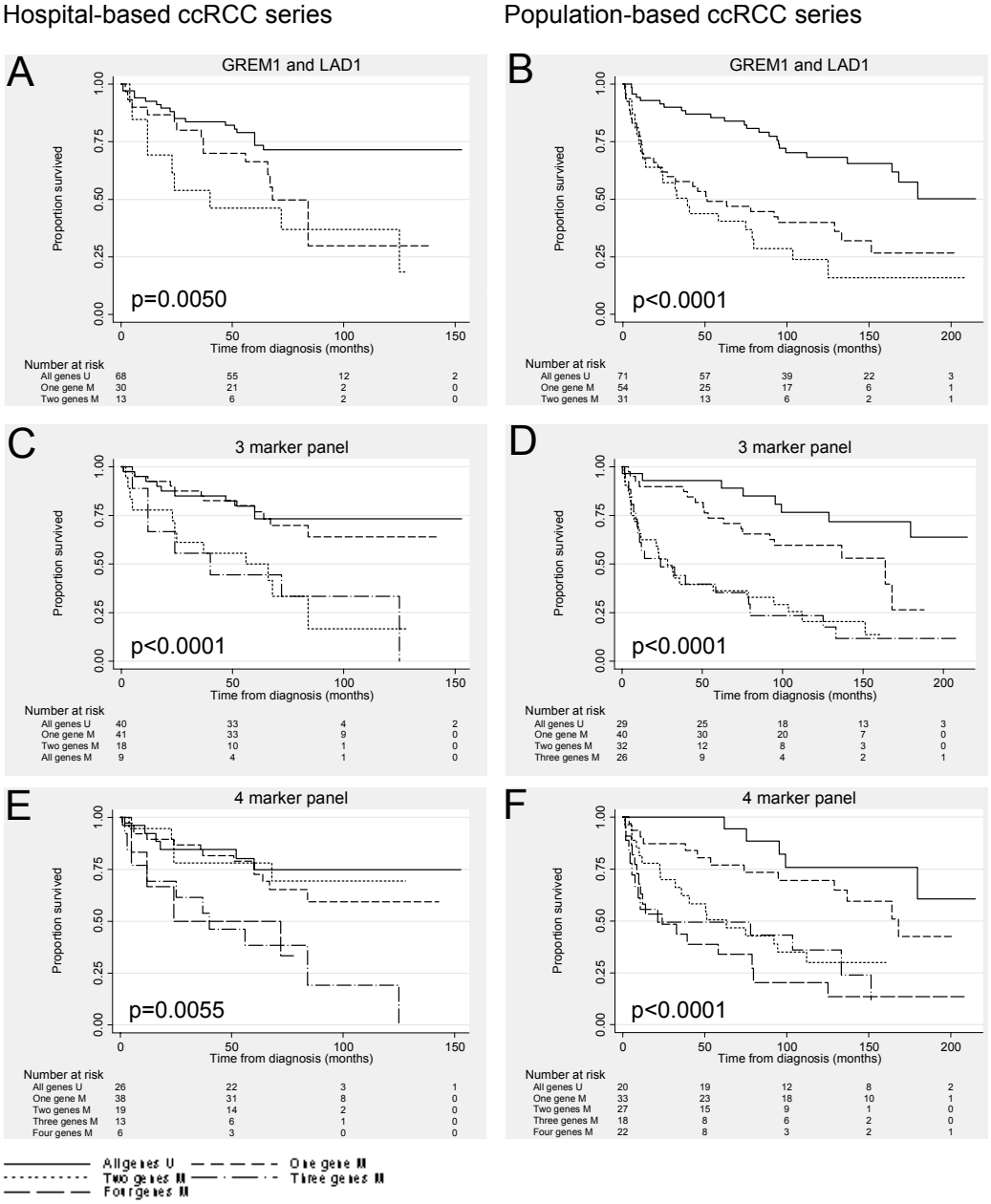


Figure 4. Overall cause-specific survival curves for marker panels in hospital- and population-based ccRCC series
Kaplan-Meier curves of the best two-, three and four marker panels in the hospital-based series: GREM1 and LAD1 (A), GREM1, LAD1, and NEURL (B), and GREM1, LAD1, NEFH and NEURL (C), respectively, and population-based series: GREM1 and LAD1 (D), LAD1, NEFH and NEURL (E), and GREM1, LAD1, NEFH and NEURL (F), respectively.

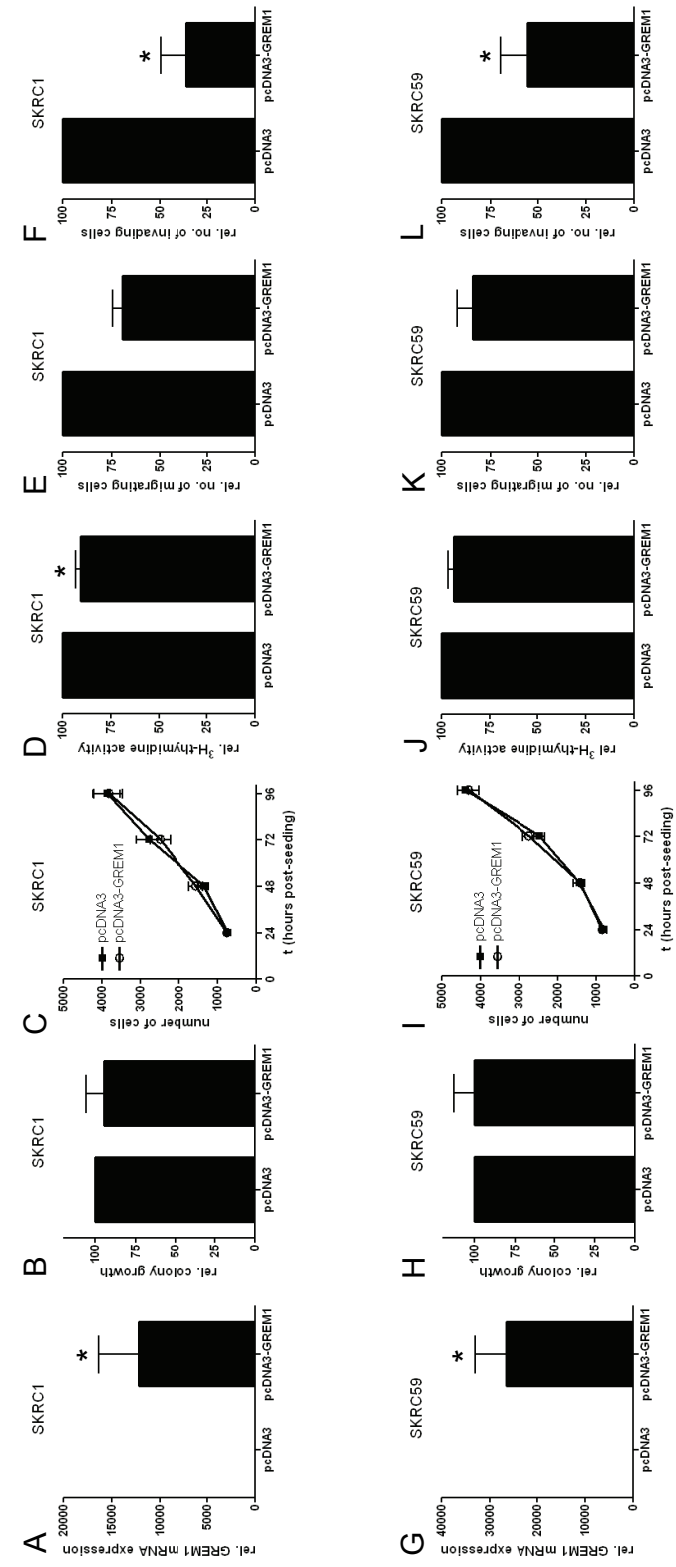


Figure 5. Functional assays of GREM1 in SKRC1 and SKRC59 cells
GREM1 mRNA expression measured by qRT-PCR in SKRC1 (A) and SKRC59 (G) cells transfected with GREM1-pcDNA3 or empty (control) vector (pcDNA3). * SKRC1 p=0.009; * SKRC59 p=0.004. Proliferation of SKRC1 and SKRC59 transfected cells is shown as mean colony formation (B, H), number of cells in time (C, I), and ^{3}H-thymidine incorporation (D, J) *SKRC1 p=0.005, measured after two weeks of growing in selection (G418) growth media. Migration (E, K) and invasion (F, L) of transfected cells is measured as the mean number of cells passed through the transwell membrane coated without or with matrigel, respectively *SKRC1 p=0.005, * SKRC59 p=0.028. All data are shown as mean values relative to control vector. Error bars correspond to the standard error of the mean.

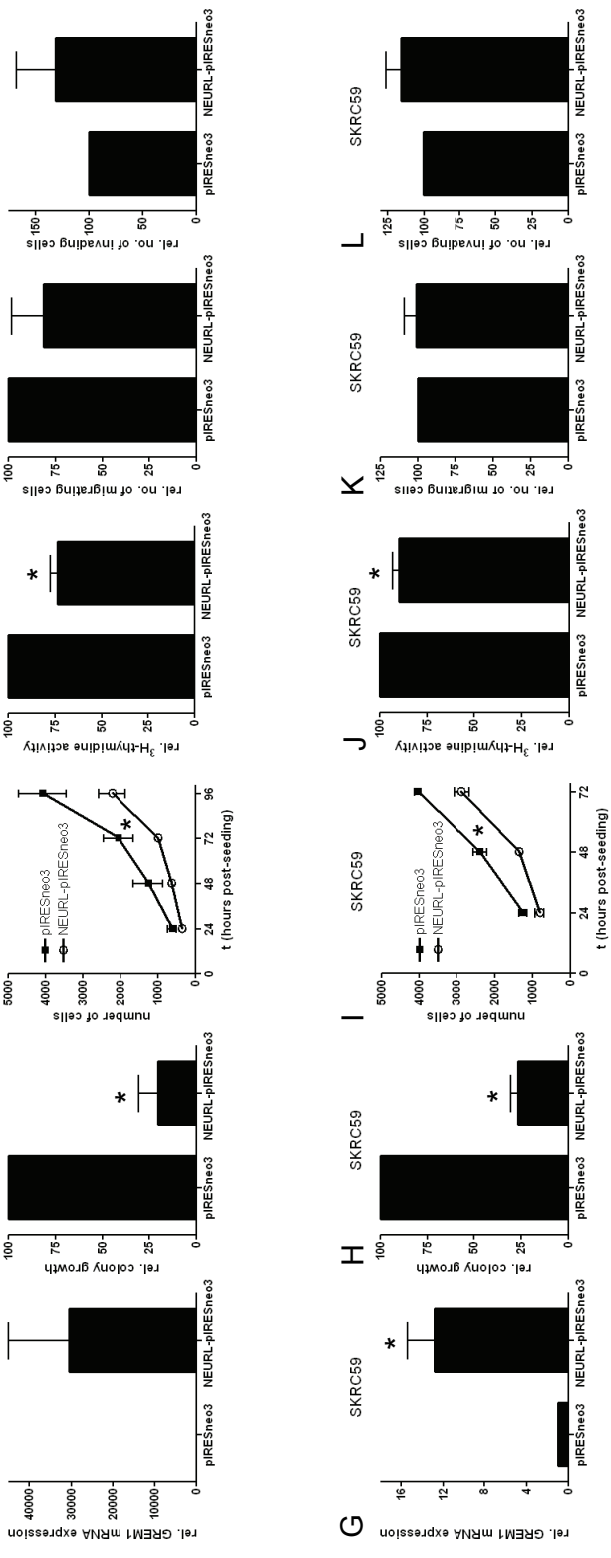


Figure 6. Functional assays of NEURL in SKRC1 and SKRC59 cells
NEURL mRNA expression measured by qRT-PCR in SKRC1 (A) and SKRC59 (G) cells transfected with NEURL-pIRESneo3 or empty (control) vector (pIRESneo3). * SKRC1 p=0.009, * SKRC59 p=0.001. Proliferation of SKRC1 and SKRC59 transfected cells is shown as mean colony formation (B, H) * SKRC1 p=0.003, * SKRC59 p=0.010, number of cells in time (C, I) * SKRC1 p<0.0001, * SKRC59 p<0.0001, and ^{3}H-thymidine incorporation (D, J) * SKRC1 p<0.0001, * SKRC59 p=0.021, measured after two weeks of growing in selection (G418) growth media. Migration (E, K) and invasion (F, L) of transfected cells is measured as the mean number of cells passed through the transwell membrane coated without or with matrigel, respectively. All data are shown as mean values relative to control vector. Error bars correspond to the standard error of the mean.

Discussion and conclusion

Here we integrated global transcript expression microarray data of pharmacologically demethylated ccRCC cells and MBD-affinity NGS to explore the ccRCC hypermethylome, thereby bypassing some of the limitations of previous technologies that prevent an unbiased truly high-throughput and exhaustive profiling of the functional methylome[15]. Although the stringent selected criteria used to identify candidate genes implies exclusion of other potentially interesting methylation markers, our strategy resulted in nine genes showing promoter CpG island hypermethylation in a substantial proportion (16%-58%) of tumors from the first (hospital-based) series, but not in normal kidney tissue. Promoter CpG island hypermethylation of four of these genes (*GREM1*, *LAD1, NEFH* and *NEURL*) was strongly associated with increased malignancy and/or poor survival in two independent series (n=150 and n=185) of ccRCC primary samples. To the best of our knowledge promoter CpG island hypermethylation of *LAD1, NEFH* or *NEURL* have not yet been described in RCC carcinogenesis.

The biological function of the candidate genes could increase our knowledge of RCC development and progression. For *GREM1* and *NEURL* a known role in embryogenesis and morphogenesis of the kidney is described[30-32] and we assessed their role in RCC *in vitro*. *GREM1* inhibits the ligands BMP2, 4, and 7, hindering downstream BMP-signaling. We have previously shown that specific *GREM1*-region-iii promoter hypermethylation was associated with decreased outcome in ccRCC patients[18]. Neuralized is a highly conserved E3 ubiquitin ligase, which acts upon Notch ligands to regulate Notch pathway signaling[33]. Promoter hypermethylation and subsequent down-regulation of the Notch pathway member *NEURL* has been reported in colorectal cancer in which over-expression caused reduced colony formation *in vitro*[19]. The results of induced over-expression of *GREM1* or *NEURL* in RCC cells suggest that these genes have complementary tumor suppressor activity in RCC. *NEURL* over-expression inhibited colony-formation and cell proliferation whereas *GREM1* over-expression inhibited migration (trend) and invasion *in vitro.* In fact, several studies have demonstrated a cross-talk between TGF-β and Notch signaling and both synergy and antagonism have been described[34-36]. Both *GREM1* and *NEURL* regulate their pathway ligands[37-41] and in that way modulate downstream signaling, with which down regulated expression interferes. Our data suggest a possible synergism between *GREM1* and *NEURL* promoter hypermethylation in RCC patients and cell lines, indicative for pathway cross-talk or additive effects of these pathways in renal cancer development. However, a synergistic effect on RCC development or progression needs to be confirmed *in vitro*. The relatively unknown LAD1 protein encoded by the *Ladinin 1* gene is an anchoring filament that is a component of basement membranes. It may contribute to the stability of the association of the epithelial layers with the underlying mesenchyme[42]. *LAD1* promoter hypermethylation has not yet been described. The *NEFH* encoding neurofilament heavy chain is known to be one of the major components of the neuronal cytoskeleton neurofilaments[43] and promoter hypermethylation of this gene has been described in esophageal carcinoma[44]. Induced over-expression of *LAD1* or *NEFH* in RCC cells did not indicate a significant proliferative, migrative or invasive effect *in vitro* (data not shown). *LAD1* and *NEFH* could possibly be involved in cell structure and stability, but their role in RCC needs to be further elucidated.

Synchronous promoter CpG island hypermethylation of *GREM1* and *NEURL* was also significantly correlated with higher risk of cancer-related death (data not shown). Interestingly, patients with tumor specific promoter CpG island hypermethylation of all four identified candidate genes had a significantly increased probability to cancer-related death as compared to patients without, or with one, two or three methylated gene promoter(s) (Table 4 and data not shown). Together with the enhancing prognostic effect of promoter hypermethylation of all four genes on survival (Figure 3 and 4), our findings indicate a highly significant prognostic gene panel predicting RCC recurrence and -related death.

Currently, no prognostic molecular markers are available for use in clinical practice for ccRCC [1]. A decade ago, a prognostic model was developed in metastatic RCC by Motzer et al. at the Memorian Sloan Kettering Cancer, which is known as the the MSKCC risk system, or informally known as the "Motzer criteria" (Karnofsky performance score <80, LDH >1,5 fold upper limit of normal (ULN), Hb < lower limit of normal (LLN), Calcium (corrected for albumine) > ULN, and interval nephrectomy < 1 year)[45]. This model is widely used as standard criteria both for selecting patients for participation in clinical trials and for recommending treatment options to patients not enrolled in trials. However, this system, only used for RCC patients with metastatic disease, was derived in the cytokine era, leading to importance of re-evaluation of prognostic markers in the current era of 'targeted' therapy. There is an urgent need for criteria predicting prognosis at time of primary RCC diagnosis for those patients (about 70%) presenting without metastasis. Identification of a distinct patient group with poor prognosis before they present with metastases, would allow medical oncologists to better manage renal cancer, although successful treatments options for RCC are currently still lacking. Therefore a Phase-III trial should clarify whether the targeted therapies, e.g. tyrosine kinase inhibitors or mTOR inhibitors, used for metastatic RCC are also effective in an adjuvant setting for subclinical metastatic disease.

