Serum Cell-Free DNA in Renal Cell Carcinoma
A Diagnostic and Prognostic Marker

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BACKGROUND: Currently, there are no established diagnostic and prognostic serum markers for renal cell carcinoma (RCC). The objective of this study was to evaluate the putative significance of serum cell-free DNA. METHODS: Preoperative serum samples from 200 consecutive patients with sporadic, solid renal tumors were analyzed (157 patients with RCC and 43 patients with benign renal tumors). Quantitative real-time polymerase chain reaction was used to assess total cell-free DNA (ring finger protein 185 [RNF185]) and CpG island methylation of Ras association domain family member 1A (RASSF1A) von Hippel-Lindau (VHL), prostaglandin-endoperoxidase synthase 2 (PTGS2), and P16 (cyclin-dependent kinase inhibitor 2A). Associations with RCC, pathologic variables, and disease-specific survival were evaluated. RESULTS: Total cell-free DNA levels and CpG island methylation of RASSF1A and VHL were highly diagnostic for RCC with an area under the receiver operating characteristic curve of 0.755, 0.705, and 0.694, respectively. VHL methylation was detected more frequently in patients with clear cell RCC than in those with other subtypes (P = .007). Total cell-free DNA levels were higher in patients with metastatic RCC (P < .001) and necrotic RCC (P = .003) and were associated with poorer disease-specific survival (P < .001). In multivariate analysis, the tumor stage, size, grade, and necrosis (SSIGN) score (P < .001) and categorized total cell-free DNA levels (P = .028) were retained as independent prognostic factors. CONCLUSIONS: The current results indicated that cell-free DNA represents a novel serum-based diagnostic and prognostic biomarker for RCC. Total serum cell-free DNA levels and CpG island methylation of RASSF1A and VHL may be useful diagnostic biomarkers for RCC. VHL methylation of cell-free DNA is suggestive of clear cell RCC. Total serum cell-free DNA may be a useful prognostic biomarker that may assist in tailoring postoperative surveillance and therapy. External prospective validation of these data will be required. Cancer 2012;118:82-90. © 2011 American Cancer Society.

KEYWORDS: renal cell carcinoma, cell-free DNA, diagnosis, prognosis, receiver operating characteristic curve.

Urologic malignancies represent a major public health problem and a significant burden of disease. It has been estimated that more than 1 million patients are newly diagnosed with a urologic malignancy annually.1 Unlike prostate cancer and testicular cancer, in which reliable diagnostic and prognostic serum markers have been identified, currently, there are no established serum markers for renal cell carcinoma (RCC).

Cell-free DNA in the blood was detected first more than 50 years ago.2 In recent decades, a great amount of knowledge has been accumulated after polymerase chain reaction (PCR) techniques became widely available. Cell-free DNA levels in serum and plasma from patients with cancer, including RCC, have been detected, and a decrease in these levels has been demonstrated after successful therapy.3,4 It is now well established that cell-free DNA is composed in part of tumor DNA,5 which opens the opportunity to detect tumor-specific signatures, including the methylation of genes in serum from patients. Thus, in addition to the analysis of total cell-free DNA and mitochondrial DNA with quantitative real-time PCR,6,7 methylation analysis of CpG islands has been performed.8 It has been demonstrated that cell-free DNA may be a sensitive and specific marker for diagnosing prostate cancer9 and that it may predict recurrence-free survival after radical prostatectomy.6 In addition, several reports have indicated its clinical usefulness in bladder cancer9 and testicular cancer.8 In kidney cancer, only limited data exist on serum cell-free DNA. Gang et al4 studied 78 patients who underwent
surgery for clear cell RCC by conventional PCR of different glyceraldehyde 3-phosphate dehydrogenase (GAPDH) fragments. Those authors observed a different fragment distribution between patients and controls and within different RCC tumor (T) classifications. Hauser et al.\(^\text{10}\) detected higher concentrations of actin beta (ACTB) fragments in 35 patients with RCC compared with 54 controls. In total, relatively few patients were included, methylation of CpG islands was not analyzed, and associations with survival were not assessed.

