Wnt Antagonist Family Genes as Biomarkers for Diagnosis, Staging, and Prognosis of Renal Cell Carcinoma Using Tumor and Serum DNA

Shinji Urakami,[1,2] Hiroaki Shiina,[2] Hideki Enokida,[3] Hiroshi Hirata,[1] Ken Kawamoto,[1] Toshifumi Kawakami,[1] Nobuyuki Kikuno,[1] Yuichiro Tanaka,[1] Shahana Majid,[1] Masayuki Nakagawa,[3] Mikio Igawa,[2] and Rajvir Dahiya[1]

Abstract **Purpose:** We hypothesized that combined methylation analysis of Wnt antagonist genes could serve as a panel of biomarkers for diagnosis, staging, and prognosis in renal cell carcinoma (RCC).
Experimental Design: Samples (n = 62) of RCC and corresponding normal renal tissue (NRT) were analyzed using methylation-specific PCR for methylation of six Wnt antagonist genes (*sFRP-1, sFRP-2, sFRP-4, sFRP-5, Wif-1*, and *Dkk-3*). To increase the sensitivity/specificity of RCC detection, the methylation score (M score) for multigene methylation analysis was developed. Receiver operator characteristic curve analysis was used to determine the optimal sensitivity/specificity of the M score. In addition, the M score was compared with the clinicopathologic outcome. Thirty-three serum DNA samples were also used to investigate the methylation status of Wnt antagonist genes.
Results: The methylation levels of all Wnt antagonists were significantly higher in RCC than in NRT. In multivariate regression analysis, the methylation level of sFRP-1 was a significant independent predictor of RCC, whereas for sFRP-2 and sFRP-4 there was a trend toward significance as independent predictors. The M score of Wnt antagonist genes was significantly higher in RCC than in NRT. Overall, the M score had a sensitivity of 79.0% and a specificity of 75.8% (area under the curve, 0.808) as a diagnostic biomarker. In addition, the M score could significantly distinguish grade, pT category, M category, and overall survival of RCC patients. The M score was independent of age and gender in predicting overall survival by the Cox proportional hazards model. In RCC patients, 72.7% of the methylation-specific PCR results had identical methylation in samples of tumor and serum DNA. No serum DNA in normal controls showed aberrant methylation of the Wnt antagonist genes. In addition, the methylation status of Wnt antagonist genes in serum DNA was significantly correlated with tumor grade and stage.
Conclusions: This is the first report showing that M score analysis of Wnt antagonist genes can serve as an excellent epigenetic biomarker panel for detection, staging, and prognosis of RCC using serum DNA.

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Renal cell carcinoma (RCC) accounts for 2% of visceral malignancies worldwide, and the incidence in North America and northern Europe has been increasing at 3% yearly (1). This increased incidence is in part due to the widespread usage of noninvasive imaging, such as computed tomography and ultrasound, for the detection of asymptomatic tumors (2). However, to our knowledge, no ideal noninvasive tumor markers with high sensitivity and specificity currently exist for early detection, monitoring, or prognostic prediction of RCC. Most RCCs are curable if detected when still confined to the renal capsule. In contrast for metastatic RCCs, only limited therapeutic options are available because of their high resistance to radiotherapy, immunotherapy, and chemotherapy. Therefore, the development of noninvasive and accurate diagnostic biomarkers for early detection of RCC is imperative and crucial to improve RCC prognosis. On the other hand, ~10% of patients with locally confined tumors die of disease progression within 5 years of nephrectomy (3). Although several prognostic variables for RCC, such as serum interleukin-6 or immunosuppressive acid protein, have been proposed, they have not been shown to be reliable tumor markers due to low specificity and sensitivity (4, 5). Therefore, accurate preoperative prognostic biomarkers are needed to distinguish highly malignant RCCs to aggressively treat them or put them under stringent surveillance.

Authors' Affiliations: [1]Department of Urology, Veterans Affairs Medical Center and University of California, San Francisco, California; [2]Department of Urology, Shimane University, Izumo, Japan; and [3]Department of Urology, Kagoshima University, Kagoshima, Japan

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Requests for reprints: Rajvir Dahiya, Urology Research Center (112F), Veterans Affairs Medical Center and University of California at San Francisco, 4150 Clement Street, San Francisco, CA 94121. Phone: 415-750-6964; Fax: 415-750-6639; E-mail: rdahiya@urology.ucsf.edu.