The frequency and stability of DNA methylation, the possibility to determine methylation in formalin fixed paraffin-embedded (FFPE) tissue, and the simple and low cost tests that are available for assessing DNA methylation, make methylation markers excellent candidate biomarkers[46]. In our series, patients with promoter hypermethylation of the four-marker panel represent a clinically distinct group with an up to 6.6 fold increased independent risk of cancer-related death as compared to patients without this hypermethylation. The 5-year survival rate drastically decreased in relation to the methylation degree from 100% in cases without hypermethylation to 77%, 50-52, and 35% in cases with one, two or three, or four gene promoters methylated, respectively.

Strikingly, patients with promoter hypermethylation of all four genes had a 40% chance of dying from RCC within the first year and identification of this high risk group could contribute significantly to better management of RCC patients.

In conclusion, our comprehensive analysis using novel MBD technology combined with re-expression arrays identified the renal cell cancer functional hypermethylome. Although we focused on a few relevant pathways and used stringent selection criteria, this hypermethylome will provide novel insights into ccRCC biology and has already identified candidate tumor suppressor genes and clinically relevant markers. This panel of methylation markers strongly predicts patient outcome, which will require addition-

al validation to determine the potential to implement this into clinical practice. Identification of high risk patients could direct adjuvant treatment trials to patients with subclinical metastatic disease to improve disease management and increase RCC survival.

Acknowledgments

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Supplemental table 1. Candidate gene promoter CpG island methylation associated with patient and tumor characteristics (hospital-based series)

		All ccRCC	Methylated *CSPG4*	Methylated *KRT7*	Methylated *QPCT*	Methylated *RGMA*	Methylated *SORL1*
		n=150 (100%)	n=25 (21%)	n=25 (19%)	n=60 (45%)	n=20 (16%)	n=81 (58%)
Sex	Male (*n*)	99 (66%)	17 (68%)	20 (80%)	41 (68%)	14 (70%)	59 (73%)
	Female (*n*)	51 (34%)	8 (32%)	5 (20%)	19 (32%)	6 (30%)	22 (27%)
	P (U versus M)		0.93	0.13	0.84	0.77	0.19
Age (mean)		60.1	63.3	60.6	60.4	58.5	60.1
		(± 12.1 SD)	(±12.6 SD)	(±12.1 SD)	(±12.6 SD)	(±13.8 SD)	(±12.5 SD)
	P (U versus M)		0.09	0.89	0.78	0.61	0.87
Tumor size in cm (mean)		6.36	7.82	7.82	6.74	7.26	6.68
		(± 3.45 SD)	(± 4.25 SD)	(± 3.92 SD)	(± 4.17 SD)	(± 4.39 SD)	(± 3.68 SD)
	P (U versus M)		0.05	0.01	0.31	0.18	0.12
Fuhrman grade (n, %)	1	15 (10%)	2 (8%)	3 (12%)	6 (10%)	3 (15%)	5 (6%)
	2	72 (48%)	7 (28%)	8 (32%)	24 (40%)	7 (35%)	38 (47%)
	3	46 (31%)	10 (40%)	7 (28%)	21 (35%)	7 (35%)	29 (36%)
	4	17 (11%)	6 (24%)	7 (28%)	9 (15%)	3 (15%)	9 (11%)
	P (U versus M)		0.13	0.03	0.16	0.51	0.22
Tumor stage (n, %)	1	8 (5%)	0 (0%)	0 (0%)	4 (6%)	0 (0%)	4 (5%)
	2	89 (60%)	11 (44%)	11 (44%)	34 (56%)	14 (70%)	41 (51%)
	3	30 (20%)	8 (32%)	7 (28%)	12 (20%)	2 (10%)	22 (27%)
	4	22 (15%)	6 (24%)	7 (28%)	10 (17%)	4 (20%)	14 (17%)
	P (U versus M)		0.090	0.088	0.894	0.316	0.100
ECP % (mean)		1.4	2.6	2.8	1.8	1.5	1.5
		(±2.17SD)	(±3.80 SD)	(±3.69 SD)	(±2.68 SD)	(±2.17 SD)	(±2.29 SD)
	P (U versus M)		0.0050	0.0011	0.1321	0.9869	0.4942
TCP % (mean)		6.7	9.7	10.7	7.5	7.3	6.9
		(±9.25 SD)	(± 14.40 SD)	(± 13.39 SD)	(± 10.33 SD)	(± 11.74 SD)	(± 10.05 SD)
	P (U versus M)		0.087	0.039	0.408	0.814	0.928
MVD per mm2 (mean)		197.5	162.4	143.4	191.33	206.21	183.3
		(± 91.0 SD)	(± 91.3 SD)	(± 81.5 SD)	(± 85.3 SD)	(± 101.8 SD)	(± 82.4 SD)
	P (U versus M)		0.078	0.0006	0.521	0.664	0.076
OS in months (mean)		63.5	59.3	61.8	59.9	63.9	61.6
		(± 36.8 SD)	(± 37.8 SD)	(±44.0 SD)	(± 35.2 SD)	(± 35.1 SD)	(± 36.5 SD)
	P (U versus M)		0.74	1.00	0.39	0.98	0.65
MFS in months (mean)		51.8	41.3	46.5	46.9	52.0	47.4
		(± 41.0 SD)	(± 36.8 SD)	(± 49.6 SD)	(± 37.1 SD)	(± 39.0 SD)	(± 39.7 SD)
	P (U versus M)		0.22	0.64	0.28	0.98	0.34
HR (sex/age)			1.7	1.6	1.3	0.7	1.4
			(CI 0.8-3.3)	(CI 0.8-3.0)	(CI 0.7-2.3)	(CI 0.3-1.8)	(CI 0.7-2.5)
HR (multi)			0.7	0.5	1.0	0.7	0.8
			(CI 0.3-1.6)	(CI 0.2-1.1)	(CI 0.5-1.9)	(CI 0.2-1.9)	(CI 0.4-1.5)

Tumor stage based on International Union against Cancer (UICC) TNM classification of malignant tumors, fourth, fully revised edition, 1987
P: p-value for comparison of methylated and unmethylated cases
ECP: endothelial cell proliferation
TCP: tumor cell proliferation
MVD: micro vessel density
OS: overall survival
MFS: metastasis-free survival
SD: standard deviation
HR (sex/age): hazard ratio (RCC related death) corrected for sex and age
HR (multi): hazard ration (RCC related death) corrected for age, sex, tumor size, stage, and grade)
CI: 95% confidence interval

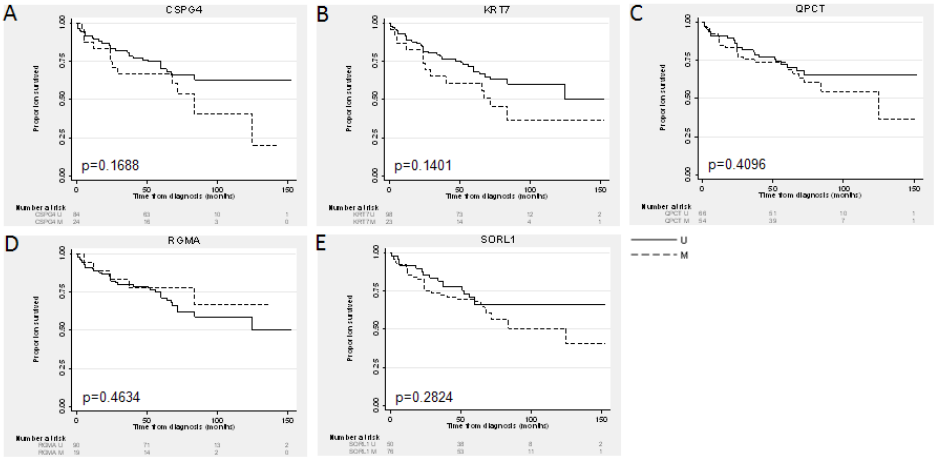

Figure S1. Overall cause-specific survival curves for candidate genes in hospital-based ccRCC series
Kaplan-Meier curves of *CSPG4* (A), *KRT7* (B), *QPCT* (C), *RGMA* (D), and *SORL1* (E) analyzed for overall survival in hospital-based series.

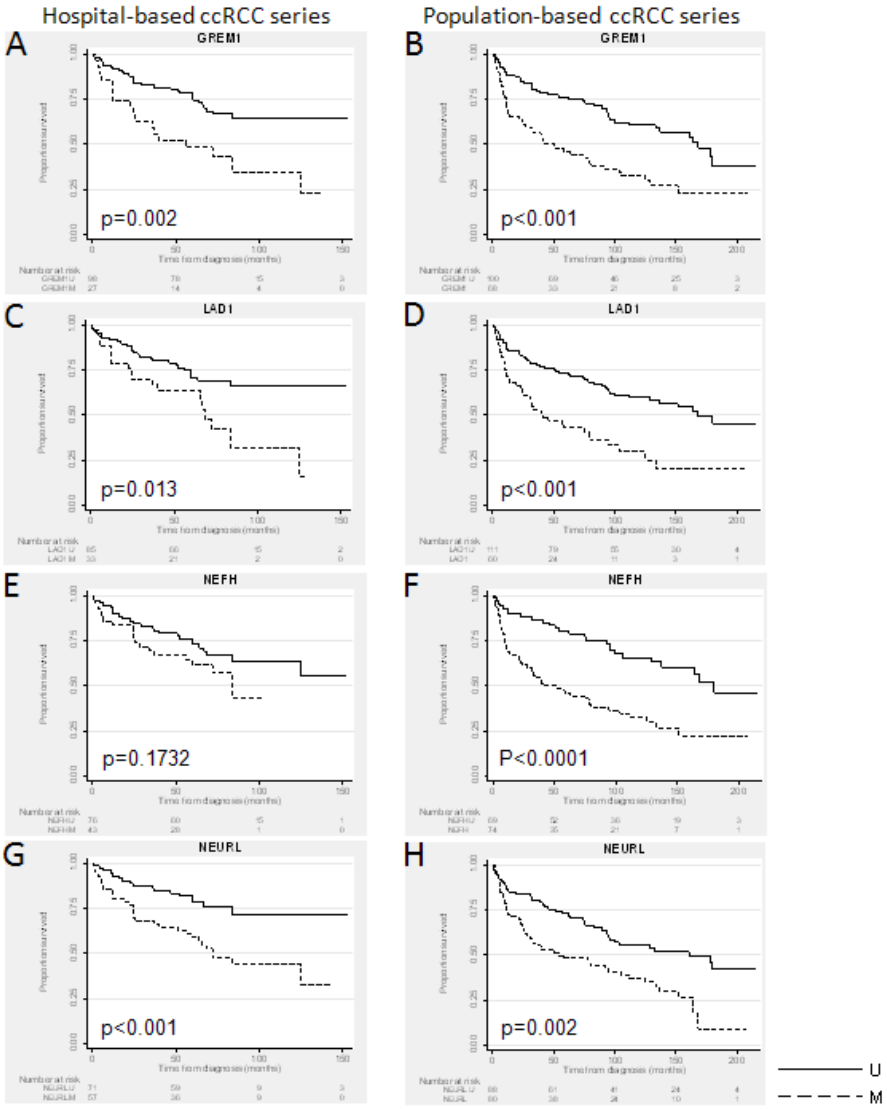

Figure S2. Overall cause-specific survival curves for *GREM1, LAD1, NEFH,* and *NEURL* in hospital- and population-based ccRCC series

Kaplan-Meier curves of *GREM1* (A, B), *LAD1* (C, D), *NEFH* (E, F) and *NEURL* (G, H) analyzed for overall survival in hospital- (left panels) and population-based series (right panels).

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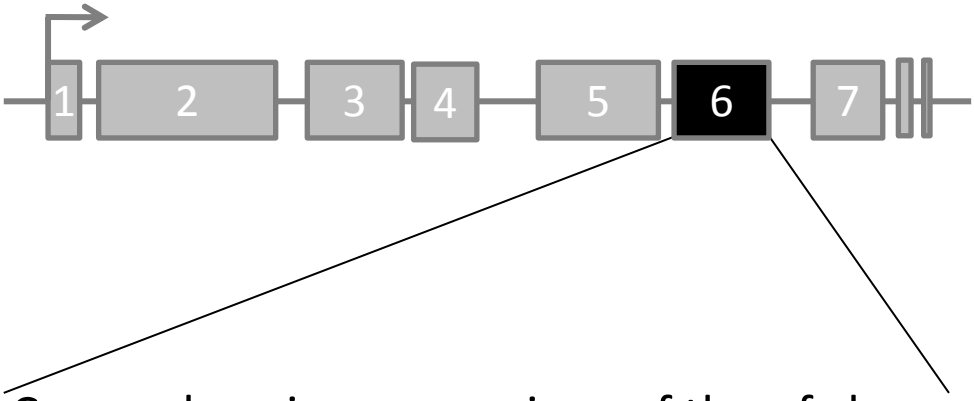

Comprehensive comparison of the of clear cell renal cell cancer methylome with the colorectal and breast cancer methylomes identifies conserved targeting of critical pathways through unique epigenetic events

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In preparation

Abstract

The renal cell carcinoma (RCC) genome is distinctive from other cancer types. To explore whether this also holds true for the RCC DNA methylome, we examined whether the clear cell RCC subtype (ccRCC) methylome differs from those of colorectal- (CRC) and breast cancer (BC). We integrated MBD-enriched massively parallel sequencing data with data from gene expression microarrays following global pharmacological DNA demethylation of ccRCC (n=4), CRC (n=6), and BC (n=4) cell lines to stringently identify functionally relevant promoter hypermethylated genes. In order to assess cancer-specific methylomes only, methylomes of healthy kidney, colon, and breast cells were determined in parallel and subtracted. A comprehensive comparison indicated that the sizes of the functional ccRCC, CRC and BC promoter hypermethylomes are approximately of the same magnitude (ccRCC: n = 237; CRC: n=327; BC: n=269) with only 61 gene promoters being mutually methylated in all three cancer types and 91, 151, and 112 genes showing ccRCC-, CRC- and BC-type specific promoter hypermethylation. Interestingly, pathway analysis indicated that biological processes such as development, immune response, apoptosis and survival are commonly affected by promoter hypermethylation, while the specific genes affected in these pathways are different in each tumor type. Genes (*ADSSL1*, *CDH1*, *CHRNB1*, *PIK3CD*, *SFN* and *TRPC3*) involved in nicotinic acetylcholine receptor (nAChR) signaling and/or linked to phosphoinositide-3-kinase (PI3K) signaling showed prominent functional hypermethylation in ccRCC and were not affected in CRC or BC, exposing novel regulations specific for ccRCC carcinogenesis.

In conclusion, our analyses provide a global view of functionally methylated gene promoters and biological processes affected in ccRCC, CRC, and BC. This leads to a deeper understanding of epigenetic implications in these cancers. We present evidence that overall the functional ccRCC methylome is not distinctive from the CRC and BC methylomes in terms of size, but in its constitution at the gene level. While major cancer hallmarks were similarly affected in all three cancer types, other pathways appear to be specifically deregulated in renal malignancy only, providing novel insights in ccRCC development, novel candidate biomarkers and potential drug targets for these diseases.

Introduction

Clear cell RCC (ccRCC) is the most common subtype of adult kidney cancer and most cases (up to 91%)[1] are characterized by von Hippel Lindau (*VHL*) gene alterations, which occur rarely in other RCC subtypes or cancer types. Recently, mutations in *PBRM1* were identified in 41% of renal cell carcinomas, making *PBRM1* the second most frequently mutated gene in these cancers after *VHL*[2]. Mutations in *PBRM1* in other malignancies have, as far as we know, only been identified in a small subgroup of breast cancers[3]. Furthermore, a recent screen identified a significant higher proportion of small insertion/deletion mutations throughout protein kinase genes in ccRCC compared to several other cancer types[4]. In contrast, cancer genes that are frequently mutated in other epithelial cancers, including *BRAF*, *CDKN2A*, *PIC3CA*, *PTEN*, the *RAS* oncogenes, or *TP53* play only a minor role in ccRCC (http://www.sanger.ac.uk/genetics/CGP/cosmic/). This suggests that ccRCC is molecularly distinct from the more common tumor types in adults. It is currently not known whether this distinction of ccRCC is similarly reflected by the epigenome.

Comprehensive exploration of the (cancer) epigenome with many of its epigenetic modifications and variations is currently feasible due to technological improvements and will be a major step towards a better understanding of the role of epigenetics in health and disease.

Here we aim to analyze the ccRCC methylome using next-generation sequencing (NGS)-based technologies. To explore whether the ccRCC promoter methylome is distinctive from the two most studied and best understood cancer methylomes, i.e. colorectal (CRC) and breast cancer (BC), we comprehensively compared these three DNA promoter hypermethylomes. We integrated data obtained by methyl-CpG binding domain protein (MBD)-affinity NGS and microarray-based expression profiling following global pharmacological DNA demethylation, as introduced in chapter 5. This integrated approach enables the identification and comparison of the functional hypermethylomes of ccRCC, CRC and BC.