Thus, the overall role of cell-free DNA as a diagnostic and prognostic RCC biomarker remains poorly understood. Therefore, the objective of the current study was to evaluate the putative significance of total cell-free DNA and CpG island methylation in diagnosing RCC and to associate these markers with pathologic variables and survival.

MATERIALS AND METHODS

**Study Population**

This prospective, institutional review board-approved study was designed to test the hypothesis that total serum cell-free DNA concentrations and cell-free DNA CpG island methylation can serve as diagnostic and prognostic biomarkers for RCC. In total, 200 consecutive patients were enrolled who underwent surgery for a unilateral, sporadic, solid renal tumor suspicious for RCC at our institution between 2008 and 2009. None of the patients had concomitant malignancy, autoimmune disease, trauma, or myocardial infarction within the last 12 months before surgery, because these diseases may impact cell-free DNA levels.\(^\text{11}\) All patients provided informed consent.

Clinical and pathologic features were obtained for each patient. Clinical features included age and sex. All pathologic features were evaluated by 1 expert uropathologist (A.H.) and included subtype according to the World Health Organization classification of tumors;\(^\text{12}\) tumor-lymph node-metastasis (TNM) classification;\(^\text{13}\) Fuhrman grade;\(^\text{14}\) the presence of microscopic coagulative tumor necrosis; and pathologic tumor size according to the greatest tumor dimension. After surgery, patients with N0M0 disease were followed according to a risk-stratified surveillance protocol\(^\text{15}\) with intermittent imaging of the chest, abdomen, and pelvis.

**Sample Collection and Isolation of Cell-Free DNA**

Whole blood was drawn from patients before surgery. Serum was obtained immediately through centrifugation at \(\times 1800\) g for 10 minutes and stored at \(-80^\circ\text{C}\) until DNA extraction. Cell-free DNA was isolated from 1 mL of serum using the QIAamp Ultrasens Virus Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. In a subgroup of 20 patients who underwent curative surgery for a clinically N0M0 tumor, serum also was obtained 4 weeks after surgery.

**Real-Time Quantitative PCR**

Real-time quantitative PCR (q-PCR) was performed to assess the total amount of cell-free DNA and CpG island methylation status of the genes Ras association domain family member 1A (RASSF1A), prostaglandin-endoperoxidase synthase 2 (PTGS2), P16 (cyclin-dependent kinase inhibitor 2A), and von Hippel-Lindau (VHL) using an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, Calif). Each sample was assayed in triplicate, and each run included water blanks and an external control.

**Determination of total cell-free DNA**

The total quantity of cell-free DNA was determined using hydrolysis probes for the ring finger protein 185 gene RNF185 (TaqMan gene expression assay RNF185; assay identification no. Hs01565008_m1 [Applied Biosystems]; amplicon length, 249 base pairs [bp]). This gene is located on chromosome 22q12, which typically is not mutated in RCC, so that concentrations in serum are not lowered by deletions in tumor DNA. We used 10 \(\mu\)L of TaqMan Universal PCR Master Mix (Applied Biosystems), 1 \(\mu\)L of Taqman Gene Expression Assay, and 30 ng of cell-free DNA, which were brought to a total volume of 20 \(\mu\)L by adding UltraPure DNase/RNase-Free Distilled Water (Invitrogen, Lofer, Austria). The PCR conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. A standard dilution curve of Human Male Genomic DNA (Novagen Merck Chemicals Ltd., Nottingham, United Kingdom) of known concentration was used to determine the copy numbers (genome equivalents [GE]) of cell-free DNA. A factor of 6.6 pg of genomic DNA was applied for conversion to GE. One GE-equivalent was defined as the amount of a specific target sequence in a single reference cell.\(^\text{16,17}\)

