Biomarkers, Genomics, Proteomics, and Gene Regulation

Prognostic Significance of *Gremlin1* (*GREM1*) Promoter CpG Island Hypermethylation in Clear Cell Renal Cell Carcinoma

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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 with unmethylated ccRCCs (hazard ratio [HR] =

2.35, 95% confidence interval [CI]: 1.29 to 4.28), but not in multivariate analysis (HR = 0.88, 95% CI: 0.45 to 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 to 3.53 and HR = 2.27, 95% CI: 1.44 to 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. (Am J Pathol 2010; 176:575–584; DOI: 10.2353/ajpath.2010.090442)

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} Fifty 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

Supported by the Kootstra-Talent Fellowship Program, Maastricht University Medical Center, Maastricht, The Netherlands.

I.J.H.v.V., M.M.L.B., and K.M.S. contributed equally to this study.

Accepted for publication October 27, 2009.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

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appear to be directly associated with tumor cell proliferation and patient prognosis,^{9,10} suggesting a complex interplay of additional genetic and epigenetic changes that 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 on 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 signaling.^{11–13} BMPs, the largest subfamily of the transforming growth factor- 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 angiogenesis²³ 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 with unmarked stem cell genes, Polycomb group 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-ad-

juvant 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 and eosin-stained slides were revised by an experienced genitourinary pathologist to confirm nuclear Fuhrman grading. Tissues were sectioned into 20- μ m 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 to 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 to 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 and 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 Netherlands¹⁰ (for patient characteristics see Table 2). This study was approved by the Medical Ethical Committee of the Maastricht University Medical Center.

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. $\approx 10\%$ confluent RCC cells were cultured in RPMI 1640 media containing 10% fetal bovine serum with 5 μ mol/L (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 300 nmol/L TSA (Sigma; stock solution: 5 mmol/L dissolved in ethanol) was performed for 18 hours, starting at $\approx 30\%$ 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 μ g of total RNA was reverse transcribed by using Ready-To-Go You-Prime First-Strand Beads

Table 1. *GREM1* Promoter CpG Island Methylation Associated with Clinical, Histopathological, and Angiogenesis-Related Patient Characteristics (Hospital-Based Series)

	All ccRCC <i>n</i> = 150 (100%)	Methylated <i>GREM1</i> -i <i>n</i> = 77 (55%)	Methylated <i>GREM1</i> -ii <i>n</i> = 33 (24%)	Methylated <i>GREM1</i> -iii <i>n</i> = 28 (20%)
Sex				
Male, <i>n</i> (%)	99 (66%)	50 (65%)	22 (67%)	22 (79%)
Female, <i>n</i> (%)	51 (34%)	27 (35%)	11 (33%)	6 (21%)
<i>P</i> , 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)
<i>P</i> , 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)
<i>P</i> , U versus M		0.01	0.08	0.02
Fuhrman grade, <i>n</i> (%)				
Grade 1	15 (10%)	5 (6%)	2 (6%)	1 (4%)
Grade 2	72 (48%)	36 (47%)	15 (46%)	11 (39%)
Grade 3	46 (31%)	26 (34%)	10 (30%)	9 (32%)
Grade 4	17 (11%)	10 (13%)	6 (18%)	7 (25%)
<i>P</i> , U versus M		0.54	0.61	0.04
Tumor stage, <i>n</i> (%)				
Stage 1	8 (5%)	3 (4%)	0 (0%)	0 (0%)
Stage 2	89 (60%)	42 (54%)	17 (53%)	12 (43%)
Stage 3	30 (20%)	16 (21%)	9 (28%)	7 (25%)
Stage 4	22 (15%)	16 (21%)	6 (19%)	9 (32%)
<i>P</i> , U versus M		0.23	0.32	
ECP %, mean	1.4 (±2.17 SD)	1.8 (±2.72 SD)	2.5 (±3.37 SD)	3.0 (±2.97 SD)
<i>P</i> , 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)
<i>P</i> , 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)
<i>P</i> , U versus M		0.01	0.09	0.001

(Amersham Biosciences/GE Health care, Piscataway, NJ) with addition of random hexamers (0.2 μ g per reaction). RNA of a subset of the hospital-based series primary ccRCCs and normal renal tissue from noncancerous pa-

tients was isolated from frozen tissue using the GenElute Total RNA kit (Sigma) according to the manufacturers' instructions. For real-time RT-PCR, 1 μ g 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.)