Materials and methods

Cell culture and drug treatment

Four ccRCC cell lines (SKRC1, SKRC10, SKRC52, and SKRC59) and the breast cancer cell lines MCF-7 and T47-D were cultured in RPMI 1640 media (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The human kidney 2 cell line (HK-2) was cultured in Keratinocyte Serum Free Medium (K-SFM, GIBCO) supplemented with bovine pituitary extract (BPE) and human recombinant epidermal growth factor (EGF) as recommended. The CRC (CACO2, COLO320, HCT116, HT29, RKO, and SW480) and breast cancer (MDA-MB468 and MDA-MB231) cell lines were cultured in DMEM medium (Invitrogen) with 10% heat-inactivated FBS. All BC media were additionally supplemented with 2 mM L-glutamine and 1 x penicilline/streptomycine (Invitrogen). BC RPMI medium was further supplemented with 1 mM sodium pyruvate (Invitrogen). Above mentioned cancer cells were treated with 5'aza-2-deoxycytidine (DAC) or Trichostatin A (TSA), which inhibit DNA methylation and histone deacetylases, respectively, as described by Schuebel et al[5]. Approximately 10% confluent RCC cells were cultured in the appropriate total media with 5µM (DAC) (Sigma; stock solution: 1mM in PBS) for 96 hours, replacing media and DAC every 24 hours. Cell treatment with 300nM TSA (Sigma; stock solution: 5mM dissolved in ethanol) was performed for 18 hours, starting at approximately 30% cell confluency. Mock-treated cells were grown in parallel with the DAC or TSA treatment by addition of equal volumes of PBS or ethanol without drugs.

Gene expression microarray analyses

Microarray expression analyses were performed on mock-, DAC-, or TSA-treated cells, as described previously[5, 6]. In brief, total RNA was isolated using TRIzol Reagent (Invitrogen) or the RNeasy kit (Qiagen) according to the manufacturers' instructions and quantified using the NanoDrop ND-100 followed by quality assessment with the 2100 Bioanalyzer (Agilent Technologies). Sample amplification and labeling procedures were carried out using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) according to the manufacturer's instructions. The labeled cRNA was purified using the RNeasy mini kit (Qiagen) and quantified. Cy3- or Cy5-labeled samples were mixed with control targets (Agilent Technologies), assembled on Oligo Microarray, hybridized, and processed according to the Agilent microarray protocol. Scanning was performed with the Agilent G2565BA microarray scanner using settings recommended by Agilent Technologies.
Array data were analyzed using R (version 2.10.0) and BioConductor, using the limma package (version 3.2.1)[7]. Median Cy5 and Cy3 signals read from the raw data and M-values, i.e. log2(Cy5/Cy3), were loess normalized. Basal expression was roughly estimated using the single channel Cy3 values, containing the mock treated samples. Based on the threshold used for basal expression, the specificity could be further increased, with obvious implications on sensitivity.

Methyl binding domain-affinity next generation sequencing

DNA from normal breast tissue, derived from BioChain Institute, Hayward, CA, USA was isolated by proprietary modified guanidine and thiocyanate techniques. DNA from normal colon tissues, derived from the tissue archive of the department of pathology at the VU University medical center, Amsterdam, The Netherlands, was isolated using the QIAamp DNA Micro Kit (Qiagen Benelux, The Netherlands) according to the manufacturer's instructions. DNA from the above described cultured cells was isolated using the Puregene DNA purification kit (BIOzym, Landgraaf, the Netherlands) according to the manufacturer's instructions. Genomic DNA was fragmented by use of a COVARIS S2 system with AFA fiber micro tubes to obtain fragments with an average length of 200 bp. Methylated DNA was captured by performing pull-down using the MethylCollector kit for the cancer cell lines and normal colon and breast samples (Active Motif) or the MethylCap kit with High-Salt elution for the normal kidney sample (Diagenode), according to the manufacturer's protocols. Both are based on autologous methyl-binding domains and independent analyses demonstrated that more than 95% of methylation cores identified by the MethylCollector kit were also identified by the MethylCap kit (manuscript submitted). Subsequently, fragments were sequenced using the Illumina Genome Analyzer II. The concentrations of the fragmented and captured DNA was determined on a Fluostar Optima plate reader with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen P7589) 480/520nm. For all samples together, the paired-end 40bp sequence reads where mapped using BOWTIE[8] on the human reference genome (NCBI build 37.3). Coverage values were summarized using the map of the human methylome, which consists of putatively independently methylated regions (methylation cores) throughout the genome (manuscript in preparation). For each sample, and each methylation core, the maximum read coverage was used for further analysis. Subsequently, we focused on the broad promoter region for each gene (-2000 to +500 bp). A Poisson background model was used to identify significantly methylated regions (alpha = 0.01), with lambda estimated as the sum of the coverage values over all promoter methylation cores divided by the number of promoter methylation cores. This approach takes into account coverage differences between samples, although generally low coverage will result in low sensitivity.

DNA methylation analyses – methylation-specific PCR

Genomic DNA (500 ng) was chemically modified by sodium bisulfite using the EZ DNA methylation kit (Zymo Research, Orange, California) according to the manufacturer's instructions. Gene promoter methylation was subsequently determined by methylation-specific PCR (MSP) as described in detail elsewhere[9-11]. Primer sequences and PCR conditions are available upon request.

Results

Exploring DNA hypermethylation in ccRCC, CRC and BC identifies functional promoter hypermethylomes of approximately the same magnitude

Methylated DNA regions of ccRCC (n=4), CRC (n=6) and BC (n=4) cell lines were identified by massively parallel sequencing of MBD-enriched fragmented DNA, a method which has been described previously[12]. In order to compare the ccRCC functional methylome with those of CRC and BC, we integrated promoter methylation data with gene re-expression data after DNA demethylation by DAC, emphasizing on genes in which promoter methylation indeed is associated with gene suppression and thus is likely biologically relevant (Figure 1A).

Using this approach, a total of 7829, 6580, and 7389 genes indicated promoter methylation by MBD-binding in ccRCC, CRC, and BC cells, respectively, ranging from 2802 to 5695 genes per cell line (Table 1). Changes in gene expression following methyltransferase or histone deacetylase inhibition identified a zone (spike) in which gene expression was not increased by TSA (TSA-negative <1.4 fold), and was absent in untreated cells, but increased >1.4 fold by DAC treatment (DAC-positive)[5]. To reduce false positive results, we used stringent selection criteria within these spike genes[6], which in total resulted in 1583, 2306, and 2117 upregulated genes in ccRCC, CRC, and BC cells, respectively, ranging from 186 to 1301 genes per cell line (Table 1). Variation in the prevalence of DNA methylation has previously been observed associated with microsatellite (MSI) or chromosomal (CIN) instability in CRC and in specific subtypes of BC. Integrating results from both approaches and excluding genes that did not show a correlation between promoter methylation and gene re-expression within the same cell line, identified 446, 595, and 492 putatively biologically relevant methylated gene promoter regions in ccRCC, CRC, and BC cells, respectively. Supplemental Figure S1 shows the overlay of genes identified by MBD-sequencing within the expression spikes per cell line. Genes within this spike overlapping with the MBD-sequencing data define the functional hypermethylome for each cell line individually. These stringent criteria for inclusion limit the further characterization to those genes functionally silenced by promoter region DNA methylation. This minimal magnitude of the ccRCC, CRC and BC functional promoter methylomes comprise 1.8-2.2%, 2.4-3.0%, and 2.0-2.5%, respectively of the number of estimated protein-coding genes in the human genome (20,000-25,000 Human Genome Project, http://www.ornl.gov/sci/techresources/Human_Genome /home.shtml). Among those genes, 149 (33%), 238 (40%) and 171 (35%) genes are cancer type-specific for ccRCC, CRC, and BC cells, respectively. Approximately 40-55% of the genes are overlapping between two cancer types (270, 246 and 210 genes for CRC-BC, ccRCC-CRC and ccRCC-BC, respectively) and approximately 25-35% (159 genes) are overlapping between the three cancer types (Figure 1B).
MBD-affinity NGS of the normal renal epithelial cell line HK-2 and normal breast and colon tissues was performed and results were pooled in order to exclude tissue type-specific promoter DNA methylation. Correction for methylated gene promoters in the normal pool resulted in a total of 237, 327, and 269 hypermethylated gene promoter regions among which 91 (38%), 151 (46%) and 112 (42%) showed ccRCC-, CRC- and BC-type specific promoter hypermethylation, respectively (see Supplemental Table S1 for

specific gene lists). Additionally, approximately 35-50% are overlapping between two cancer types (124, 113 and 94 genes for CRC-BC, ccRCC-CRC and ccRCC-BC, respectively) and approximately 20-25% (61 genes; Supplemental table S1) are overlapping between the three cancer types (Figure 1C).

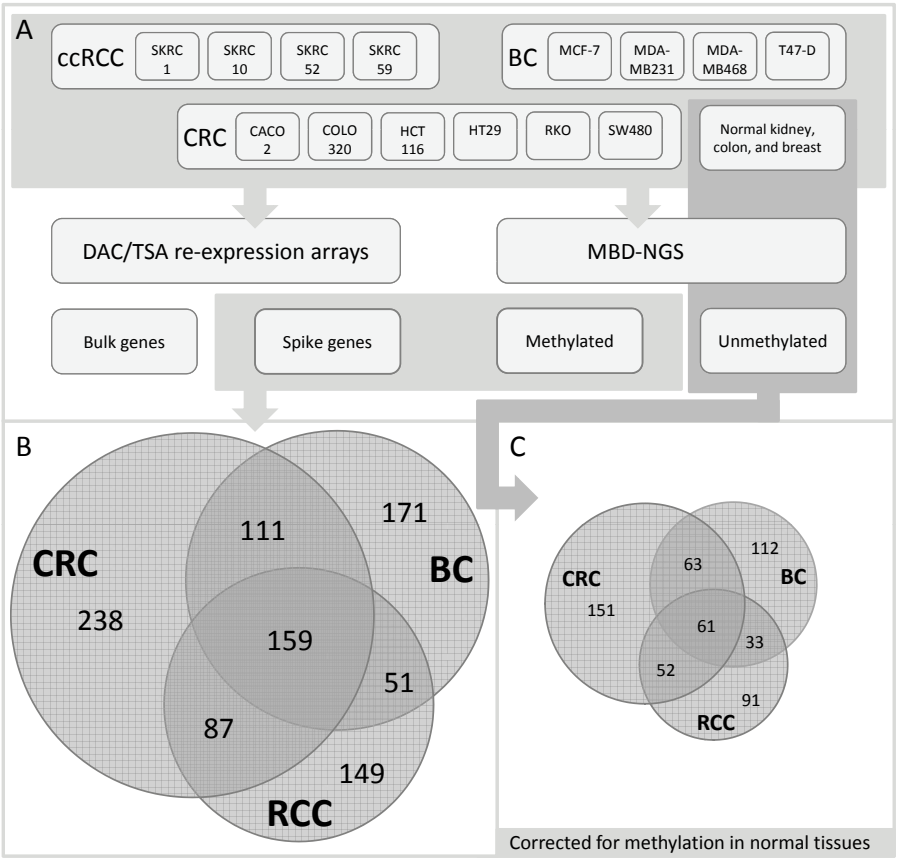


Figure 1. Gene discovery flowchart of ccRCC, CRC and BC functional methylomes
Schematic view of the approach applied for the stringent identification of putatively functional ccRCC, CRC and BC methylomes. Four ccRCC, six CRC and four BC cell lines were analyzed for methylation indirectly by DAC/TSA re-expression arrays and by MBD-NGS. Gene promoters that indicated methylation by MBD-binding within the spike of re-expressed genes after DNA demethylation were stringently selected (A) and analyzed for overlap between the three cancer types (B). Genes identified in at least one cell line per cancer type as specifically up-regulated after DNA demethylation and enriched by MBD-protein(s), but not in normal healthy cells, were considered as the differentially methylated functional methylome (C).

Table 1. Number of genes in ccRCC, CRC and BC methylomes

	ccRCC				CRC						BC			
	SKRC1	**SKRC 10**	**SKRC 52**	**SKRC 59**	**CaCo2**	**Co-lo320**	**HCT 116**	**SW 480**	**HT 29**	**RKO**	**MDA-MB-468**	**MDA-MB-231**	**T47D**	**MCF7**
MBD/ cell line	5438	4786	5695	2802	3433	3922	3430	3192	3560	3355	3118	4670	4762	3285
DAC↑/ cell line	571	508	731	651	227	663	1301	186	491	668	612	1230	234	795
MBD/ cell type	7829				6580						7389			
DAC↑/ cell type	1583				2306						2117			
Overlap MBD+ DAC↑	541				778						669			
Overlap cell line specific	446				595						492			

Pathways enriched for promoter hypermethylation in ccRCC, CRC and BC

As the cancer-specific functional hypermethylomes of the three cancer types are apparently in approximately the same order of magnitude, we subsequently examined whether cellular pathways were also similarly affected by functional promoter hypermethylation, or rather in a cancer type-specific manner. Since the net effect of a pathway can be the same independent of the specific activating or other inhibitory components affected, all genes were considered together. The pathway analyses were performed by using the well annotated MetaCore databases (GeneGo). We selected the 50 most relevant pathways associated with functional hypermethylation, first for genes mutually involved in all three cancer types (n=61; Supplemental Table S2) and then separate for the cancer type-specific genes (RCC: n=91; CRC: n=151; BC: n=112; Supplemental Tables S3-S5).

Overall, in the four pathway lists, pathways significantly enriched for promoter hypermethylation and subsequent gene silencing included those controlling development, immune response, apoptosis and survival, transcription, cell cycle, G-protein signaling, neurophysiological process, muscle contraction, cytoskeleton remodeling, cell adhesion and signal transduction (Figure 2). As expected, genes functionally hypermethylated in all three cancer types heavily represented cellular processes related to carcinogenesis. In particular, genes encoding various components of pathways involved in immune response, development and cell adhesion were strongly enriched for functional hypermethylation (32 out of 61 genes, Figure 2B).

Interestingly, the cancer type-specific genes also profoundly represented pathways involved in development and immune response (ccRCC: 34/91 (37%), CRC: 51/151 (34%) and BC: 31/112 (28%), Figure 2A), indicating that these particular pathways are affected both by shared genes in ccRCC, CRC and BC, as also by different genes in a more cancer type-specific fashion. In contrast, cell adhesion was mainly affected by common genes in all three cancer types, but less in a cancer type-specific fashion. Furthermore, the majority of genes were unique for each cancer type. However, they represent mostly the same pathways and processes (Figure 2). Top-ranked pathways involved in development, immune response, G-protein signaling, apoptosis and survival, cell adhesion, cell cycle, cytoskeleton remodeling, and transcription are affected by

the vast majority of genes in each of the three cancer type-specific gene lists (62%, 50% and 44% for ccRCC, CRC and BC, respectively), and therefore by different functionally methylated genes.

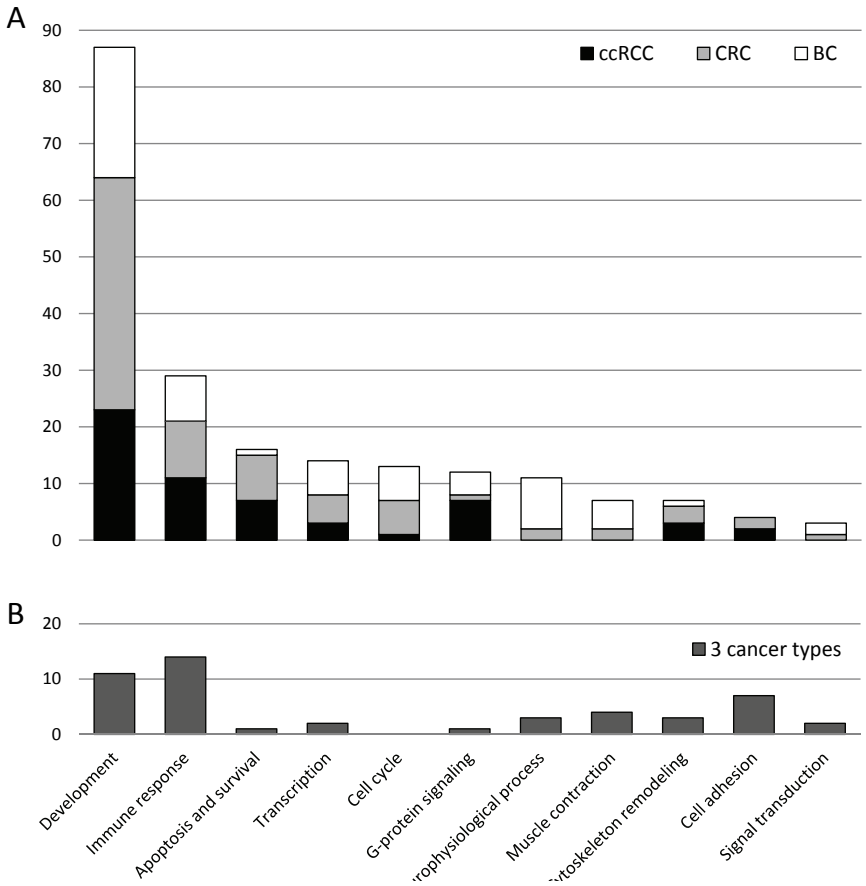


Figure 2. Number of genes involved per pathway in ccRCC, CRC, BC or all three cancer types
Methylation enriched pathways with number of genes affected per cancer type (A) and number of mutually affected genes in all three cancer types (B).