**Determination of CpG island methylation status**

Restriction endonuclease q-PCR\(^\text{18}\) was applied to assess CpG island methylation of RASSF1A, PTGS2, P16, and VHL. In this concept, cell-free DNA is incubated
with restriction enzymes that recognize and cut restriction sites with suspected methylation. Unmethylated DNA is cut by the enzyme, and no products are detected after PCR amplification. In contrast, the enzymes do not cut the methylated form of the sequences. To accomplish this, 50 ng of cell-free DNA solution were incubated for 4 hours at 37°C with 3 μL Buffer Tango 10X (Fermentas, St. Leon-Rot, Germany), 15 U restriction enzyme, and UltraPure DNase/RNase-Free Distilled Water up to total volume of 30 μL. The restriction enzymes were Hin6I for PTGS2 and P16, and HpaII for RASSF1A and VHL (Fermentas), which cuts GCGC and CCGG, respectively. For a control, cell-free DNA was incubated with buffer and water without restriction enzyme according to the same protocol. After incubation, q-PCR was performed. The PCR mixture contained 5 μL SYBR2X (Applied Biosystems), 3 μL water, 0.5 μL of each reverse and forward primer, and 10 μL of the digested mix. Forward and reverse primers are provided in Table 1. The q-PCR conditions were: 10 minutes at 95°C, 40 cycles for 30 seconds at 95°C and for 30 seconds at annealing temperature specific for each primer pair (Table 1), and 40 seconds at 72°C.

### Table 1. Primers Used for Amplification of the RASSF1A, PTGS2, P16, and VHL Genes to Detect Methylation After Incubation With Restriction Enzymes

| Gene   | Direction | Primer Sequence | Annealing Temperature, °C |
|--------|-----------|-----------------|---------------------------|
| RASSF1A| Forward   | 5’-GCTTGCTAGGGCCCAAG-3’ | 59                      |
|        | Reverse   | 5’-GAGCTCCGCACTCAT-3’  |                         |
| PTGS2  | Forward   | 5’-GGAAGAGAAGCCCAAGTGTC-3’ | 60                      |
|        | Reverse   | 5’-GGTTTGCCGCAAGTGCTTT-3’ |                         |
| P16    | Forward   | 5’-ACGCCGGAAGAAAGAG-3’  | 60                      |
|        | Reverse   | 5’-CTGCGTCCTCTCCCTCTC-3’ |                         |
| VHL    | Forward   | 5’-AGGAGGCTGCAAGAGT-3’  | 60                      |
|        | Reverse   | 5’-GGGCTCCATCTGCCTT-3’   |                         |

Abbreviations: A, adenine; C, cytosine; G, guanine; RASSF1A, Ras association domain family member 1A; P16, cyclin-dependent kinase inhibitor 2A; PTGS2, prostaglandin-endoperoxidase synthase 2; T, thymine; VHL, von Hippel-Lindau.

Statistical Analysis
Design and analysis of this study were performed according to reporting recommendations for tumor marker prognostic studies (ReMARK) guidelines. The chi-square test and the nonparametric Kruskal-Wallis test were used to compare categorical data and continuous data, respectively. The Wilcoxon signed-rank test was used to compare preoperative and postoperative levels of total cell-free DNA. Methylation status was coded as a categorical variable. Correlations were determined using the Pearson coefficient. The diagnostic performance (sensitivity and specificity) of serum cell-free DNA and methylated DNA was analyzed with a receiver operating characteristic curve (ROC). The area under the ROC curve (AUC) was applied to evaluate diagnostic accuracy. AUCs were compared as described previously.

Disease-specific survival was calculated from the date of surgery to the date of either death from RCC or last follow-up. Survival analyses were performed with univariate Cox proportional hazards models. For visualization of Kaplan-Meier survival estimates, a cutoff point for subcategorization was detected with recursive partitioning-based tree analysis. Because there should be at least 10 to 15 events for each variable that is included in a multivariate model to avoid over fitting, multivariate Cox models that included all pathologic variables were not fit. Instead, we used the tumor stage, size, grade, and necrosis (SSIGN) score, which incorporates TNM classifications, tumor size, nuclear grade, and tumor necrosis. The statistical package R 2.10.1 (R Foundation for Statistical Computing, Vienna, Austria; http://cran.r-project.org. [Accessed on March 1, 2011]) was used for all analyses. The level of statistical significance was set at P < .05.