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Current diagnostic and prognostic research is focused on the molecular pathology of RCC and the identification of molecular mechanisms that contribute to differences in tumor pathogenesis and progression. Aberrant promoter hypermethylation of known or putative tumor suppressor genes occurs frequently during tumor pathogenesis and progression and has been found to be one of the primary mechanisms of gene down-regulation (6, 7). Recent development of methylation-specific PCR (MSP) can readily detect these epigenetic changes, offering hope for early cancer detection (8). In addition, detection of promoter CpG methylation in body fluid DNA is feasible and noninvasive (9); therefore, combined MSP analyses of multiple genes in serum DNA can potentially serve as an excellent tumor marker for diagnosis and prognosis of RCC.

In this regard, several antagonists of Wnt signaling have been identified (10) and can be divided into two functional classes: the secreted frizzled-related protein (sFRP) class and the Dickkopf (Dkk) class. The former class, which includes the sFRP gene family (sFRP-1, sFRP-2, sFRP-3, sFRP-4, and sFRP-5), Wnt inhibitory factor-1 (Wif-1), and Cerberus, inhibits Wnt signaling by directly binding to Wnt molecules instead of Fz (10). The latter class, which comprises certain Dkk family proteins (Dkk-1, Dkk-2, Dkk-3, and Dkk-4), inhibits Wnt signaling by binding to the LRP5/LRP6 component of the Wnt receptor complex. Thus, the functional loss of Wnt antagonists can contribute to activation of the Wnt pathway and result in carcinogenesis through dysregulation of cell proliferation and differentiation. Recent publications from our laboratory and others have shown that impaired regulation by hypermethylation of Wnt antagonists was found in many cancers (11 – 15). In addition, decreased expression of Dkk-3 in RCC has been reported (13). However, no comprehensive analysis of Wnt antagonist gene methylation has been reported for RCC.

Therefore, in the current study, we assessed (*a*) how the methylation status of Wnt antagonist genes change during RCC pathogenesis and progression and (*b*) whether multigene methylation analysis of Wnt antagonists could serve as a potential epigenetic biomarker panel for diagnosis, staging, and prognosis of RCC. In addition, we also investigated whether hypermethylation of these genes could be detected in serum DNA from RCC patients before surgery.

Materials and Methods

Tissue samples. Sixty-two samples of primary renal cancer and corresponding normal renal tissue (NRT) were obtained by either radical or partial nephrectomy. All kidneys with RCC were diagnosed based on histopathologic findings. They were graded and staged according to the WHO criteria and Union Internationale Contre le Cancer tumor-node-metastasis classification (1997), respectively. The age of the patients ranged from 29 to 84 years, with a median age of 67.5 years. The details of the patients' clinicopathologic characteristics are summarized in Table 1. The cohort included 48 cases of grade 1 and 2 and 14 cases of grade 3 RCC, 37 cases of pT1 (pT1a and pT1b), and 25 cases of pT2 to pT4 RCC. Half of each surgical specimen was fixed in 10% buffered formalin (pH 7.0) and embedded in paraffin wax. Sections (5 μm) were used for H&E staining for histologic evaluation. Snap-frozen samples were stored at −80°C until analyzed. Controls consisted of 10 samples of peripheral blood lymphocytes from normal healthy volunteers. Informed consent was obtained from each patient.

Table 1. Characteristics of 62 patients with RCC

	n (%)
Age (mean ± SD)	63.9 ± 12.8
Gender	
Male	42 (67.7)
Female	20 (32.3)
Pathology	
Clear cell	56 (90.3)
Granular cell	3 (4.8)
Clear cell and granular cell mixed	3 (4.8)
Tumor grade	
1	13 (21.0)
2	35 (56.4)
3	14 (22.6)
pT category	
pT1a	25 (40.3)
pT1b	12 (19.4)
pT2	9 (14.5)
pT3a	9 (14.5)
pT3b	6 (9.7)
pT4	1 (1.6)
pN category	
pN0	57 (91.9)
pN1-2	5 (8.1)
M category	
M0	55 (88.7)
M1	7 (11.3)

Serum samples. A total of 33 serum samples was collected from 22 paired RCC patients and from additional 11 RCC patients before surgery. Peripheral blood (10 mL) was collected in clot activator tubes, and the serum was separated by centrifugation. Samples were subjected to two consecutive centrifugations at 1,500 × *g* for 10 minutes at room temperature to remove the cellular components. Circulating cell-free DNA was extracted from 800 μL serum using a QIAmp DNA Mini Blood kit (Qiagen, Valencia, CA) and resuspended in 50 μL of distilled water. In addition, 20 serum samples from age- and sex-matched normal healthy volunteers with no smoking history were included.