Pathways enriched for functional promoter hypermethylation in ccRCC

Although the uniquely functionally methylated genes in the three cancer types affected similar pathways in general, the specific target(s) within these pathways was (were) mostly different per cancer type. Focusing on ccRCC, the top-ranked pathway, involved in apoptosis and survival, specifically affected the pathway of the nicotinic acetylcholine receptor (nAChR) in apoptosis inhibition and cell cycle progression. In this pathway, the acetylcholine receptor subunit beta (*CHRNB1*) and phosphoinositide-3-kinase, catalytic, delta polypeptide (*PIK3CD*) were functionally methylated in ccRCC

only. Examining the top 50 pathways remarkably revealed several other pathways linked to the nAChR pathway involving various components of the PI3 kinase and calcium (Ca^{2+}) pathway which in turn are linked to the VEGF, MAPK, and EGFR pathway. *PIK3CD*, stratafin (*SFN/14-3-3σ*), the transient receptor potential cation channel subfamily C member 3 (*TRPC3*) and adenylosuccinate synthase like 1 (*ADSSL1*) are genes involved in one or more of these pathways which indicated functional hypermethylation specifically in ccRCC. Interestingly, six of the top 50 ccRCC pathways were categorized to be linked with epithelial-to-mesenchymal transition (EMT). Again, the *PIK3CD* gene and in addition the cadherin 1 (*CDH1*) gene, known to be involved in this mechanism, showed functional hypermethylation in ccRCC.

These affected pathways in RCC are also (to a greater or lesser extend) affected in CRC and BC, however by different genes (Supplemental Table S4 and S5). The integrated approach correctly indicated the methylation status as analyzed by MSP in ccRCC (Table 2). If methylation was indicated by both MBD-affinity NGS and the re-expression data, MSP confirmed the methylation status and *vice versa* in 100%. However, in four cases methylation was only indicated by MBD, without re-expression after demethylation, and two of these were methylated by MSP (Table 2). This underscores the significance of integrating both technologies to correctly identify functional promoter methylation.

Table 2. Validation of MBD-affinity NGS and re-expression microarray data by MSP in ccRCC cell lines

	ADSSL1			***CDH1***			***CHRNB1***			***PIK3CD***			***SFN***			***TRPC3***		
	MBD	DAC	MSP	MBD	DAC	MSP	MBD	DAC	MSP	MBD	DAC	MSP	MBD	DAC	MSP	MBD	DAC	MSP
SKRC1	√	√	M	√	√	M	√	√	M	√	√	M	√	√	M	√	√	M
SKRC10	√	√	M	√	√	M	√		M	√		U	√	√	-	√		M
SKRC52			U	√		U			U	√	√	M	√		-	√	√	M
SKRC59			U			U			U			U			U	√	√	M

MBD √: methylation indicated by MBD-affinity NGS
DAC √: methylation indirectly indicated by re-expression microarray data following demethylation treatment
M: methylated by MSP, U: unmethylated by MSP
Gray highlight indicates MBD or DAC data validated by MSP

Discussion and conclusion

The aim of this study was to compare the functional ccRCC hypermethylome with those of CRC and BC. The analysis that we present here provides a global picture of both the tumor-specific methylomes of ccRCC, CRC and BC and the methylome shared by these three tumor types. Numbers of affected gene promoters within these three cancer types, identified using very stringent selection criteria, appear to be approximately similar, indicating that the size of the methylomes is in the same order of magnitude for renal, colorectal and breast cancer cells. Using this stringent approach, the number of genes affected by promoter hypermethylation forming the minimally sized functional cancer hypermethylomes, comprises about two to three percent of the number of estimated protein-coding genes in the human genome (20,000-25,000 Human Genome Project). Objective comparison of data estimating methylome(s) (sizes) in literature is complicated as it is highly technique- and cell line/tissue-dependent. The data are based on the currently existing annotation databases and because of the stringent selection criteria used in the presented analyses, many interesting candidate genes, such as methylated genes that not become re-expressed upon DAC treatment etc, are being excluded from further analysis. Furthermore, variation between cell lines, like MSI and CIN in CRC, could significantly influence functional methylome sizes as observed for the MSI positive cell lines HCT116 and RKO which show the largest number of re-expressed genes. For ccRCC and BC this is less clear. Further analyzing these data encloses possibilities to unravel such issues.

Our integrated approach has never been applied before, but we believe that the functional methylomes we identified are smaller in size yet more biologically significant than those methylomes identified in other epigenome-wide and whole epigenome studies which did not take gene re-expression into account. In this first analysis we focused on promoter hypermethylation thereby excluding other regions like gene body methylation. Evidence is emerging that regions outside the promoter also could regulate gene expression[13], suggesting that our identified functional hypermethylome is incomplete. Again such information could be unraveled by further analyzing the data acquired by our approach, although this first requires a full elucidation of the functional locus (promoter, exon1, intron(s), gene body) and its specific effects of DNA methylation on gene expression.

Almost half of the identified genes also showed promoter methylation in normal kidney, colon or breast cells, which is indicative of tissue-specific epigenetic regulation in normal development or aging. On the other hand, the methylome was analyzed in only a small number of normal samples which preferentially needs to be extended. This underlines the importance to correct for methylation in non-cancerous tissues and to validate the hypermethylation status of identified genes in a large number of normal tissues by specific techniques, such as MSP.

26%, 19%, and 23% of the identified genes in ccRCC, CRC, and BC respectively, overlapped the three cancer types and were enriched in pathways related to carcinogenesis affecting many, if not all, hallmarks of cancer, like evading growth suppression and sustaining proliferative significance, resisting cell death, and activating invasion and metastasis[14]. Interestingly, one of the recently emerged cancer hallmarks, a defective immune destruction[15], comprised a common feature in all three cancer types. Immune

response appeared to be the most affected pathway (13/50; 26%) in the top 50 pathway list of commonly functionally methylated genes in ccRCC, CRC and BC (Figure 2B and Supplemental Table S2). In addition to the commonly affected gene promoters, specific components related to immune response were also heavily enriched for functional hypermethylation in each cancer type separately with 11, 10 and 8 genes involved in ccRCC, CRC and BC respectively (Figure 2A and Supplemental Table S3-5). Aberrant DNA methylation may influence signaling and expression of genes important for functioning of the innate and acquired immune systems[16, 17]. Promoter hypermethylation of several genes has been suggested to help tumor cells escape from an immunological attack either by preventing an immune response (*CIITA*, *HLA-A* and *-B* genes)[18-21] or by acquired resistance to products of the activated immune system or its activation self (*IRF-7*, *STAT1*, *2*, and *3* genes, *DAPK* and multiple INF pathway genes)[22-26]. Important to note, our analysis based on cell lines excludes the possibility that stromal contamination from infiltrating immune cells, which may be present when analyzing primary tissues, could have distorted this result. Thus, our data underscore the importance of epigenetic deregulation of this novel hallmark in cancer by DNA hypermethylation and provide a basis for future cancer research.

Comprehensive analyses of the cancer-specific functional methylomes resulted in rather similar compositions of functional categories for ccRCC, CRC and BC. We observed largely comparable enriched pathways within the three cancer types, with the two largest comprising development and immune response. Several pathways were epigenetically altered by promoter hypermethylation in all three cancer types, however by different genes, suggesting that several genes within the same group or pathway serve the same goal. In general, these analyses show conserved targeting of critical pathways through unique epigenetic events. For all these gene groups, new genes were identified that had not been implicated in epigenetic (DNA methylation) tumorigenesis in these cellular processes. These data forms a basis for future exploration of the cancer specific methylome, which contributes to the understanding of the cancer pathogenesis. This could also provide novel candidate biomarkers and potential drug targets, although clinical validation in multiple large series of cancers is required.

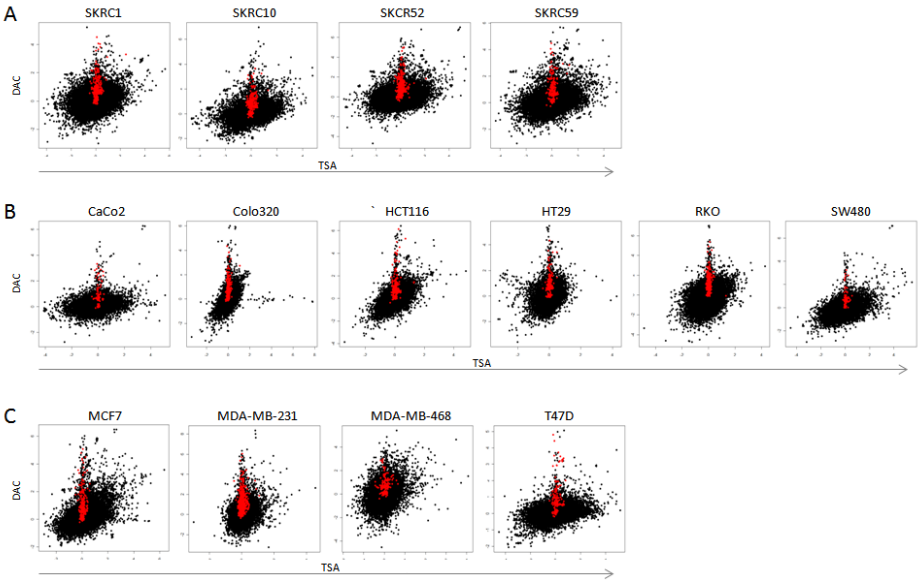
Here we focused on the renal cell cancer promoter hypermethylome hypothesizing that, based on the genetic differences, it could be distinguished from the CRC and BC hypermethylomes. In contrast to what we hypothesized, the functional ccRCC methylome was quite comparable to the CRC and BC ones, in terms of size, but also by equally affected pathways, as mentioned previously. However, the specific target(s) within those pathways was (were) mostly different per cancer type. Based on the histology and genetics of ccRCC, we expected involvement of genes related to hypoxia and angiogenesis. Strikingly, for ccRCC the most prominent identified pathway involved in apoptosis and survival specifically affected the nAChR pathway in apoptosis inhibition and cell cycle progression. In addition, nine of the top 50 pathways were enriched for functional hypermethylation with gene promoters linked to this pathway, like the PI3K and the calcium signaling pathway, implicating cross-talk of the PI3K/Akt pathway with other pathways relevant to ccRCC. One of these connections may result in EMT[27, 28], which is one of the characteristics of ccRCC[29]. The PI3K/Akt pathway has been emerged as a central feature of EMT and repression of the E-cadherin gene (*CDH1*) is one of its major characteristics[27]. During tumorigenesis EMT may increase the motility and inva-

siveness of cancer cells, and malignant transformation may be associated with signaling pathways promoting EMT, however this is less characterized and represents an area of active investigation[27, 30]. EMT has been suggested to play a role in RCC, however its mechanism remain elusive. Our analyses might indicate a novel path connecting epigenetic (indirect) regulation of the PI3K pathway and/or its cross-talk to other signaling pathways with EMT, which has not been described before.
It is becoming clear that genes involved in a pathway interact with a number of other pathways to create an extremely complex network. Because of this network it could be explained why a single inhibition can easily be circumvented by up-regulation of these interconnections, ultimately leading to failure of therapeutic strategies. The complexity of genetic and epigenetic alterations in human cancer increases when considering several changes. The genomic landscape has been described as a few commonly affected 'gene mountains' scattered among a much larger number of 'gene hills' that are infrequently genetically altered[31, 32]. The vast majority of aberrations appear to occur in the 'hills', which is likely to explain the wide variation in tumor behavior and response to therapy. After establishing the frequency of hypermethylation of these genes in large series of primary tumors, we can determine whether this also holds true for the methylome of these cancers. Concerning DNA methylation aberrations (in ccRCC, CRC and BC), altered pathways rather than individual genes appear to direct tumorigenesis. Nevertheless, a similar design can be considered regarding DNA methylation profiles in cancer. Certain processes (mountains) are commonly deregulated during carcinogenesis in general, however due to different affected genes which eventually serve the same goal. Additionally multiple extra processes (hills) will be affected in each cancer type contributing to the heterogeneity and complexity of human neoplasia.

Although many of the known cancer genes that are frequently mutated in other than RCC cancers have not been reported to be affected otherwise in RCC, it might be that the pathways of which those genes are a component of, are disrupted in other ways, for instance silenced by DNA hypermethylation. Gene members of the PI3K/Akt signaling pathway for example, which is crucial to many aspects of cell growth and survival, are frequently mutated or amplified in a wide variety of solid tumors. Receptor tyrosine kinases upstream of PI3K, the p110α catalytic subunit of PI3K, the downstream kinase, AKT, and the negative regulator, PTEN, are all frequently altered in cancer[33], but information about the involvement in RCC is sparse. Although the PI3K pathway has been reported to be deregulated in renal cell carcinoma[34, 35], mutations have been detected only scarcely. *PTEN* gene mutation in ccRCC has been reported to be a rare event[36-38]. The molecular basis for these observations has not been determined. Our analyses revealed several genes (*ADSSL1*, *CHRNB1*, *PIK3CD*, *SFN* and *TRPC3*) directly or indirectly linked to this pathway to be functionally methylated, which might alternatively deregulate this pathway in ccRCC. Whether inactivation of these genes by promoter hypermethylation indeed affects this signaling pathway, remains to be investigated further.

In conclusion, these analyses provide a first global view of functionally methylated genes and processes affected by this epigenetic feature in ccRCC, CRC and BC. The combination of methylation-specific sequencing and re-expression analysis appeared powerful and permits the identification of genes and pathways that may not be easily detected by either analysis alone. These results lead to a deeper understanding of the methylation data and its implications for renal cell, colorectal and breast neoplasia and form a basis for future research.

Supplemental figure and tables



Supplemental Figure 1. Overlay of genes by MBD-binding and DAC re-expression per ccRCC, CRC and BC cell line
Gene expression changes for the ccRCC cell lines SKRC1, SKRC10, SKRC52 and SKRC59 (A), the CRC cell lines CACO2, COLO320, HCT116, HT29, RKO, and SW480 (B), and the BC cell lines MCF-7, MDA-MB468, MDA-MB231 and T47-D (C) treated with TSA (x-axis) or DAC (y-axis) are plotted by log-fold change. Each dot represents an individual probe, which represents a gene (some genes were represented by several probes as a control) on the array. Overlapping genes within the spike, identified by MBD-sequencing are indicated in red, genes without overlap are depicted in black.

Supplemental Table S1. Gene lists of ccRCC, CRC and BC functional methylomes

3 cancer types (n=61)	**ccRCC** (n=91)	**CRC** (n=151)	**BC** (n=112)
PRTFDC1	*NEBL*	*UNC13D*	*HTR7*
LIMD2	*LOXL4*	*THBD*	*BDNF*
ZIK1	*C10orf81*	*CRYGD*	*RRAD*
L1TD1	*CDC42BPG*	*C3orf14*	*CCDC68*
PHACTR3	*PLEKHG6*	*EPS8L2*	*CNN1*
CRYBA2	*ADSSL1*	*EFS*	*CNTD2*
CAND2	*RASGRP1*	*FOXA1*	*RCSD1*
FBLN2	*ALDH1A3*	*GALC*	*GOLT1A*
FOXF2	*CDH1*	*EVL*	*SLC35F3*
ANK1	*CHRNB1*	*LTK*	*C2orf44*
CYB5R2	*IGFBP4*	*C15orf48*	*ELOVL2*
OVOL1	*COLEC12*	*FBN1*	*LAMA2*
BNC1	*C18orf1*	*ISG20*	*AKAP12*
KRT16	*ICAM4*	*SLC16A5*	*SCIN*
TBX21	*KCNN1*	*SPHK1*	*HDAC9*
CIDEA	*SFN*	*LAMA3*	*C7orf38*
ELAVL3	*NES*	*COL16A1*	*MKX*
PPP1R14A	*CRABP2*	*TEKT2*	*TLL2*
DLL3	*CACNA2D3*	*OXCT2*	*C10orf82*
NPAS1	*MFI2*	*S100A6*	*PPAPDC1A*
ZNF542	*MYO1G*	*C20orf103*	*FAM24B*
AK5	*LAT2*	*FKBP1B*	*C10orf88*
ACTA1	*ADHFE1*	*EGR4*	*ZNF214*
SLC32A1	*CA3*	*CD8B*	*DEPDC7*