RESULTS

#### Study Population

One hundred fifty-seven of 200 patients (78.5%) had RCC, and 43 patients (21.5%) had benign tumors. The mean (±standard deviation) age at the time of surgery was 64.3 ± 13.0 years (median, 65.0 years; interquartile range [IQR], 55.4-74.9 years). One hundred twenty-seven patients (63.5%) were men. The age and sex distribution between patients with RCC and benign tumors was similar (P = .333 and P = .804, respectively). Among the RCCs, 112 were clear cell (71.3%), 31 were papillary...
(19.7%), and 14 were chromophobe (8.9%). Twenty-four patients (15.3%) presented with metastatic disease. Patient characteristics are summarized in Table 2.

The mean total serum cell-free DNA level, represented by RNF185, was 2881 GE/mL (median, 1931 GE/mL; IQR, 1074-3968 GE/mL). Methylation of VHL, RASSF1A, PTGS2, and P16 was detected in 83 of 200 patients (41.5%), 75 of 200 patients (37.5%), 75 of 200 patients (37.5%), and 92 of 200 patients (46%), respectively.

**Association With RCC**

Total cell-free DNA levels were higher in patients with RCC than in patients with benign tumors (mean ± standard deviation: 3319 ± 3181 GE/mL vs 1288 ± 913 GE/mL; *P* < .001) (Fig. 1). In patients who underwent curative surgery, mean total cell-free DNA levels dropped significantly after surgery (from 3089 ± 1964 GE/mL to 1446 ± 1053 GE/mL; *P* = .003; n = 20).

Of the genes that we analyzed for methylation, VHL and RASSF1A were methylated more frequently in patients with RCC: Methylated VHL was detected in 79 patients (50.3%) with RCC but in only 4 patients (9.3%) with benign tumors (*P* < .001). Similarly, RASSF1A was methylated in 72 patients with RCC (45.9%) versus 3 patients (7%) with benign tumors (*P* < .001). P16 methylation and PTGS2 methylation were observed in similar percentages among patients with benign tumors and RCC (*P* = .788 and *P* = .689, respectively).

The AUC was 0.755 for RNF185, 0.705 for VHL methylation, 0.694 for RASSF1A methylation, 0.517 for PTGS2 methylation, and 0.512 for P16 methylation (Table 3). RNF185, VHL methylation, and RASSF1A methylation had high specificity but low sensitivity for RCC diagnosis. Comparing the different AUCs, RNF185, VHL methylation, and RASSF1A methylation had significantly higher diagnostic accuracy than PTGS2 methylation (*P* = .001, *P* = .003, and *P* = .006, respectively) and P16 methylation (*P* = .001, *P* = .003, and *P* = .005, respectively); whereas the accuracies of RNF185, VHL methylation, and RASSF1A methylation all were similar (each *P* > .2). For clear cell RCC, the AUC was 0.760 for RNF185, 0.744 for VHL methylation, and 0.711 for RASSF1A methylation.

**Association With Pathologic Variables**

RNF185 levels were higher in necrotic tumors (*P* = .003) and in patients with lymph node metastasis (*P* = .028) and distant metastasis (*P* < .001), whereas no significant