Cell culture. Two human RCC cell lines, Caki-1 and Caki-2, were obtained from the American Type Culture Collection (Manassas, VA). The Caki-1 cell line was maintained in RPMI 1640 with L-glutamine and sodium pyruvate, whereas the Caki-2 cell line was maintained in McCoy's 5A medium with L-glutamine. FCS (10%) was added to all the media, and cells were maintained in a humidified atmosphere of 5% CO_2, 95% air at 37°C.

5-Aza-2′-deoxycytidine treatment. The Caki-1 and Caki-2 cell lines were treated with demethylating agent 5-aza-2′-deoxycytidine (5-Aza-dC; 10 μmol/L) for 3 days in triplicate and harvested. The genomic DNA and total RNA were extracted from the cell lines before and after 5-Aza-dC treatment and used for MSP and one-step reverse transcription-PCR (Titanium One-Step Reverse Transcription-PCR kit, BD Biosciences, Palo Alto, CA). Primer sequences for reverse transcription-PCR of the Wnt antagonist genes were previously reported by us (11).

Nucleic acid extraction. Genomic DNA and total RNA were extracted from frozen RCC and matched NRT samples using a Qiagen kit after microdissection (16). The concentrations of DNA and RNA were determined with a spectrophotometer, and their integrity was assessed by gel electrophoresis.

Methylation-specific PCR. Genomic DNA was modified with sodium bisulfite using a commercial kit (Invitrogen, Carlsbad, CA). The targeted genes used in this study were *sFRP-1*, *sFRP-2*, *sFRP-4*, *sFRP-5*, *Wif-1*, and *Dkk-3*. The first universal primer set covered no CpG sites in either the forward or reverse primer and amplified a DNA fragment of the promoter region containing several sites. Then, a second round of nested MSP or unmethylation-specific PCR (USP) was done using the

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universal PCR products as templates. Primer sequences designed for MSP and USP of the Wnt antagonist genes have been reported previously (11). The methylation status of the *sFRP-3* gene was not examined because its promoter does not contain any CpG islands. For semiquantitative MSP analysis, a preliminary suitable number of PCR cycles for each primer set were carried out to determine the linear range of the reaction. The PCR products were separated by electrophoresis in a 1.5% agarose gel containing ethidium bromide, and DNA bands were visualized by UV light. In samples with a positive MSP band, the relative methylation ratio was determined after the MSP or USP product was electrophoresed in nondenaturing 12% polyacrylamide gels. The area under the curve (AUC) corresponding to each band was calculated using ImageJ software,[4] and the relative methylation level was determined [MSP ratio = MSP band density / (MSP band density + USP band density)] as reported previously (17, 18).

Quantitative methylation analysis by real-time MSP. To validate our semiquantitative MSP analysis, we did quantitative real-time MSP. The primers used for quantitative real-time MSP were the same ones used for semiquantitative MSP. MYOD1 served as an internal reference. Primer sequences for MYOD1 were obtained from previously published data (19). All PCR experiments were carried out in a volume of 20 μL with 96-well plates and the ABI PRISM 7000 (Applied Biosystems, Foster City, CA). Samples (3 μL bisulfite-treated DNA) were run in triplicate containing 10 μL SYBR Green Master Mix (Applied Biosystems), 0.16 μL TaqStart Antibody (Clontech, Mountain View, CA), and 10 pmol of each forward and reverse primer. Every PCR experiment included serial dilutions of universal methylated DNA (Chemicon, Temecula, CA) as a positive control for construction of the calibration curve and water blanks. PCR amplification was done by means of the following procedure: 95°C for 15 minutes followed by 45 cycles at 95°C for 15 seconds, 55°C for 20 seconds, and 30-second extension step at 74°C (MYOD1) and 77°C (sFRP-1). A subsequent dissociation curve analysis checked the specificity of products. The methylation ratio was defined as the ratio of the fluorescence emission intensity values for the sFRP-1 PCR products to those of the MYOD1 PCR products obtained by quantitative real-time MSP as suggested by Jeronimo et al. (19).

Bisulfite DNA sequencing. Bisulfite-modified DNA was amplified using a pair of universal primers. Direct bisulfite DNA sequencing of the PCR products using either forward universal primer or reverse primer was done according to the manufacturer's instructions (Applied Biosystems).