Supplemental Table S1. continued

3 cancer types (n=61)	**ccRCC** (n=91)	**CRC** (n=151)	**BC** (n=112)
KCNS1	*RIMS2*	*CCDC37*	*RASGRP2*
TNNC2	*MAL2*	*SLC7A14*	*SUV420H1*
EYA2	*SHC3*	*KCTD8*	*GPR83*
POMC	*BSPRY*	*HERC5*	*SCN4B*
KCNG3	*BARHL1*	*DDIT4L*	*TMTC1*
DLX1	*C10orf110*	*SHROOM1*	*HOXC9*
HOXD13	*FLRT2*	*HSPA1A*	*C12orf42*
CCK	*SHC4*	*IL20RA*	*GLT8D2*
ZIC1	*SLC6A4*	*TWIST1*	*NID2*
EGFLAM	*C1QTNF1*	*AKR1B1*	*SEMA6D*
OTP	*GNA15*	*TP53INP1*	*ISL2*
BHMT	*CD34*	*NOTCH1*	*SALL1*
B3GALT4	*LAMA5*	*C9orf167*	*SLFN13*
GDF6	*C21orf63*	*FAM133A*	*FBXL20*
GABBR2	*EDIL3*	*NAP1L3*	*SKAP1*
PRRX2	*DGKI*	*LRCH2*	*HOXB4*
ME3	*KLRG2*	*PTF1A*	*CACNA1G*
TMEM25	*MAP3K8*	*PPP1R3C*	*KCNJ2*
KL	*ELOVL3*	*EMX2*	*CCDC11*
CSPG4	*PSTK*	*RIC3*	*CFD*
CHRNA3	*AMPD3*	*NRIP3*	*TNNI3*
MT1M	*SYT13*	*ABCC8*	*ZNF329*
PCSK9	*SLC43A3*	*WIT1*	*GFI1*
RASSF5	*LRMP*	*KCNE3*	*NTNG1*
DYSF	*PTPRR*	*JAM3*	*TBX15*
MAL	*SLC27A2*	*CCND2*	*C1orf114*
TMEM22	*PRSS22*	*MANSC1*	*RYR2*
CXCL14	*DNAH3*	*BCAT1*	*PLCB4*
PRDM13	*PRRT2*	*GLS2*	*L3MBTL*
PON3	*HS3ST3A1*	*DPY19L2*	*MATN4*
PAX6	*SLFN11*	*SALL2*	*DPYSL5*
POU2AF1	*HOXB13*	*RIPK3*	*DLX2*
PAPLN	*PSTPIP2*	*GPR68*	*HOXD8*
ZNF167	*HPN*	*ADAL*	*PROK2*
RBP1	*PIK3CD*	*TM6SF1*	*PCSK1*
NPFFR2	*PAX7*	*ANPEP*	*C6orf114*
C8G	*C1orf172*	*MPV17L*	*FAM8A1*
	GJB3	*MYH11*	*HIST1H1A*
	ST6GALNAC5	*YPEL3*	*MDGA1*
	PRMT6	*LRRC36*	*PLA2G7*
	NMNAT2	*FOXF1*	*CD109*
	G0S2	*NLRP1*	*C6orf59*
	MARK1	*RASD1*	*TBX18*
	PXDN	*SSTR2*	*MCHR2*
	IGFBP2	*MATK*	*ULBP1*
	SLC16A14	*ZNF439*	*HOXA4*
	CD200	*PSG9*	*DPY19L2P1*
	TRPC3	*FUZ*	*C7orf10*
	CDH18	*KIF17*	*PGAM2*
	SMOC2	*RAB42*	*PON1*
	BMPER	*KIAA1324*	*VGF*
	RELN	*PCP4L1*	*KCND2*
	CA8	*TDRD5*	*LZTS1*
	TRAF1	*AKT3*	*MATN2*
	PNPLA4	*RIMS4*	*GLDC*
	MAGEB2	*SLC2A10*	*RAB39B*
	EFHC2	*OLIG1*	*FGFR2*
	MAGIX	*CBR1*	*KCTD14*
	WNK3	*KCNJ4*	*KIF5A*
	TSPAN15	*EHD3*	*PITPNM2*
	EFEMP2	*ITGA4*	*NEK9*
	LRRC32	*ACADL*	*GRAMD2*

Supplemental Table S1. continued

3 cancer types (n=61)	**ccRCC** (n=91)	**CRC** (n=151)	**BC** (n=112)
	PTPRO	*NMUR1*	*PLLP*
	ABCC9	*ECEL1*	*DNM3*
	RHOV	*ENTPD3*	*PKP1*
	ATF7IP2	*ZNF501*	*CTSZ*
	GPD2	*CD38*	*ST6GAL2*
		KIAA1239	*PROC*
		NSUN7	*AGPS*
		BASP1	*TMBIM1*
		PTGER4	*WNT10A*
		ZNF354C	*CPZ*
		FLT4	*CARTPT*
		TUBB2B	*VCAN*
		SNAP91	*CPNE5*
		MOXD1	*RGS22*
		EYA4	*GNA14*
		FNDC1	*ANGPTL2*
		HOXA11	*GSTP1*
		ELMO1	*SLC30A2*
		C8orf42	*BTG3*
		XKR6	*B3GNT5*
		EFHA2	*IGF2BP2*
		NEFL	*PROM1*
		DOCK8	*LRFN2*
		IGFBPL1	*TRIM55*
		RASEF	*ANKS6*
		DFNB31	*RPS6KA3*
		COL5A1	
		OLFM1	
		BNIP3	
		KIAA1772	
		ZNF568	
		CNN3	
		DUOX2	
		NDRG4	
		MGAT5B	
		RAB31	
		CBS	
		TIMP3	
		CPE	
		TRMT12	
		C9orf53	
		NGFRAP1	
		ALOX5	
		OGDHL	
		HHEX	
		DPYSL4	
		C13orf15	
		RGMA	
		ATP2A3	
		NGFR	
		GALR2	
		LASS4	
		PADI2	
		C1orf103	
		C1orf92	
		WNT3A	
		SULF2	
		KCNQ2	
		ADRB2	
		SNCB	
		GFPT2	
		ENG	
		FNBP1	
		FAM69B	
		MAGEA8	

Supplemental Table S2. Pathway top 50 mutually involved in the three cancer types (ccRCC, CRC, BC)

#	Pathway	p-value	Ratio	FDR
1	Development_S1P2 and S1P3 receptors in cell proliferation and differentiation	2,765E-03	2 26	15%
2	Immune response_Antigen presentation by MHC class I	3,203E-03	2 28	15%
3	Cell adhesion_Cell-matrix glycoconjugates	5,848E-03	2 38	15%
4	Development_TGF-beta-dependent induction of EMT via RhoA, PI3K and ILK.	8,483E-03	2 46	15%
5	Development_Melanocyte development and pigmentation	9,585E-03	2 49	15%
6	Neurophysiological process_GABAergic neurotransmission	9,966E-03	2 50	15%
7	Chemotaxis_Leukocyte chemotaxis	2,155E-02	2 75	
8	wtCFTR and delta508 traffic / Clathrin coated vesicles formation (norm and CF)	5,634E-02	1 19	
9	Immune response_TLR3 and TLR4 induce TICAM1-specific signaling pathway	5,922E-02	1 20	
10	Cytoskeleton remodeling_Regulation of actin cytoskeleton by Rho GTPases	6,780E-02	1 23	
11	Immune response_IL-27 signaling pathway	7,065E-02	1 24	
12	Development_Leptin signaling via JAK/STAT and MAPK cascades	7,349E-02	1 25	
13	Muscle contraction_Delta-type opioid receptor in smooth muscle contraction	7,632E-02	1 26	
14	Cell adhesion_Cadherin-mediated cell adhesion	7,632E-02	1 26	
15	Muscle contraction_nNOS Signaling in Skeletal Muscle	7,914E-02	1 27	
16	Development_Mu-type opioid receptor regulation of proliferation	8,195E-02	1 28	
17	Apoptosis and survival_nAChR in apoptosis inhibition and cell cycle progression	8,475E-02	1 29	
18	Muscle contraction_S1P2 receptor-mediated smooth muscle contraction	8,755E-02	1 30	
19	Chemotaxis_CCR4-induced leukocyte adhesion	8,755E-02	1 30	
20	Development_Slit-Robo signaling	8,755E-02	1 30	
21	Neurophysiological process_Mu-type opioid receptor-mediated analgesia	8,755E-02	1 30	
22	Cytoskeleton remodeling_RalA regulation pathway	8,755E-02	1 30	
23	Cell adhesion_Gap junctions	8,755E-02	1 30	
24	Signal transduction_Activin A signaling regulation	9,589E-02	1 33	
25	Immune response_Role of the Membrane attack complex in cell survival	9,865E-02	1 34	
26	Methionine metabolism	9,865E-02	1 34	
27	Development_TGF-beta-dependent induction of EMT via SMADs	1,014E-01	1 35	
28	Cytoskeleton remodeling_Keratin filaments	1,042E-01	1 36	
29	Cell adhesion_Tight junctions	1,042E-01	1 36	
30	Immune response_IL-12-induced IFN-gamma production	1,042E-01	1 36	
31	G-protein signaling_G-Protein alpha-s signaling cascades	1,042E-01	1 36	
32	Cell adhesion_Role of tetraspanins in the integrin-mediated cell adhesion	1,069E-01	1 37	
33	Development_Mu-type opioid receptor signaling	1,096E-01	1 38	
34	Transcription_Sin3 and NuRD in transcription regulation	1,096E-01	1 38	
35	Immune response_Alternative complement pathway	1,123E-01	1 39	
36	Transcription_NF-kB signaling pathway	1,123E-01	1 39	
37	Immune response_Th1 and Th2 cell differentiation	1,151E-01	1 40	
38	Immune response_PGE2 in immune and neuroendocrine system interactions	1,232E-01	1 43	
39	Cell adhesion_Histamine H1 receptor signaling in the interruption of cell barrier integrity	1,285E-01	1 45	
40	Immune response_Bacterial infections in normal airways	1,391E-01	1 49	
41	Immune response_Lectin induced complement pathway	1,391E-01	1 49	
42	Mucin expression in CF via TLRs, EGFR signaling pathways	1,444E-01	1 51	
43	Signal transduction_Activation of PKC via G-Protein coupled receptor	1,470E-01	1 52	
44	Immune response_Classical complement pathway	1,470E-01	1 52	
45	Ganglioside Metabolism p1	1,522E-01	1 54	
46	Muscle contraction_ACM regulation of smooth muscle contraction	1,574E-01	1 56	
47	Immune response_TLR signaling pathways	1,574E-01	1 56	
48	Bacterial infections in CF airways	1,626E-01	1 58	
49	Immune response _Immunological synapse formation	1,652E-01	1 59	
50	O-glycan biosynthesis	1,753E-01	1 63	

Supplemental Table S3. Pathway top 50 clear cell renal cell cancer-specific genes

#	Pathway	p-value	Ratio		FDR
1	Apoptosis and survival_nAChR in apoptosis inhibition and cell cycle progression	8,642E-03	2	29	31%
2	Development_Role of CDK5 in neuronal development	1,176E-02	2	34	31%
3	G-protein signaling_H-RAS regulation pathway	1,383E-02	2	37	31%
4	Development_Delta-type opioid receptor mediated cardioprotection	1,383E-02	2	37	31%
5	Apoptosis and survival_BAD phosphorylation	1,761E-02	2	42	31%
6	Development_EPO-induced PI3K/AKT pathway and Ca(2+) influx	1,841E-02	2	43	31%
7	Transcription_CREB pathway	1,923E-02	2	44	31%
8	Immune response_Inhibitory action of Lipoxins on pro-inflammatory TNF-alpha signaling	2,007E-02	2	45	31%
9	Immune response_ICOS pathway in T-helper cell	2,091E-02	2	46	31%
10	Development_TGF-beta-dependent induction of EMT via RhoA, PI3K and ILK.	2,091E-02	2	46	31%
11	Development_PIP3 signaling in cardiac myocytes	2,178E-02	2	47	31%
12	Development_HGF signaling pathway	2,178E-02	2	47	31%
13	ATP/ITP metabolism	2,211E-02	3	124	31%
14	Development_IGF-1 receptor signaling	2,537E-02	2	51	31%
15	Some pathways of EMT in cancer cells	2,537E-02	2	51	31%
16	Development_Role of HDAC and calcium/calmodulin-dependent kinase (CaMK) in control of skeletal myogenesis	2,822E-02	2	54	31%
17	Immune response_TREM1 signaling pathway	3,019E-02	2	56	31%
18	Development_Gastrin in cell growth and proliferation	3,640E-02	2	62	31%
19	Immune response_CD40 signaling	3,857E-02	2	64	31%
20	Cardiac Hypertrophy_NF-AT signaling in Cardiac Hypertrophy	3,967E-02	2	65	31%
21	Cell adhesion_Role of CDK5 in cell adhesion	4,301E-02	1	9	31%
22	Hypoxia-induced EMT in cancer and fibrosis	4,301E-02	1	9	31%
23	Development_MicroRNA-dependent inhibition of EMT	4,767E-02	1	10	31%
24	Retinol metabolism	5,013E-02	2	74	31%
25	Development_NOTCH-induced EMT	8,868E-02	1	19	31%
26	wtCFTR and delta508 traffic / Clathrin coated vesicles formation (norm and CF)	8,868E-02	1	19	31%
27	Cytoskeleton remodeling_ESR1 action on cytoskeleton remodeling and cell migration	9,313E-02	1	20	31%
28	Translation_IL-2 regulation of translation	9,313E-02	1	20	31%
29	Development_FGF2-dependent induction of EMT	9,313E-02	1	20	31%
30	Atherosclerosis_Role of ZNF202 in regulation of expression of genes involved in Atherosclerosis	9,756E-02	1	21	31%
31	Cell cycle_Role of 14-3-3 proteins in cell cycle regulation	1,020E-01	1	22	31%
32	Transcription_Role of heterochromatin protein 1 (HP1) family in transcriptional silencing	1,020E-01	1	22	31%
33	Development_EGFR signaling via PIP3	1,064E-01	1	23	31%
34	G-protein signaling_Cross-talk between Ras-family GTPases	1,064E-01	1	23	31%
35	Apoptosis and survival_NO signaling in survival	1,107E-01	1	24	31%
36	Cytoskeleton remodeling_Role of PDGFs in cell migration	1,107E-01	1	24	31%
37	G-protein signaling_M-RAS regulation pathway	1,107E-01	1	24	31%
38	Development_Dopamine D2 receptor transactivation of EGFR	1,107E-01	1	24	31%
39	Immune response_IL-23 signaling pathway	1,151E-01	1	25	31%
40	G-protein signaling_R-RAS regulation pathway	1,151E-01	1	25	31%
41	Cytoskeleton remodeling_Neurofilaments	1,151E-01	1	25	31%
42	G-protein signaling_TC21 regulation pathway	1,151E-01	1	25	31%
43	G-protein signaling_K-RAS regulation pathway	1,151E-01	1	25	31%
44	Cell adhesion_Cadherin-mediated cell adhesion	1,194E-01	1	26	31%
45	Immune response_IL-10 signaling pathway	1,194E-01	1	26	31%
46	Apoptosis and survival_NGF signaling pathway	1,194E-01	1	26	31%
47	Immune response_CD137 signaling in immune cell	1,322E-01	1	29	31%
48	NGF activation of NF-kB	1,322E-01	1	29	31%
49	Apoptosis and survival_p53-dependent apoptosis	1,322E-01	1	29	31%
50	DNA damage_Brca1 as a transcription regulator	1,365E-01	1	30	31%

Supplemental Table S4. Pathway top 50 colorectal cancer-specific genes

#	Pathway	p-value	Ratio	FDR
1	Development_MAG-dependent inhibition of neurite outgrowth	1,243E-04	4 37	5%
2	Development_Notch Signaling Pathway	2,251E-04	4 43	5%
3	Development_NOTCH-induced EMT	2,989E-04	3 19	5%
4	Cell cycle_Regulation of G1/S transition (part 2)	7,744E-04	3 26	5%
5	Transcription_Role of Akt in hypoxia induced HIF1 activation	8,668E-04	3 27	5%
6	Hypoxia-induced EMT in cancer and fibrosis	1,709E-03	2 9	10%
7	Development_NOTCH1-mediated pathway for NF-KB activity modulation	1,712E-03	3 34	10%
8	Regulation of metabolism_Bile acids regulation of glucose and lipid metabolism via FXR	2,190E-03	3 37	10%
9	Development_Regulation of epithelial-to-mesenchymal transition (EMT)	1,027E-02	3 64	
10	Apoptosis and survival_Beta-2 adrenergic receptor anti-apoptotic action	1,127E-02	2 23	
11	Proteolysis_Role of Parkin in the Ubiquitin-Proteasomal Pathway	1,224E-02	2 24	
12	Cytoskeleton remodeling_Neurofilaments	1,325E-02	2 25	
13	Immune response_Antigen presentation by MHC class I	1,647E-02	2 28	
14	NGF activation of NF-kB	1,761E-02	2 29	
15	Apoptosis and survival_Granzyme B signaling	2,123E-02	2 32	
16	Apoptosis and survival_Role of CDK5 in neuronal death and survival	2,379E-02	2 34	
17	Development_Role of CDK5 in neuronal development	2,379E-02	2 34	
18	Development_TGF-beta-dependent induction of EMT via SMADs	2,512E-02	2 35	
19	Development_SSTR2 in regulation of cell proliferation	2,648E-02	2 36	
20	Cell cycle_Regulation of G1/S transition (part 1)	2,929E-02	2 38	
21	Translation_Non-genomic (rapid) action of Androgen Receptor	3,220E-02	2 40	
22	Neurophysiological process_ACM1 and ACM2 in neuronal membrane polarization	3,220E-02	2 40	
23	Development_Neurotrophin family signaling	3,220E-02	2 40	
24	Development_VEGF-family signaling	3,371E-02	2 41	
25	Apoptosis and survival_Apoptotic TNF-family pathways	3,523E-02	2 42	
26	Immune response_IL-7 signaling in B lymphocytes	3,679E-02	2 43	
27	Signal transduction_AKT signaling	3,679E-02	2 43	
28	Development_VEGF signaling and activation	3,679E-02	2 43	
29	Transcription_CREB pathway	3,837E-02	2 44	
30	Development_Ligand-independent activation of ESR1 and ESR2	3,837E-02	2 44	
31	Development_VEGF signaling via VEGFR2 - generic cascades	3,997E-02	2 45	
32	Development_Leptin signaling via PI3K-dependent pathway	4,326E-02	2 47	
33	Development_PIP3 signaling in cardiac myocytes	4,326E-02	2 47	
34	Development_Melanocyte development and pigmentation	4,664E-02	2 49	
35	Immune response_IL-13 signaling via PI3K-ERK	4,837E-02	2 50	
36	Development_IGF-1 receptor signaling	5,012E-02	2 51	
37	Some pathways of EMT in cancer cells	5,012E-02	2 51	
38	Immune response_PGE2 common pathways	5,190E-02	2 52	
39	Cell adhesion_ECM remodeling	5,190E-02	2 52	
40	PGE2 pathways in cancer	5,735E-02	2 55	
41	Immune response_CD40 signaling	7,482E-02	2 64	
42	G-protein signaling_Rap1B regulation pathway	7,504E-02	1 11	
43	CFTR folding and maturation (norm and CF)	9,453E-02	1 14	
44	Cell cycle_Nucleocytoplasmic transport of CDK/Cyclins	9,453E-02	1 14	
45	Beta-2 adrenergic-dependent CFTR expression	1,009E-01	1 15	
46	wtCFTR and deltaF508 traffic / Late endosome and Lysosome (norm and CF)	1,009E-01	1 15	
47	DNA damage_Role of SUMO in p53 regulation	1,136E-01	1 17	
48	Muscle contraction_ GPCRs in the regulation of smooth muscle tone	1,162E-01	2 83	
49	Cytoskeleton remodeling_Alpha-1A adrenergic receptor-dependent inhibition of PI3K	1,261E-01	1 19	
50	wtCFTR and delta508 traffic / Clathrin coated vesicles formation (norm and CF)	1,261E-01	1 19	