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**Table 2. Patient and Tumor Characteristics**

| Variable               | RCC, n=157 | Benign, n=43 |
|------------------------|------------|--------------|
| Sex                    | 99 (63.1)  | 28 (65.1)    |
| Men                    | 58 (36.9)  | 15 (34.9)    |
| Age: Mean±SD, y        | 64.7±12.8  | 62.5±13.6    |
| Tumor size: Mean±SD, cm| 4.5±3.1    | 3.8±2.8      |
| Tumor classification   |            |              |
| T1-T2                  | 92 (58.6)  |              |
| T3                     | 65 (41.4)  |              |
| Pathologic lymph node status | 154 (98.1) |              |
| pNx/N0                 | 3 (1.9)    |              |
| Metastasis classification|           |              |
| M0                     | 133 (84.7) |              |
| M1                     | 24 (15.3)  |              |
| Nuclear grade          |            |              |
| 1-2                    | 119 (75.8) |              |
| 3-4                    | 38 (24.2)  |              |
| Subtype                |            |              |
| Clear cell             | 112 (71.3) |              |
| Papillary              | 31 (19.7)  |              |
| Chromophobe            | 14 (8.9)   |              |
| Tumor necrosis         | 72 (45.9)  |              |
| SSIGN score            |            |              |
| 0-1                    | 58 (36.9)  |              |
| 2-5                    | 62 (39.4)  |              |
| 6-9                    | 20 (12.7)  |              |
| ≥10                    | 17 (10.8)  |              |

Abbreviations: RCC, renal cell carcinoma; SD, standard deviation; SSIGN score, tumor stage, size, grade, and necrosis score.
An association was observed with T classification \((P = .087)\) or tumor grade \((P = .101)\). In addition, there was a weak but significant correlation with tumor size \((P = .010)\).

\(\text{VHL}\) methylation status was associated with histologic subtype. In total, 65 of 112 clear cell RCCs (58%), 11 of 31 papillary RCCs (35.5%), and 3 of 14 chromophobe RCCs (21.4%) had methylated \(\text{VHL}\) detected in serum \((P = .007)\). No significant association was observed with other pathologic variables \((P > .2)\). Similarly, methylation of \(\text{RASSF1A}\), \(\text{PTGS2}\), and \(\text{P16}\) was not associated with any pathologic variable \((P > .2)\). Similar results were obtained only when clear cell RCCs were analyzed (data not shown).

**Association With Disease-Specific Survival**

The mean follow-up duration was 28 months (median, 28 months; IQR, 22-35 months), during which 24 patients (15.3%) died of RCC. There was a significant association of total cell-free DNA levels \((\text{RNF185})\) with survival \((P = .004)\); with each 1000-GE/mL increase in the level of total cell-free DNA, the risk of RCC-specific death increased by 10% \((\text{hazard ratio}, 1.10; 95\% \text{CI}, 1.03-1.17)\). In recursive partitioning-based tree analysis, the cutoff point for subcategorization \((\text{high/low})\) was 2400 GE/mL, which corresponded to the value obtained for \(\text{RNF185}\) in the diagnostic ROC curve analysis. There were 76 patients (48.4%) with high total cell-free DNA levels and 81 patients (51.6%) with low total cell-free DNA levels. The difference in survival between both groups was statistically significant \((\text{hazard ratio}, 15.03; 95\% \text{CI}, 3.52-64.21; P < .001)\) (Fig. 2). Methylation of \(\text{VHL}\), \(\text{RASSF1A}\), \(\text{PTGS2}\), and \(\text{P16}\) was not associated with survival.

In multivariate analyses, SSIGN score \((P < .001)\) and categorized total cell-free DNA levels \((P = .028)\), but not continuous total cell-free DNA levels \((P = .192)\), were identified as independent prognostic factors (Table 4).

**DISCUSSION**

In the current study, we evaluated the diagnostic and prognostic role of serum cell-free DNA concentration and methylation of \(\text{RASSF1A}, \text{VHL}, \text{PTGS2}\), and \(\text{P16}\) in RCC. Our data indicate that total cell-free DNA and methylation of \(\text{VHL}\) and \(\text{RASSF1A}\) may serve as diagnostic biomarkers for patients with RCC. Furthermore, total serum cell-free DNA appears to predict disease-specific survival.