Table 2. *GREM1* Promoter CpG Island Methylation Associated with Clinical, Histopathological, and Angiogenesis-Related Patient Characteristics (Population-Based Series)

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

Tumor stage based on International Union against Cancer (UICC) TNM classification of malignant tumors, fourth, fully revised edition, 1987.

P indicates *P* value for comparison of methylated and unmethylated cases.

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://emboss.sourceforge.net/>) 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 1A), 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 1A). *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, CA) 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

Table 3. PCR Primers and Conditions for *GREM1*

Method	Forward	Reverse	T _m (°C)	Cycles (n)	Length (bp)
RT	5'-CAGCCTACACGGTGGGAGC-3'	5'-CTGCTCTGAGTCATTGTGCTGG-3'	60	40	128
MSP i					
FI	5'-TGGYGATAGGTTTGTGTTGG-3'	5'-CCCCCTCTAAAAAACRAAACTA-3'	56	35	156
U	5'-GTTTTTGTGTTGGTGTAGTGAGTTT-3'	5'-AACAAACTACAAAATCTCTAATCAAACA-3'	62	30	134
M	5'-TGTTGGCGGTTAGCGAGTTC-3'	5'-GAAACTACGAAATACTCTAATCGAACG-3'	62	30	126
MSP ii					
FI	5'-TTTTGGGGTGATTTTTTTTAGT-3'	5'-CACCCCTCCTACTTCCCC-3'	56	35	96
U	5'-GTGATTTTTTTTAGTTGGTTTGGGT-3'	5'-ACCCCTCCTACTTCCCCACA-3'	64	35	88
M	5'-ATTTTTTTTAGTCGGTTCGGGC-3'	5'-CCCTCCTACTTCCCCGCG-3'	64	35	83
MSP iii					
FI	5'-GGGGTTTTTTTTTGTGTTAGGT-3'	5'-CTACTACCACCAACACCAAAAAC-3'	56	35	161
U	5'-TTTTTTTGTGTTAGGTTGTGGATATT-3'	5'-CAAAAACATATTCAAAAACCTCCA-3'	65	25	140
M	5'-TTTGTGAGGTGCGGATATC-3'	5'-AAAACGTATTGCGAAACCTCCG-3'	65	25	134
BSseq i	5'-TGGYGATAGGTTTGTGTTGG-3'	5'-CCCAAATCTAACTTTCCCTATC-3'	60	35	279
BSseq ii	5'-GATAGGAAAGTTTAGATTGGG-3'	5'-CACCCCTCCTACTTCCCC-3'	64	35	222
BSseq iii	5'-AGGGTYGTAAATTAATTAGGATT-3'	5'-CTACTACCACCAACACCAAAAAC-3'	60	40	268
BSseq TSS	5'-GGYGTGTTGGTTAATGGAGAGG-3'	5'-CRCCCACTCACATCCCTACC-3'	62	35	279

RT indicates 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, nonmethylation sensitive primers; BSseq i, ii, iii, TSS, bisulfite sequencing *GREM1*-region i, ii, iii, and around transcription start site.

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, 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 μ l of each MSP reaction was directly loaded onto 2% agarose gels containing GelStar Nucleic Acid Gel Stain (Cambrex, NJ), and visualized under UV illumination. The methylation status of the hospital-based ccRCC series was successfully obtained in 93% to 95% (138 to 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. Nonconcordant MSP results were analyzed a third time, and a two 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.