Supplemental Table S5. Pathway top 50 breast cancer-specific genes

#	Pathway	p-value	Ratio	FDR
1	Transcription_CREB pathway	9,817E-04	3 44	10%
2	Neurophysiological process_Long-term depression in cerebellum	1,345E-03	3 49	10%
3	Development_EDNRB signaling	1,426E-03	3 50	10%
4	Muscle contraction_Regulation of eNOS activity in cardiomyocytes	1,783E-03	3 54	10%
5	Transcription_Role of heterochromatin protein 1 (HP1) family in transcriptional silencing	4,310E-03	2 22	
6	Development_ERK5 in cell proliferation and neuronal survival	4,707E-03	2 23	
7	Neurophysiological process_NMDA-dependent postsynaptic long-term potentiation in CA1 hippocampal neurons	5,449E-03	3 80	
8	Cell cycle_Role of Nek in cell cycle regulation	8,997E-03	2 32	
9	Muscle contraction_Role of kappa-type opioid receptor in heart	9,551E-03	2 33	
10	Immune response_Role of the Membrane attack complex in cell survival	1,012E-02	2 34	
11	Development_Activation of ERK by Kappa-type opioid receptor	1,130E-02	2 36	
12	Development_PACAP signaling in neural cells	1,318E-02	2 39	
13	Immune response_PIP3 signaling in B lymphocytes	1,518E-02	2 42	
14	Neurophysiological process_ACM regulation of nerve impulse	1,805E-02	2 46	
15	Immune response_MIF - the neuroendocrine-macrophage connector	1,805E-02	2 46	
16	Development_PEDF signaling	2,033E-02	2 49	
17	Signal transduction_PKA signaling	2,192E-02	2 51	
18	Development_Beta-adrenergic receptors signaling via cAMP	2,274E-02	2 52	
19	Development_Role of HDAC and calcium/calmodulin-dependent kinase (CaMK) in control of skeletal myogenesis	2,440E-02	2 54	
20	Immune response _Immunological synapse formation	2,877E-02	2 59	
21	Plasmalogen biosynthesis	3,342E-02	2 64	
22	Cardiac Hypertrophy_NF-AT signaling in Cardiac Hypertrophy	3,439E-02	2 65	
23	Reproduction_GnRH signaling	4,144E-02	2 72	
24	Transport_Rab-9 regulation pathway	4,417E-02	1 10	
25	Phosphatidylinositol metabolism	5,817E-02	2 87	
26	Transcription_Transcription factor Tubby signaling pathways	7,396E-02	1 17	
27	Protein folding_Membrane trafficking and signal transduction of G-alpha (i) heterotrimeric G-protein	8,230E-02	1 19	
28	Cell cycle_Chromosome condensation in prometaphase	9,057E-02	1 21	
29	Cell cycle_Sister chromatid cohesion	9,468E-02	1 22	
30	Development_S1P4 receptor signaling pathway	9,468E-02	1 22	
31	Glycolysis and gluconeogenesis p.3 / Human version	1,028E-01	1 24	
32	Development_GDNF signaling	1,028E-01	1 24	
33	Development_Mu-type opioid receptor signaling via Beta-arrestin	1,028E-01	1 24	
34	Glycolysis and gluconeogenesis p.3	1,028E-01	1 24	
35	Cell cycle_Initiation of mitosis	1,069E-01	1 25	
36	G-protein signaling_R-RAS regulation pathway	1,069E-01	1 25	
37	Cytoskeleton remodeling_Neurofilaments	1,069E-01	1 25	
38	G-protein signaling_TC21 regulation pathway	1,069E-01	1 25	
39	G-protein signaling_K-RAS regulation pathway	1,069E-01	1 25	
40	Neurophysiological process_GABA-A receptor life cycle	1,149E-01	1 27	
41	Development_Regulation of CDK5 in CNS	1,189E-01	1 28	
42	Development_Delta-type opioid receptor signaling via G-protein alpha-14	1,189E-01	1 28	
43	Chemotaxis_CCR4-induced leukocyte adhesion	1,269E-01	1 30	
44	Apoptosis and survival_Granzyme A signaling	1,269E-01	1 30	
45	Regulation of lipid metabolism_FXR-dependent negative-feedback regulation of bile acids concentration	1,308E-01	1 31	
46	Development_HGF-dependent inhibition of TGF-beta-induced EMT	1,348E-01	1 32	
47	Development_Angiotensin signaling via STATs	1,348E-01	1 32	
48	Cell cycle_Start of DNA replication in early S phase	1,348E-01	1 32	
49	Development_Angiotensin activation of ERK	1,387E-01	1 33	
50	G-protein signaling_N-RAS regulation pathway	1,387E-01	1 33	

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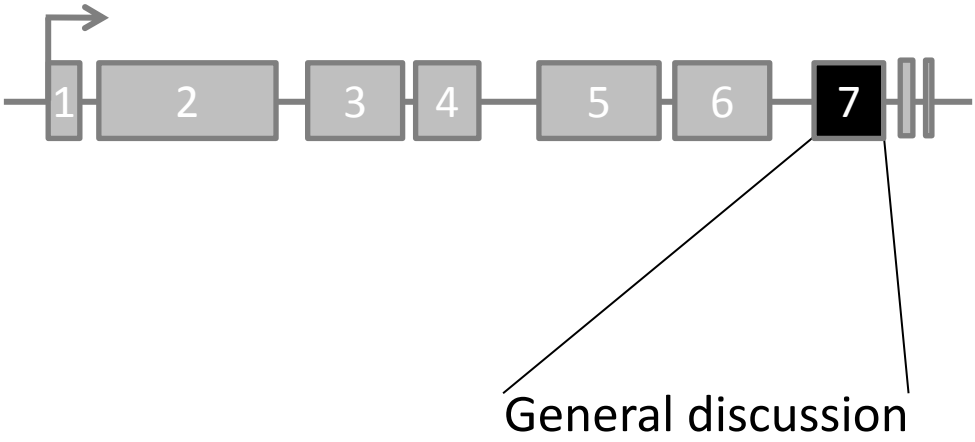
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General discussion

General discussion and future research

RCC is the most frequent malignant tumor of the kidney in adults. It has an unpredictable course and is the most lethal of all genitourinary malignancies, mostly because of a lack of clinical symptoms and diagnosis in a late stage. The classical triad (flank pain, hematuria and an abdominal mass) only occurs in about 10% of cases, and is generally indicative of advanced disease. The majority of renal tumors is asymptomatic and is detected incidentally by abdominal imaging, usually for an unrelated cause.

In the 1980s, genitourinary oncologists expressed skepticism about any success in the treatment of advanced RCC, as none of the current therapeutic options at that time could provide any clinical benefit[1, 2]. Since then, major advances have been accomplished in the field of RCC, especially in the last two decades. In 1992, the use of high-dose Interleukine-2 was approved for the treatment of advanced RCC patients. Just one year later, the *VHL* gene was identified and linked to some forms of hereditary and sporadic renal cancer[3], which, as we now know, drastically increased the knowledge on renal carcinogenesis and provided new therapeutic options for advanced RCC patients.

Developing knowledge regarding genetic and epigenetic changes in oncogenesis and tumor progression is becoming increasingly important as a prerequisite for improved efficacy of disease management. While abnormal *VHL* functioning plays a key role, the variable nature of RCC is most likely determined by the complex interplay of additional (epi)genomic modifications, among which the role of epigenetic alteration of gene expression is becoming more and more acknowledged. *VHL* promoter hypermethylation and subsequent *VHL* silencing in RCC was one of the first examples of this phenomenon[4] and so far approximately 60 genes have been suggested to be epigenetically dysregulated in RCC[5]. Our intention was to further elucidate functional DNA methylation modifications, as one of the major epigenomic players in clear cell renal cancer. This holds great promise to increase the knowledge on tumor biology which might translate into epigenetic biomarkers for early detection and diagnosis, and prediction of progression of the most common type of RCC.

As reviewed in chapter 2, RCC does not constitute one single entity since RCCs are highly heterogeneous in terms of histology, clinical behavior, genotype, prognosis and response to therapy. Therefore, subtyping of RCC is essential, specifically in basic and clinical studies, to elucidate biological characteristics and use these for marker- and therapy development for each of the RCC subtypes. Most studies are focused on the clear cell RCC as this is the most common subtype, allowing easier patient and sample collection. Other subtypes, like papillary type I and II or chromophobe RCC, are much less prevalent and therefore less frequently studied. As a result, guidelines for patient management are only available for ccRCC and the other subtypes are preferably treated in a trial and if such a trial is not available they are mostly treated with treatments initially developed for ccRCC. Nevertheless, in order to elucidate the specific characteristics of their biology study of subtypes needs to be undertaken. In this thesis, we focused on the clear cell subtype in view of the characteristics of the case series available.

In the past few years, investigators in the field of renal cancer mostly have focused, and will continue to focus, on the recently developed and approved targeted therapies. Dozens of randomized clinical trials (RCTs) have been conducted or are underway

to improve response rates. New therapeutic approaches target the high angiogenic potential of ccRCC which is a result of the abnormal functioning of the *VHL* gene. Since the discovery of *VHL* and its linkage to RCC in 1993, few additional abnormal genes or pathways have been identified in significant subgroups of (cc)RCC patients, despite recent technical developments that allow massive sequencing. Research targeting the *VHL* pathway continues, but validated biomarkers to guide decisions on treatment of RCC patients through prognostic or predictive biomarkers are painfully lacking.

The genetic profile of (cc)RCC appears distinctive compared to other epithelial carcinomas, with aberrant *VHL* signaling as a characteristic particular for ccRCC and lack of mutations in most known cancer genes. Except for the recently discovered *PBRM1* gene[6], previous exploration of the ccRCC genome has not yielded new major discoveries in the RCC biology or molecular biomarkers. Whether the epigenetic profile of RCC is also distinctive remains elusive and the epigenome has yet to be fully discovered. We decided to explore the so-called promoter hypermethylome and its role in ccRCC development and potentially clinical management of this neoplasm. In chapter 6 we initiated the comparison of the renal, colorectal and breast cancer methylomes. To this point, similar processes seem to be affected by DNA hypermethylation in cancer in general, which can be specifically regulated by several genes in different cancer types. The RCC methylome does not appear to be different in terms of size or affected processes, as analyzed thus far. Additional analyses will shed more extensive light on these issues.

The study of aberrant DNA methylation might yield novel biological insight as well as provide new biomarkers. In our initial experimental study (chapter 3), we used a pharmacological approach to indirectly identify putative tumor suppressor genes silenced by promoter CpG island hypermethylation in ccRCC. We identified the highly conserved *Gremlin1* (*GREM1*) as a candidate gene involved in the development of ccRCC. Further investigation of *GREM1* in renal cancer patients suggested that *GREM1* has characteristics of a tumor suppressor gene in ccRCC, and that region-specific promoter hypermethylation of *GREM1* has an impact on the biology and outcome of this type of cancer with good prospects for use as a prognostic biomarker. However, the details of the role of *GREM1* inactivation in tumor progression needs to be further clarified. A first indication of its role in ccRCC carcinogenesis is described in chapter 5. We found that *GREM1* attenuates the invasive behavior of the tumor cells, while cell proliferation did not seem to be affected. Expression of *GREM1* in the ccRCC cells reduced their ability to (migrate and) invade *in vitro*. Silencing of *GREM1* by promoter CpG island hypermethylation therefore might induce invasiveness and thus be involved in the progression of the ccRCC.

The observation that region-specific hypermethylation correlates with clinical parameters and prognosis for *GREM1* suggests an extra layer of complexity in DNA methylation research. In order to elucidate the general importance of this phenomenon, we evaluated the current literature on the effects of location-specific DNA methylation on gene expression and/or associations with clinicopathological characteristics in cancer (chapter 4). The exact location of biologically and clinically relevant hypermethylation has not been identified for the majority of methylation markers, as the choice of the region to be studied was based upon considerations of primer design rather than biological relevance of the region. For validation and relevant interpretation of results,

and for thoughtful design of future DNA methylation analyses, the classical dogma of promoter CpG island hypermethylation and gene silencing needs to be revised and detailed. In chapter 4 we clarify the misconception and oversimplification of hypermethylation and gene silencing in cancer, in an attempt to better understand the complexity and significance of location in DNA methylation. We expect that this approach will ultimately lead to accurate identification of biologically and clinically relevant location of DNA methylation and will enable translation of data into validated biomarker assays.

Once we realized the importance of location and disposed of novel technologies to study DNA methylation genome-wide, we extended our approach to identify biologically relevant DNA methylation markers in ccRCC. In chapter 5 we integrated the indirect pharmacological re-expression approach and the novel next-generation sequencing-based method to identify biological relevant progression markers. Methylated tumor DNA pulled down by methyl-binding proteins was massively sequenced and compared with methylation enriched DNA of normal kidney cells. Using this approach of gene selection, in combination with the expression status after demethylation of tumor DNA resulted in a list of candidate genes highly probably tumor-specifically silenced by DNA hypermethylation. In addition to *GREM1*, we identified promoter CpG island hypermethylation of *Neuralized* (*NEURL*), *Ladinin1* (*LAD1*), and *Neurofilament heavy polypeptide* (*NEFH*) as potential biomarkers with prognostic value in ccRCC. Because of the known role of the Notch pathway in embryogenesis and nephrogenesis[7], we investigated the role of *NEURL* (which is a relative minimal unraveled member of the Notch-pathway) in ccRCC, as we had done for *GREM1* which is also known for its role in nephrogenesis[8, 9]. Using several methods to assess tumor growth we found that expression of *NEURL* in ccRCC cells significantly reduced cell proliferation *in vitro* (chapter 5), but did not modify migration or invasion of the cells. Silencing of *NEURL* by promoter CpG island hypermethylation might stimulate proliferation and thus induce tumor growth, explaining the correlation with poor prognosis. The combined results of tumor suppressive activity of *GREM1* (affecting (migration and) invasion) and *NEURL* (affecting proliferation) in ccRCC cells suggests a complementary role for these genes in tumor progression. Indeed, survival of ccRCC patients with both *GREM1* and *NEURL* promoter hypermethylation in the tumor was significantly worse as compared to ccRCC patients without or with only one of the gene promoters methylated. Similarly, the risk of cancer-related death was increased in case of hypermethylation of both promoters. This suggests that the simultaneous hypermethylation of multiple promoters has a synergistic effect on tumor progression. Hypermethylation of both gene promoters was independent of *VHL* mutation or hypermethylation, suggesting that VHL abnormalities can initiate neoplastic transformation, but additional aberrations are responsible for growth and further progression. Accumulation of disrupted pathways in the tumor can enhance the neoplastic propensity and create a microenvironment beneficial for the tumor to grow and metastasize. Furthermore, pathways could integrate downstream to regulate other or additional genes that enhance tumor progression. Although both Notch and the BMP signaling pathways are important in embryogenesis, details about the mechanisms involved and possible interactions between Notch and BMP signaling need to be clarified. In many cases the two pathways act similarly, but it is not known whether this is by distinct mechanisms or by interplay

between Notch and BMP signaling. Few papers reported a cross-talk in downstream signaling and both synergy and antagonism has been described between these pathways[10-12]. It is an emerging concept that cellular behavior not only depends on the effect of a particular signaling pathway, but possibly also on the integration of signals from multiple pathways. This enables the cell to respond to a more complex repertoire of signals, and to integrate this information into the large variety of responses a cell can elaborate. Our analyses in chapter 6 suggest a novel path connecting epigenetic (indirect) regulation of the PI3K pathway and/or its cross-talk to other signaling pathways with epithelial to mesenchymal transition (EMT), which has not been described before. The top 50 of pathways with genes specifically regulated by promoter hypermethylation in ccRCC, represented enrichment for genes in or linked to PI3K pathway, implicating cross-talk of the PI3K/Akt pathway with other pathways relevant to ccRCC. One of these connections may result in EMT[13, 14], which is one of the characteristics of ccRCC[15]. The PI3K/Akt pathway has been emerged as a central feature of EMT and repression of the E-cadherin gene (*CDH1*) is one of its major characteristics[13]. This finding needs to be unraveled in the future.