It has been noted for decades that patients with cancer have elevated cell-free circulating DNA levels compared with controls.\(^3\)\(^10\) These elevated levels may be

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**Table 3. Diagnostic Accuracy of Serum Markers in Detecting Renal Cell Carcinoma in a Cohort With Sporadic Solid Renal Tumors**

| Marker     | AUC     | Sensitivity, % | Specificity, % | PPV, % | NPV, % | Correctly Classified, % |
|------------|---------|----------------|----------------|--------|--------|-------------------------|
| Total (\(\text{RNF185}\)) | 0.755   | 51             | 93             | 96.4   | 34.2   | 60                      |
| \(\text{VHL}\) methylation | 0.705   | 50.3           | 90.7           | 95.2   | 33.3   | 59                      |
| \(\text{RASSF1A}\) methylation | 0.694   | 45.9           | 93             | 96     | 32     | 56                      |
| \(\text{PTGS2}\) methylation | 0.517   | 38.2           | 65.1           | 80     | 22.4   | 44                      |
| \(\text{P16}\) methylation | 0.512   | 46.5           | 55.8           | 79.3   | 22.2   | 48.5                    |

Abbreviations: AUC, area under the curve; NPV, negative predictive value; \(\text{RASSF1A}\), Ras association domain family member 1A; \(\text{RNF185}\), ring finger protein 185; \(\text{P16}\), cyclin-dependent kinase inhibitor 2A; PPV, positive predictive value; \(\text{PTGS2}\), prostaglandin-endoperoxidase synthase 2; \(\text{VHL}\), von Hippel-Lindau.

\(^a\)\(\text{RNF185}\), \(\text{VHL}\) methylation, and \(\text{RASSF1A}\) methylation had good diagnostic accuracy, whereas \(\text{PTGS2}\) and \(\text{P16}\) methylation had no diagnostic value. In this analysis the cutoff point for total cell-free DNA \((\text{RNF185})\) was 2400 genome equivalents per milliliter.
caused by higher degrees of necrosis, apoptosis, and spontaneous active release of DNA in cancer patients, but they also may be caused by therapy.\textsuperscript{11} It is noteworthy that nonmalignant diseases, such as trauma, systemic inflammatory disorders, pulmonary embolism, and acute myocardial infarction, also can elevate levels of cell-free circulating DNA.\textsuperscript{11} It has been documented previously and confirmed in the current study that a subset of healthy individuals has methylation of cell-free DNA,\textsuperscript{8} which may be related to these nonmalignant conditions. Our current results further confirm the role of cell-free circulating DNA as a diagnostic RCC marker that may assist in the differential diagnosis of solid renal masses. Two previously published studies on this topic assessed different sized DNA fragments, thus, different fragmentation patterns, and the results indicated that larger DNA fragments are derived mainly from tumor necrosis, and smaller fragments (<200 bp) are derived from apoptosis.\textsuperscript{23} Gang et al.\textsuperscript{4} observed a large 397-bp fragment in 71 of 78 patients with RCC and a 456-bp fragment in 69 of 78 patients with RCC, but no such fragments were observed in controls. In addition, larger fragments were detected less frequently after surgery.\textsuperscript{4} Another study quantified 106-bp and 384-bp fragments of ACTB by q-PCR.\textsuperscript{10} Levels of both fragments were higher in patients with RCC than in controls. The AUC values ACTB-106 and ACTB-384 were 0.686 and 0.725, respectively. The current study quantified total cell-free circulating DNA by amplification of a 249-bp fragment of RNF185. The fragment size suggests that it is derived from necrosis, which is supported further by the finding that higher levels were observed in patients who had necrotic RCCs. The AUC of 0.755 is very similar to the AUC of ACTB-384 reported by Hauser et al.\textsuperscript{10} For the current study, we did not analyze small apoptotic fragments, which appear to be ubiquitous in all patients and seem to have lower power to diagnose RCC than the larger fragments; however, a combination of both may further increase diagnostic accuracy.\textsuperscript{10}