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; **grey 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 representative BSseq (B), MSP, **asterisk** represents clones for bisulfite sequencing of the cell lines SKRC1, SKRC10, SKRC52, and SKRC59 for region i, ii, TSS and iii. (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 indicates 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.

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/Ki-67 double immunohistochemical staining³⁴ 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, one hot-spot (most vascularized microscopic field) was selected and four areas were chosen randomly. Vessel counts were performed at $\times 200$ 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 mm². Next, a total number of at least 500 intratumoral endothelial cells and 500 tumor cells were evaluated in consecutive fields at a $\times 400$ 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 Ki-67–stained nuclei/total number of endothelial cells evaluated) $\times$ 100; TCP% = (the number of tumor cells with Ki-67–stained nuclei/total number of tumor cells evaluated) $\times$ 100.

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 *t* tests and Pearson 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. HRs and corresponding 95% 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 1C). 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 1B). As shown in Figure 1D, 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 to –462), 24% (33/139) for region ii (–236 to –140), and 20% (28/138) for region iii (+311 to +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 noncancer patients, 0 to 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 to 143) versus 64.5 (range, 2 to 153) and 58.5 (1 to 143) versus 41 months (0 to 143), respectively. The Kaplan–Meier curve for *GREM1*

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** and **D**), with **A**, **B**, and **C** for patients of the hospital-based series and **D** for patients of the population-based series.

region i methylation (Figure 2A) 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 to 3.32, but not for MFS ($HR_i = 1.08$; 95% CI, 0.76 to 1.54). Multivariate analyses, however, show no association between *GREM1* region i and OS or MFS ($HR_i = 0.66$; 95% CI, 0.33 to 1.33, and $HR_i = 0.82$; 95% CI, 0.55 to 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 to 153) versus 76 (range, 1 to 153) and 56 (1 to 153) versus 60 months (0 to 153), respectively. Figure 2B 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 to 1.80, and $HR[MFS]_{ii} = 0.74$; 95% CI, 0.49 to 1.12) nor in the multivariate analyses ($HR[OS]_{ii} = 0.55$; 95% CI, 0.26 to 1.18, and $HR[MFS]_{ii} = 0.56$; 95% CI, 0.36 to 0.87).

In contrast, ccRCCs methylated at region iii are statistically significantly 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 with 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 to 153) versus 56 (range, 2 to 138), and 60 (1 to 153) versus 23 months (0 to 138), respectively. Figure 2C 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 with 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 to 4.28); the association with MFS was borderline significant ($HR = 1.54$; 95% CI: 0.99 to 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 to 1.74, and $HR(MFS) = 1.04$, 95% CI: 0.65 to 1.68.

Because *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 with 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 to 218) versus 42 (range, 1 to 212), respectively. Figure 2D 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 with 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 to 3.53) and a multivariate HR of 2.27 (95% CI: 1.44 to 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, whereas 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 bp 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 (ie, 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 then spreads downstream toward the TSS and region iii or starts in region iii and then retroverts toward region i cannot be deduced from these data.

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

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 antiangiogenesis 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 proangiogenic 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 ($\text{Rho} = 0.82$, $P < 0.001$). 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 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 and 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.

Except for sclerostin domain-containing-1 (SOSTDC1), another BMP antagonist that 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 (ie, 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 S1, at <http://ajp.amjpathol.org>). Several explanations can explain these results. First, overall the basal expression level of *GREM1* in normal kidney is very low, an observation that 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 S1, at <http://ajp.amjpathol.org>). 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 with 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 inter-

action 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 in 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.

Acknowledgments

We thank Dr. Egbert Oosterwijk from the Nijmegen Center for Molecular Life Sciences (NCMLS), Radboud University Nijmegen Medical Center (RUNMC), Nijmegen, The Netherlands for providing the renal carcinoma cell lines. We thank Sean Smith for technical support.

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