Possible roles of *LAD1* and *NEFH* in RCC carcinogenesis or progression remain to be investigated. The neurofilament heavy chain encoded by *NEFH* is known to be one of the major components of the neuronal cytoskeleton neurofilaments[16] and the relatively unknown protein *Ladinin1* encoded by the *LAD1* gene is an anchoring filament that is a component of basement membranes[17]. These genes may contribute to cell structure and stability and/or the association with the surrounding mesenchyme. EMT has been suggested to be involved in progressed renal cancer via several processes[15, 18-20]. Whether EMT as a putative consequence of loss of *LAD1* and/or *NEFH* could be involved in RCC progression needs to be explored further. Together with the induction of proliferation, migration and invasion by *NEURL* and *GREM1* promoter hypermethylation, respectively, hypermethylation of *LAD1* and *NEFH* might modify cell characteristics to stimulate motility. Combined, these pathways then enable the tumor to progress and metastasize with profound effects on patient survival.

LAD1 and *NEFH* promoter hypermethylation indeed strongly amplified the association of *GREM1* and *NEURL* promoter hypermethylation with prognosis. Hypermethylation of all four genes defines a highly significant prognostic event which is potentially clinically applicable for the management of ccRCC. As two-third of the ccRCC patients present without metastasis, these will undergo resection of the primary tumor with curative intention. Half of these patients will relapse, but currently there is no indicator that can predict how an individual patient will fare after surgery. Using the four marker panel, we might be able to divide ccRCC in different molecular diseases. This segmentation will make it possible to select those patients who have a strongly increased risk to cancer-related death, probably due to the existence of undetectable micrometastases. This simple molecular test could select those patients which are likely to benefit from adjuvant (targeted) therapy, improving renal cancer management. The significance of the four marker panel in daily clinical practice needs to be validated in tumor material from a prospectively collected cohort of ccRCC patients after surgery and subsequently in a large RCT.

Other potential biomarkers, related to the *VHL* pathway (*VHL*, *HIF*, *VEGF(R)*, and *CAIX*) or the *mTOR* pathway (*pS6*, *PTEN*, and *pAKT*), have been studied for their prognostic or

predictive value but all lack clinical impact and have not contributed to current clinical decision-making[21]. This is not only observed in renal cancer biomarker research but is a general phenomenon. Despite hundreds of reports on tumor markers, the number of markers that have emerged as clinically useful is small. Initial studies often show great promise, but inconsistent data of subsequent studies contradict the promising results. It is challenging to face the obstacles in translational research. Some researchers suggest that most published research findings are false, due to poor study design, which is sometimes unavoidable[22]. Methodological, biological and pathological hurdles hinder successful biomarker identification and clinical implementation. A robust biomarker should be specific, biologically relevant and expressed homogeneously. Firstly, the study design should include adequate and appropriate controls, sample collection, storage, and manipulation must be standardized, and statistical limitations by an underpowered study should be avoided. Furthermore, initial findings should be validated in independent cohorts or subsequent studies. The National Cancer Institute-European Organization for Research and Treatment of Cancer (NCI-EORTC) reported guidelines which have been written to enable uniform design and reporting of biomarker studies; the so-called REMARK (REporting recommendations for tumor MARKer prognostic studies)[23].

Secondly, newly developed predictive or prognostic assays will need to be multiparametric, as tumor biology often relies on complex signaling pathways with extensive crosstalk and feedback and -forward control.

Thirdly, heterogeneity must be taken into account. It is quite clear that various histological subtypes are very different oncological entities. However, there is also inter- and intra-tumor heterogeneity within one subtype. Tumors from different patients with similar pathological stage and grade can be very heterogeneous and patients with similar presentations can have very different molecular tumor profiles at genomic or transcriptomic level[24, 25]. Within one tumor morphological heterogeneity is also observed. There are areas of dedifferentiation or sarcomatoid changes, but also more subtle areas of morphological variability can occur, which can lead to sampling bias.

Promoter hypermethylation of the four genes we identified in the clear cell subtype of RCC could be a robust prognostic biomarker panel for ccRCC patients. Methylation of these genes is highly tumor specific, expected to be biologically relevant and because of the high-sensitivity of the methylation specific PCR to assess the methylation status, part of the heterogeneity problem becomes irrelevant. Furthermore, whereas a mutation spectrum causing tumor suppressor gene (TSG) inactivation is usually diverse (limiting the utility of mutation-specific detection strategies for tumor screening programs), TSG inactivation by promoter hypermethylation provides a more homogeneous target for molecular screening strategies. Though, sampling error cannot be completely prevented and must always be taken into account in biomarker research. As our initial findings could be validated in an independent population-based cohort and the four genes are involved in various pathways, it is feasible that this panel could succeed into clinical practice. Furthermore, Andre et al. suggested a molecular triage program for biology-driven clinical trials which should fulfill some criteria: 1) the test should be accurate and easy to implement, 2) molecular characterization of several molecular alterations, 3) molecular screening should be independent of the screening phase of the clinical trial, and finally high-throughput technologies are beneficial to

identify such molecular events[26]. Again, our identified four-marker panel meets these criteria, suggesting a robust selection tool.

In current practice, the Leibovich risk model[27] can be used to predict disease progression to some extent after patients undergo radical nephrectomy for clinically localized ccRCC. This model represents a scoring algorithm based on tumor stage, regional lymph node status, tumor size, nuclear grade, and histological tumor necrosis and can be used to stratify patients for adjuvant therapy. However, this model just broadly stratifies patients in a low, intermediate, or high risk group.
The currently available systemic therapies for ccRCC, such as the tyrosine kinase inhibitors (TKIs) sorafenib, sunitinib, and pazopanib, the anti-VEGF monoclonal antibody bevacizumab (in combination with IFNα), and the mTOR inhibitors temsirolimus, everolimus are mostly used for treatment of patients with advanced disease. These palliative treatments elicit clinical benefit (stable disease or response) up to about 50%. As so far predicting factors remain elusive, the different systemic therapies are used in a 'one shoe fit all'-based manner. Therefore, patients are often exposed to multiple treatments. Systemic RCC treatment is accompanied by (sometimes severe) toxicity. In VEGF-directed targeted therapy adverse effects like diarrhea, fatigue, nausea, edema, hypertension, hand-foot syndrome, hematological abnormalities or hypothyroidism are being reported[28-30]. Due to these adverse events a dose reduction is required in 35-50%[31-37] of the patients and discontinuation of the treatment is necessary in 15%[32, 35] of the patients. Non-responding patients encounter a reduction in quality of life without compensation of disease stabilization, partial or complete response or improvement in terms of progression-free or overall survival.
Furthermore, TKIs used in mRCC also target a number of tyrosine kinases that are not specifically associated with angiogenesis. The importance of these 'off target' effects in the efficacy and toxicity of the drug are unclear[38].
Patient- and/or tumor heterogeneity and 'off target' effect might explain the diverse responses to therapy observed in patients with apparently similar tumors. This illustrates the urge to identify biomarkers of response and resistance as well as toxicity to RCC therapy. These will enable identification of those patients who will benefit from specific targeted therapy, thus giving tailored or personalized treatment. The proof of concept for prognostic marker identification using the novel MBD-NGS puts forward a promising tool to identify predictive markers, analyzing tumor tissue from responding- versus non-responding (to a specific therapy) patients.

In conclusion, over the last 20 years researchers have initiated a change in the paradigm of RCC as a disease that was perceived as resistant to any systemic treatment and had very poor outcomes. However, despite great improvements, renal cancer management as currently implemented in the year 2011 needs to be brought to the next level. Exploring the tumor biology will facilitate a basis to indentify biologically and clinically relevant biomarkers. Adequate biomarkers should guide the medical oncologist in decision-making for subsequent treatment after nephrectomy. Specific tumor markers, like our identified four marker panel of promoter methylated genes as described in chapter 5, can predict patient outcome and patients with a high risk of recurrence or relapse should be selected for adjuvant therapy based on additional mark-

ers (or marker panels) to predict response and treatment toxicity in a personalized manner. Integration of somatic mutations and/or aberrant DNA methylation and genetic variances could contribute to individualized management of RCC. This thesis provides novel insights in the biology of ccRCC and identified promising biomarkers that predict prognosis of ccRCC. Future *in vitro*-, *in vivo*- and marker validation studies are required to confirm the tumor suppressor function of the identified candidates, the biological role of these markers and their potential as biomarkers in ccRCC.

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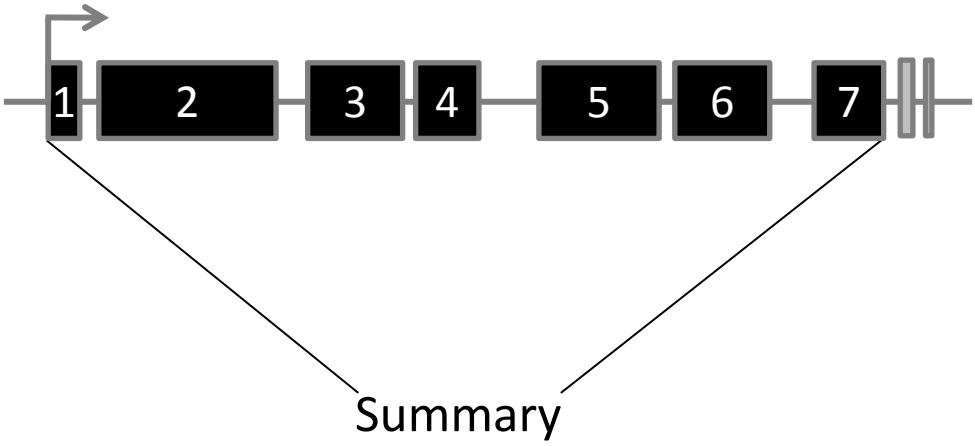
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Summary

Renal cell cancer (RCC) represents the vast majority (80-85%) of all kidney tumors in adults. Although it is the most common and best studied type of kidney cancer, it still has an unpredictable course. In case of localized disease, RCC can be curable with surgery, but for those patients with distant metastases the prognosis is poor. Because of lack of clinical symptoms RCC may be difficult to detect in an early stage. Approximately 30% of all RCC patients have metastatic disease at time of diagnosis and in another 30% of patients recurrence develops after complete resection of the primary tumor. Current surgical and pharmaceutical interventions have limited success in treating patients with advanced RCC. Thanks to improved understanding of the molecular biology of metastatic RCC (mRCC), since 2005, drugs have been developed specifically targeting angiogenesis. Developing knowledge of genetic and epigenetic changes implicated in tumor development and behavior is becoming increasingly important for advancing the efficacy of disease management. **Chapter 1** enlightens the current situation in RCC management.

As the biology of RCC is incompletely understood and biomarkers for RCC are lacking, our main aim of this thesis was to address these issues. Defective VHL functioning appears to be the key event, in both hereditary and sporadic cases. However, the variable nature of the resulting neoplasm is most likely strongly determined by the complex interplay of additional downstream modifications, among which the role of epigenetic alteration of gene expression is becoming more and more acknowledged.
In **chapter 2** we provide an overview of RCC, which comprises a group of tumors of renal epithelial origin, each with a different histology, displaying a different clinical course and caused by different genetic alterations. The histologically classified clear cell subtype (ccRCC) is the most frequent form (60-80%) of RCC followed by papillary type I and type II (pRCC, 10-15%) and the chromophobe subtype (chrRCC, 5-10%). The background of RCC, as well as the genetics, epigenetics and the clinical applications are discussed.
Elucidation of epigenetic modifications in RCC holds great promise for novel insights in biology and identification of biomarkers. DNA methylation is the best known epigenetic alteration with promising biomarker potential. Substantial proof of principle for the clinical value of hypermethylation markers in cancer has been reported, with some already having demonstrated their importance in (pre)clinical practice. We hypothesized that genome-wide studying the DNA methylation changes in the most common type of RCC, the so-called ccRCC methylome, will gain insight into ccRCC biology and identify methylation marks that are associated with prognosis of RCC patients and can be developed into clinically useful disease markers. Using state of the art technologies to analyze tumor specific DNA methylation in ccRCC, we identified promoter hypermethylation of candidate tumor suppressor genes as described **chapter 3 and 5**. The four identified genes, *Gremlin1* (*GREM1*), *Ladinin1* (*LAD1*), *neurofilament heavy polypeptide* (*NEFH*), and *Neuralized* (*NEURL*), of which promoter CpG island hypermethylation was strongly predictive for ccRCC survival in two independent series (n=150 and n=185) of ccRCC primary samples, also indicated an increased prognostic effect when methylated simultaneously. The four markers combined are strongly associated with risk for cancer-related death in the test series as well as, independently of other clinicopathological characteristics, in the validation series. This results in a promising methylation

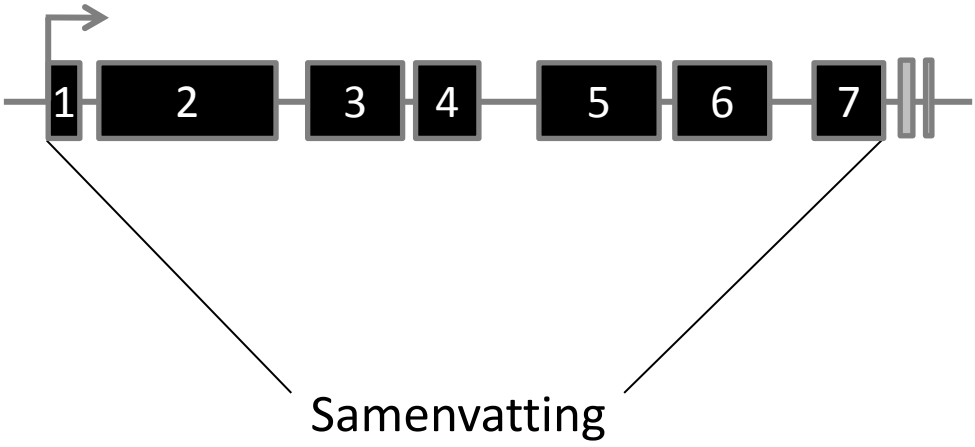
marker panel to predict prognosis of ccRCC patients after surgery. Identification of this group of high risk patients could direct adjuvant treatment trials to patients with subclinical metastatic disease to improve disease management and increase RCC survival. Besides the prognostic effects of these genes, we also investigated their functional role in ccRCC *in vitro* (**chapter 5**), as robust biomarkers should be specific and biologically relevant. Biological assessment of *GREM1* and *NEURL* in ccRCC cells suggested that these genes have complementary tumor suppressor activity in RCC. *NEURL* inhibited colony-formation and cell proliferation whereas *GREM1* inhibited migration and invasion. Biological assessment of *LAD1* and *NEFH* in RCC cells gave ambiguous results about the effect on proliferation, migration or invasion *in vitro*, providing only weak evidence for a tumor suppressor effect when compared to the data for *GREM1* and *NEURL*. Therefore we should be careful in drawing conclusions from these latter data.

Comprehensively studying the promoter methylation pattern of *GREM1* as described in **chapter 3** showed that only region-specific hypermethylation correlates with clinical parameters and prognosis. This suggested an important extra layer of complexity in DNA methylation research. In order to elucidate the general importance of this phenomenon, we evaluated the current literature on the effects of location-specific DNA methylation on gene expression and/or associations with clinicopathological characteristics in cancer. In **chapter 4** we clarify the misconception and oversimplification of hypermethylation and gene silencing in cancer, in an attempt to better understand the complexity and significance of location in DNA methylation. We expect that this approach will ultimately lead to accurate identification of biologically and clinically relevant location of DNA methylation and will enable translation of data into validated biomarker assays.

In the final experimental study of this thesis (**chapter 6**) we initiated to unravel the question whether the ccRCC methylome is distinctive from the methylomes of other epithelial cancers, as the genetic profile of (cc)RCC appears to be rather unique. Using the novel technologies as described in chapter 5 we compared the renal cell, colorectal and breast cancer methylomes. The ccRCC methylome does not appear to be different in terms of size or affected processes, as analyzed thus far. We identified conserved targeting of critical pathways through unique DNA hypermethylated events in the three analyzed cancer types. Additional analyses will shed more extensive light on these issues, however these initial analyses appeared powerful and permits the identification of genes and pathways that may not be easily detected by other approaches.