Because cell-free DNA is composed in part of tumor DNA,\textsuperscript{11} tumor-specific genetic aberrations like methylation may be detected and, thus, may improve diagnostic sensitivity and specificity. Therefore, we studied CpG island methylation of RASSF1A, VHL, PTGS2, and P16, which are methylated frequently in several cancer entities. We further hypothesized that VHL methylation is helpful to diagnose the clear cell subtype, in which inactivation is noted in approximately 50% to 60% of tumors.\textsuperscript{24} In contrast to previous studies in patients with testicular, prostate, and bladder cancers, we did not observe any diagnostic or prognostic value of PTGS2 methylation or P16 methylation.\textsuperscript{8,25,26} RASSF1A methylation, however, was detected in 45.9% of patients with RCC and in 7% of patients with benign tumors, which corresponded to an AUC of 0.694. This frequency of RASSF1A methylation is in agreement with a report on patients with testicular cancer.\textsuperscript{8} It is noteworthy that VHL methylation was detected in 50.3% of patients with RCC but in only 9.3% of controls. The AUC was 0.705 but increased to 0.744, when only clear cell tumors were analyzed. The observed frequency of VHL methylation was higher than that reported in previous studies of RCC tissue samples.\textsuperscript{27,28} There are several factors that may account for this variation. Most researchers have used methylation-specific PCR to detect VHL methylation, and it appears to be more specific but less sensitive than restriction-endonuclease q-PCR.\textsuperscript{8} Furthermore, the methylation pattern of serum and tumor DNA has not always had a large overlap.\textsuperscript{29} Finally, cell-free DNA consists of portions other than tumor DNA, which has an impact on the serum methylation pattern. VHL methylation was detected more frequently in clear cell RCC than papillary or chromophobe RCCs, as hypothesized and mirroring results obtained in tumors. Surprisingly, a proportion of papillary and chromophobe tumors had VHL methylation, which may have been caused by the existence of hybrid tumors that had genetic alterations similar to those observed in as clear cell RCC.\textsuperscript{30} Papillary RCC with clear cell features accounts for approximately 30 to 40% of papillary RCCs. In this entity, genetic alterations similar to those observed in clear cell RCC have been reported.\textsuperscript{30} The prevalence of papillary RCC with clear cell features correlates with the frequency of VHL methylation.

### Table 4. Multivariate Cox Proportional Hazards Analysis of Disease-Specific Survival\textsuperscript{a}

| Variables                        | HR   | 95% CI       | P     |
|----------------------------------|------|--------------|-------|
| **Model A**                      |      |              |       |
| SSIGN score (continuous)         | 1.31 | 1.19-1.44    | <.001 |
| Cell-free DNA >2400 GE/mL        | 5.68 | 1.21-26.71   | .028  |
| **Model B**                      |      |              |       |
| SSIGN score (continuous)         | 1.38 | 1.25-1.51    | <.001 |
| Cell-free DNA (continuous)       | 1.07 | 0.97-1.17    | .192  |

Abbreviations: CI, confidence interval; GE/mL, genome equivalents per milliliter; HR, hazard ratio; SSIGN score, tumor stage, size, grade, and necrosis score.

\textsuperscript{a}SSIGN scores and categorized total cell-free DNA levels were independent prognostic factors (Model A). If the total cell-free DNA level was evaluated as a continuous variable, then statistical significance was not reached (Model B).
observed in our papillary RCC patients. Methodological differences, such as the use of restriction-endonuclease q-PCR, also may contribute to the finding that VHL methylation was observed in a subset of patients who had non-clear cell RCC. For future studies, tumor material should be available for analysis of somatic VHL status. Thus, VHL status both in cell-free DNA and in the tumor can be evaluated. Taken together, detection of methylation status may increase the sensitivity and specificity of cell-free DNA for diagnosing RCC in the clinical setting of a solid renal mass. Furthermore, VHL methylation may be a novel, noninvasive tool for pretherapeutic subtyping of renal tumors. However, the specificity of total cell-free DNA levels and the methylation of cell-free DNA was high, but the sensitivity was very low. Because of the biologic heterogeneity of RCC, a single marker may not be sufficient to assist in clinical decision making. Instead, a combination of multiple markers, such as total-cell free DNA levels and VHL methylation, likely will increase sensitivity and overall predictive accuracy.