Statistical analysis. Sensitivity was calculated as the proportion of samples with disease (e.g., RCC) or with a specified condition present (e.g., high grade) that were correctly identified by a screening test or case definition as having disease or the condition. Specificity was calculated as the proportion of samples without disease or without a specified condition present that were correctly identified by a screening test or case definition as not having disease or the condition. Initially, the optimal cutoff values for the relative methylation level necessary to distinguish RCC from normal NRT were determined for all investigated Wnt antagonist genes using receiver operator characteristic (ROC) curve analysis. Next, using our analytic technique reported previously (11, 20, 21), we calculated the methylation score (M score) for each sample, defined as the sum of the corresponding log hazard ratio (HR) coefficients for each gene, which were derived from multivariate logistic regression analysis in the RCC and corresponding NRT samples. The optimal sensitivity and specificity of the M score for diagnosis and staging of RCC were determined by ROC curve analysis using MedCalc software (MedCalc Software, Mariakerke, Belgium). A pair-wise comparison was used to test for significance using the AUC analysis. The M score was divided into two groups above or below the median level in RCC samples, survival curves (time to progression and overall survival time) were generated using Kaplan-Meier analysis, and the difference between the curves was analyzed univariately using the Mantel-Cox (log rank) test. In addition, multivariate analysis in overall survival and progression-free survival was done using Cox proportional hazards regression model. All data, except for the ROC curve analysis, were analyzed by the StatView V statistical package (SAS Institute, Inc., Cary, NC). The relationship between clinicopathologic variables and each relative methylation level or M score was analyzed using Fisher's exact test, Mann-Whitney *U* test, and Kruskal-Wallis test. A P value of <0.05 was regarded as statistically significant.

Results

Methylation status of Wnt antagonist genes in clinical tissue samples. Figure 1A illustrates representative results of methylation analysis of the six Wnt antagonist genes. MSP-positive bands for the Wnt antagonist genes were visually present in many RCC samples and were less evident in the NRT samples. USP-positive bands were present in most RCC and NRT samples. The results of conventional MSP analysis were also confirmed by bisulfite DNA sequencing. As shown in Table 2A, the methylation levels of all investigated Wnt antagonist genes were significantly higher in RCC than in NRT. As a next step, we did quantitative real-time MSP in *sFRP-1* gene, one of Wnt antagonist genes used in this study, to validate our conventional semiquantitative MSP results. Representative 10 samples of RCC and corresponding NRT were analyzed by both the semiquantitative MSP and quantitative real-time MSP. As shown in Fig. 1B, sFRP-1 methylation ratio using quantitative real-time MSP in RCCs was significantly higher compared with that in NRTs (P = 0.0002). In addition, correlation analysis revealed a significant positive association of relative sFRP-1 methylation level by conventional semiquantitative MSP and sFRP-1 methylation ratio by quantitative real-time MSP (P < 0.005). The grade, pT, pN, and M were each divided into two groups consisting of higher and lower categories. The methylation levels of *sFRP-1* and *Dkk-3* genes were higher in the higher grades and higher pT categories of RCC than in the lower grades or lower pT categories (Table 2A and B). In addition, metastatic RCC (M1) showed significantly higher relative methylation levels in *sFRP-1* and *sFRP-5* genes than nonmetastatic RCC (M0). However, the methylation levels of Wnt antagonist genes showed no significant association with pN category. In addition, no MSP-positive bands were found in 10 samples of human peripheral blood lymphocytes from normal healthy volunteers.

Bisulfite genomic sequencing. Bisulfite DNA sequencing was carried out to confirm whether the relative MSP levels reflected the true methylation status of CpG sites. Ten pairs of matched normal/cancer PCR products obtained using the universal primer set were sequenced. The majority of cytosines within CpG sites were completely converted to thymines (unmethylated) after bisulfite modification in the normal samples, indicating a low relative MSP ratio. On the other hand, in the tumor samples where relative methylation levels were higher, the majority of cytosines remained unaltered (methylated). Therefore, the bisulfite sequencing results were consistent with the MSP and USP results (relative methylation levels; data not shown).

Expression of Wnt antagonist gene mRNA in RCC cell lines. As shown in Fig. 1C, expression of mRNA transcripts for most Wnt antagonists was increased in the two RCC cell lines after treatment with the demethylating agent 5-Aza-dC

[4] http://rsb.info.nih.gov/ij.