Chapter 7 summarizes the major findings of the presented studies and future directions are discussed. In conclusion, this thesis provides an overview of the current knowledge on RCC genetics and epigenetics with its implications for disease management. Despite great improvements, renal cancer management urgently needs to be brought to the next level. The work herein described provides novel insights in DNA methylation analyses, in the biology of ccRCC and identified promising biomarkers that predict prognosis of ccRCC. Future *in vitro*-, *in vivo* and marker validation studies are required to confirm the tumor suppressor function of the identified candidates, the biological role of these markers and their potential as biomarkers in ccRCC. The ulti-

mate challenge will be to truly personalize RCC treatment using markers to predict not only prognosis but also response as well as toxicity to therapy.



Samenvatting

Niercelkanker is bij volwassenen de meest voorkomende (80-85%) tumorsoort van de nier. Hoewel deze vorm van nierkanker het meest bestudeerd is, blijft het verloop onvoorspelbaar. Wanneer de ziekte beperkt blijft tot de nier zelf kan het genezen worden door chirurgie. Echter, patiënten met gemetastaseerde ziekte hebben een slechte prognose. Doordat klinische symptomen vaak afwezig zijn is het moeilijk om niercelkanker in een vroeg stadium te ontdekken. Ongeveer 30% van alle niercelkankerpatienten hebben al metastases op het moment van diagnose en daarnaast zal nog eens 30% van de patiënten recidiveren nadat de gehele primaire tumor chirurgisch verwijderd is. De huidige chirurgische en farmaceutische behandelingen zijn niet erg succesvol bij de behandeling van niercelkanker in een (ver)gevorderd stadium. Echter, dankzij recentelijk nieuwe inzichten in de moleculaire biologie van niercelkanker zijn sinds 2005 nieuwe therapieën ontwikkeld, die specifiek de vorming van nieuwe bloedvaten in de tumor remmen en daarmee de groei van de tumor belemmeren. Nieuwe ontwikkelingen op het gebied van de kennis over genetische en epigenetische veranderingen die van belang zijn bij het ontstaan en het gedrag van kanker worden steeds belangrijker voor het verbeteren van de behandeling van tumoren. Een uiteenzetting van de huidige situatie met betrekking tot de behandeling van niercelkanker is beschreven in **hoofdstuk 1** van dit proefschrift.

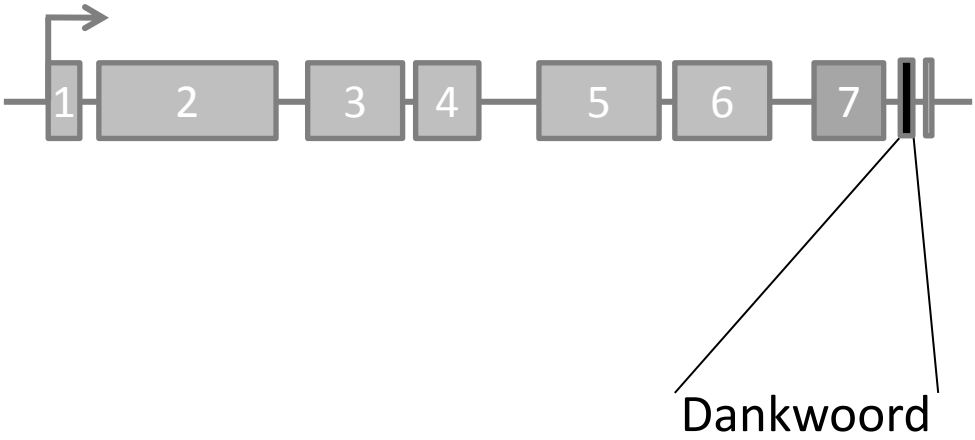
De biologie van niercelkanker is nog niet volledig bekend en momenteel zijn er nog geen biomarkers beschikbaar om het beloop van de ziekte te voorspellen. De belangrijkste doelstelling van dit proefschrift was dan ook om dit te onderzoeken. Een verminderde of defecte functie van het VHL eiwit blijkt een sleutelrol te spelen in het ontstaan van zowel de erfelijke als de niet-erfelijke vorm van niercelkanker. Echter, het wisselend verloop van de ziekte bij patiënten die allen tumoren hebben die zijn ontstaan nadat VHL is uitgeschakeld, geeft aan dat een complex samenspel van bijkomende veranderingen gaande is. Meer en meer wordt hierbij een rol van de epigenetica erkend.
In **hoofdstuk 2** wordt een overzicht gegeven van niercelkanker, dat uit een groep van tumoren bestaat die allen afkomstig zijn van het nierepitheel. Elke afzonderlijke groep heeft een andere histologie, heeft een ander beloop van de ziekte en wordt door verschillende genetische afwijkingen veroorzaakt. Het histologisch geclassificeerde heldercellig niercelcarcinoom is het meest voorkomende type niercelkanker, gevolgd door het papillaire type I en type II en het chromofobe type van niercelkanker. De achtergrond van niercelkanker, de genetica en de epigenetica wordt beschreven en de klinische toepasbaarheid hiervan wordt bediscussieerd.
Veelbelovende nieuwe inzichten in de epigenetische afwijkingen van niercelkanker kan de biologische achtergrond verduidelijken en kan leiden tot de identificatie van biomarkers. Afwijkingen in DNA methylering is de meest bekende en onderzochte epigenetische verandering in kanker en heeft veelbelovende mogelijkheden om als biomarker te dienen. Methylering van de promoter van een gen kan leiden tot uitschakeling van dit specifieke gen. Bevestiging van de theorie dat deze kenmerken in kanker van klinisch belang kunnen zijn is al uitgebreid beschreven in de literatuur en een aantal worden zelfs al in de kliniek gebruikt. Wij veronderstellen dat het bestuderen van alle veranderingen aan het DNA door methylering in het heldercellig niercelcarcinoom, het zogenaamde methyloom, zal zorgen voor een beter inzicht in de tumorbiologie en voor

het ontdekken van kenmerken die geassocieerd zijn met prognose van niercelkankerpatiënten. Gebruikmakend van de nieuwste technologieën om tumorspecifieke DNA methylering in niercelkanker te analyseren hebben we genen ontdekt die specifieke methylering vertonen in de promoteren van deze genen, die mogelijk ook een tumoronderdrukkende functie bezitten. De identificatie van deze genen is beschreven in **hoofdstuk 3 en 5**. Van de vier genen die op deze manier ontdekt zijn, *Gremlin1* (*GREM1*), *Ladinin1* (*LAD1*), *neurofilament heavy polypeptide* (*NEFH*), and *Neuralized* (*NEURL*), heeft promoter methylering een sterk voorspellende waarde voor de overleving van nierkankerpatiënten. Dit is bewezen in twee grote onafhankelijke groepen (n=150 en n=185) van primair nierkankerweefsel. Wanneer de promotoren van deze vier genen tegelijkertijd gemethyleerd zijn hebben patiënten een sterk verhoogd risico om aan de ziekte te overlijden. Dit is zowel in de eerste groep als ook, onafhankelijk van andere klinische en pathologische tumorkenmerken, in de tweede groep gevonden. Het resultaat is een veelbelovend methylerings-marker panel dat de prognose van niercelkankerpatiënten voorspelt nadat deze chirurgie hebben ondergaan. Het herkennen van deze groep patiënten met een hoog risico op overlijden kan bijdragen aan de ontwikkeling van nieuwe onderzoeken met patiënten die na de chirurgische ingreep adjuvant behandeld worden om de overlevingskansen te vergroten.
Aangezien we aannemen dat goede biomarkers ook biologisch relevant zijn, hebben we de functionele rol bestudeerd van deze genen in het heldercellig niercelcarcinoom (**hoofdstuk 5**). Deze data suggereren een complementaire tumoronderdrukkende rol van *NEURL* en *GREM1*. *NEURL* zorgt voor het remmen van de tumorgroei en *GREM1* zorgt ervoor dat de tumorcellen minder goed zullen migreren en invaderen. Het uitschakelen van deze genen in de tumor door bijvoorbeeld promoter methylering zorgt er dus voor dat de tumor kan groeien en zich kan verspreiden. Het bestuderen van de biologische functie van *LAD1* en *NEFH* in cellen van het heldercellig niercelcarcinoom gaf geen eenduidig resultaat betreffende hun rol in tumorgroei en/of verspreiding. Hierdoor kunnen momenteel geen duidelijke conclusies getrokken worden met betrekking tot hun functie in het heldercellig niercelcarcinoom.

Het uitgebreid bestuderen van de promoter methylering van het gen *GREM1*, zoals beschreven in **hoofdstuk 3**, laat zien dat regiospecifieke methylering van belang is bij de associatie met klinische parameters en prognose. Deze belangrijke observatie suggereert dat onderzoek doen naar methylering complexer blijkt dan voorheen gedacht. Om het belang van deze bevinding beter te bestuderen, hebben we de bestaande literatuur doorzocht op soortgelijke effecten van locatiespecifieke DNA methylering met betrekking tot genexpressie en/of associaties met klinische en pathologische kenmerken van kanker. In **hoofdstuk 4** verklaren we de misvatting en het versimpelen van de link tussen methylering en het onderdrukken van de genexpressie, zoals die in de huidige literatuur vaak aanwezig is. Hierdoor willen we het begrip van de complexiteit verbeteren en het belang van locatie in onderzoek naar methylering toelichten en benadrukken. Uiteindelijk zal dit leiden tot zeer accurate identificatie van biologisch en klinisch relevante locaties van DNA methylering, wat deze data geschikt maakt voor gebruik in de kliniek.

In het laatste experimentele onderzoek, beschreven in **hoofdstuk 6**, willen we analyseren of het methyloom van het heldercellig niercelcarcinoom verschilt van het methyloom van andere epitheliale kankersoorten, daar het genetische profiel van het heldercellig niercelcarcinoom uniek blijkt te zijn ten opzichte van andere tumoren. Gebruikmakend van dezelfde technologieën als beschreven in hoofdstuk 5, vergelijken we het methyloom van het heldercellig niercelcarcinoom met dat van dikke darm- en endeldarmkanker en borstkanker. Zover we op dit moment kunnen concluderen, blijkt dat deze methylomen niet van elkaar verschillen wat betreft grootte of de processen waar ze bij betrokken zijn. We hebben belangrijke biologische cascades gevonden die in alle drie de kankersoorten aangedaan worden, echter door unieke veranderingen. Bijkomende analyses zullen nog gedaan moeten worden om dit te verduidelijken, maar uit deze eerste analyses zijn al diverse ziekte-gerelateerde genen geïdentificeerd.

Hoofdstuk 7 vat de belangrijkste bevindingen uit de gepresenteerde studies samen en bespreekt hoe deze data in de toekomst gebruikt kunnen worden in de klinische praktijk. Concluderend geeft dit proefschrift een overzicht van de huidige kennis betreffende de genetica en de epigenetica van het niercelcarcinoom en daarbij de toepassingen zoals die momenteel in de kliniek beschikbaar zijn. Ondanks de wetenschappelijke vooruitgang de afgelopen jaren blijft de behandeling van niercelkankerpatiënten verre van volmaakt en is er veel ruimte voor verbetering. Het werk beschreven in dit proefschrift biedt nieuwe inzichten in de manier van het verrichten van DNA methyleringonderzoek, in de biologie van het heldercellig niercelcarcinoom en heeft geleid tot de identificatie van veelbelovende biomarkers die de prognose van patiënten met een heldercellig niercelcarcinoom kunnen voorspellen. In de toekomst zullen deze markers *in vitro* en *in vivo* gevalideerd dienen te worden teneinde hun mogelijke tumoronderdrukkende rol alsook hun waarde voor de kliniek te bevestigen. Het blijft een uitdaging om een volledig individueel behandelingstraject compleet afgestemd op het (epi)genoom van de patiënt te realiseren om niet alleen de prognose te kunnen voorspellen, maar ook de effectiviteit van de behandeling evenals de te verwachte ernst van de bijwerkingen.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Dankwoord

'Opeens' ben je zomaar aan het eind van je promotietraject, dan kijk je terug en dan besef je hoeveel mensen hier direct, indirect, links of rechts bij betrokken waren (en zijn). Nu is het de hoogste tijd om iedereen hiervoor te bedanken!

Uiteraard wil ik eerst het woord richten aan mijn gehele vrouwelijke promotieteam:
Beste promotor Prof. van Engeland, beste Manon, jij hebt mij alle kansen gegeven en altijd een weg gevonden om een 'echt' AIO-bestaan voor mij te creëren.
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Beste co-promotor Dr. Baldewijns, beste Marcella, ik mocht bij jou beginnen als student, werd je paranimf en ben nu jouw promovenda. Fijn dat jij mijn hele weg hebt bijgestaan! Bedankt en alle geluk voor de toekomst!

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Dear Prof. Herman, dear Jim, thanks for the opportunity to start my project in your lab! Thanks for staying in touch with the project and for your great input, so valuable! Of course also many thanks to all other 'Hopkins-people': Prof. Baylin, Kornel, Nita, Jana, Joo Mi, Marilia, Sabine and Kathy.

Veerle, Kim, Hanneke en Jürgen; de post-docs. Naast wetenschappelijke dank, ontzettend bedankt voor jullie gezelligheid en luisterend oor! We hebben heel wat leuke momenten gehad en heel wat afgekletst. Ik zal het niet snel vergeten!
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Natuurlijk zijn er buiten het werk ook heel wat mensen die een woord van dank verdienen op deze pagina's. Waar te beginnen?? → SAAI en SAAI+ zonder I….. dit heeft geen uitleg nodig. Heerlijk om alles van elkaar zonder blikken of blozen te begrijpen en om te klagen over die kleine teen die je gestoten hebt ☺ ! Simone en Daan, Anne en Marc, Annick en Tein en de rest van de kern van Nijmegen, Weerwolvers, (wanneer is eigenlijk weer een avondje?) BMW-ers (of overlopers), Maaike en Justus, Lieke en Sjoerd, Evelien en Rob, Koen en Marike, Willeke en Patrizio en Harm. Alles begon in Nijmegen en hoewel verspreid door heel NL, de groep blijft gewoon; bijzonder! Bedankt voor alle leuke avondjes, uitstapjes, feestjes, weekendjes, etentjes, gala's, en wat wel nog niet meer allemaal! Super! Dat nog maar veel mag volgen.

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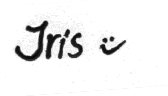

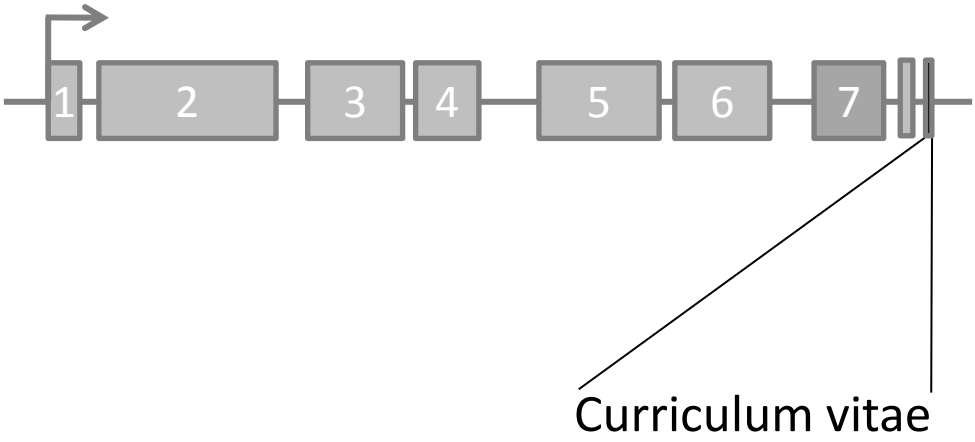
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Curriculum vitae

About the author

Iris van Vlodrop was born on February 20th 1983 in Heerlen, the Netherlands. She grew up in Landgraaf were she attended elementary school. After she graduated from secondary school (VWO, profile Nature and Health) at the Bernardinus college Heerlen in 2001, she went to the Radboud University Nijmegen to study Biomedical Sciences from 2001 until 2007. She finished her Bachelors study by completing her first internship in renal cell cancer at the Nijmegen Center for Molecular Life Sciences (NCMLS) in 2004 under supervision of Dr. Jongmans.

During her Masters study, Human Patho-biology, she completed a first internship at the department of Pathology of the Radboud University Medical Center Nijmegen, under supervision of Dr. Leenders. For her second and graduation internship, she moved back to the South of the Netherlands in 2006, where she worked at the department of Pathology of the Maastricht University Medical Center, under supervision of Dr. Baldewijns and Prof. dr. van Engeland who became her co-promotor and promotor for this dissertation. During this internship she got the opportunity to visit the laboratory of Prof. Herman of the Johns Hopkins University Baltimore, USA, which resulted in the start of her PhD-project.

In summer 2007, Iris obtained her Master's degree and was awarded with a 'Kootstra Talent Fellowship', which allowed her to continue her research in a PhD program. During her PhD studies, she worked on a project to unravel the renal cell cancer methylome for fundamental and clinical purposes, which resulted in this dissertation. In fall 2010 she was fortunate to work in the laboratory of Prof. Weidhaas at the Yale University School of Medicine to start a side-project on renal cell cancer. In December 2011 Iris started her training in Clinical Chemistry at the Albert Schweitzer Hospital in Dordrecht.

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