The current results demonstrate that cell-free circulating DNA is associated with TNM classification and may serve as a prognostic marker. Gang et al noted that the detection of large fragments was associated with higher T classification. Hauser et al reported an association of vascular invasion with DNA integrity. In both of those studies, however, survival was not assessed as an endpoint. We observed that higher total cell-free circulating DNA levels were associated with metastatic disease, tumor necrosis, and poorer survival. If these findings are confirmed by others in multivariate models, then cell-free circulating DNA levels may be integrated in prognostic models and may further enhance the predictive accuracy of established pathologic prognostic factors as it has for other biomarkers. These models may assist in tailoring postoperative surveillance and therapy. High-risk populations that may benefit from adjuvant therapies may be defined; equally, low-risk groups may be identified in which repeated surveillance imaging and hospital visits can be avoided. Because our follow-up was of short duration and the number of deaths from RCC was relatively low, results from our multivariate analyses should be viewed with reservation. To avoid over fitting of the Cox model, TNM classification, tumor size, necrosis, and grade were not entered as separate variables but were summarized as the SSIGN score. In addition, cell-free DNA was significant only as a categorical variable, and prognostic information would be lost if it was categorized as a continuous variable. A categorical variable, however, may be more appropriate for routine clinical use. Taken together, our preliminary study generates the hypothesis that total cell-free DNA may play a role as a prognostic marker. This hypothesis has to be tested in external, prospective datasets. Our analyses also demonstrated that total cell-free DNA levels decrease after curative nephrectomy. It would be important to determine whether the persistence of cell-free DNA levels after nephrectomy correlates with the risk of recurrence. Our small subgroup cohort with relatively short follow-up, however, did not allow reliable statistical analyses regarding this issue. Future studies should address this, confirm our data in more patients, and focus on the prognostic value of cell-free DNA as a continuous variable.

Methodological inconsistencies regarding studies on cell-free DNA need to be acknowledged, including the source of DNA (plasma/serum), the impact of different kits on DNA purification, the mode of detection and the inconsistency of control populations. DNA concentrations in serum are about 6 times higher than in plasma, although the reason for this difference is unknown. Second, different DNA extraction kits may offer different purification efficacies. Third, different techniques have been applied to detect cell-free DNA and methylation, including fluorometric assays, spectrophotometry, bisulfite sequencing, conventional PCR, and q-PCR. Thus, procedures are not standardized, and this limits our ability to compare studies. Furthermore, we want to emphasize that the composition of the control group is of paramount importance for interpreting results. Many studies use healthy individuals, some studies use relevant control groups like those with elevated prostate-specific antigen levels and negative prostate biopsies, and other studies do not specify the control group in detail. This may explain in part the enormous range of cell-free DNA specificity (58%-98%), sensitivity (58%-85%), and AUC values (0.708-0.881) that have been reported for the diagnosis of prostate cancer. A control group should consist of patients to whom the diagnostic test would be applied in clinical practice, such as patients with renal masses of unknown histology. Healthy individuals, such as blood donors, are less appropriate.

In conclusion, the results from this study suggest that cell-free DNA represents a novel, serum-based diagnostic and prognostic biomarker for RCC: Total circulating cell-free DNA concentration and CpG island methylation of RASSF1A and VHL may be useful diagnostic biomarkers for RCC; VHL methylation may be a novel, noninvasive tool for pretherapeutic subtyping of
renal tumors; and higher total circulating cell-free DNA concentrations may be associated with metastatic disease, tumor necrosis, and diminished survival. However, we must emphasize that this was a study on a limited number of patients with short follow-up duration. Thus, external validation of these data will be required before serum cell-free DNA can be implemented as routine biomarker. Furthermore, a longer follow-up will be necessary to evaluate the role of serum cell-free DNA as a prognostic factor.

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