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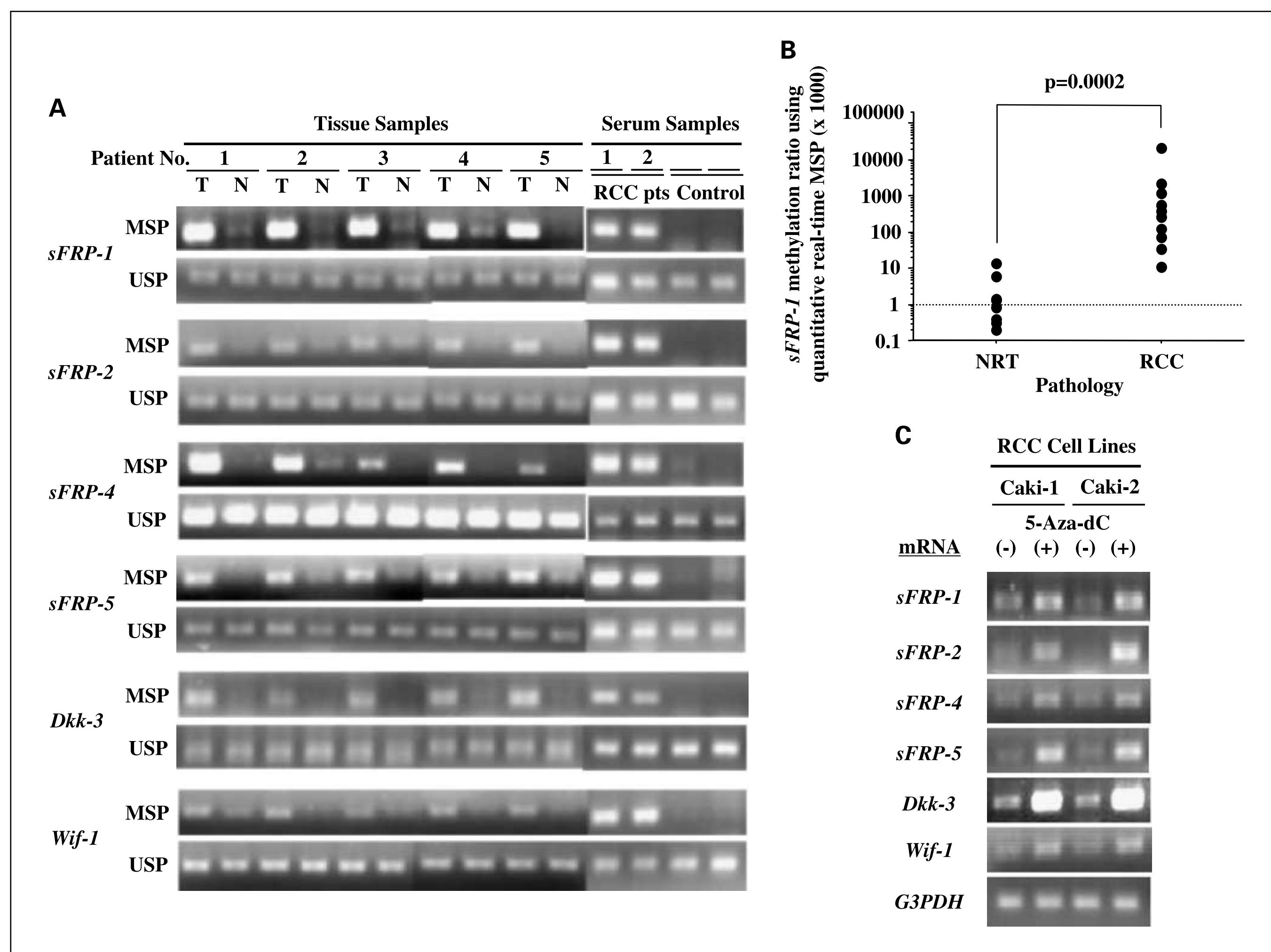

Fig. 1. *A,* methylation status of Wnt antagonist genes (*sFRP-1, sFRP-2, sFRP-4, sFRP-5, Wif-1,* and *Dkk-3*) in matched tissue (n = 5) and serum (n = 2) samples from RCC patients and controls. T, renal tumor tissue; N, matched NRT of RCC patients (numbers 1-5). MSP and USP bands of each gene. The intensity of the MSP bands was increased in RCC tissue compared with NRT. *B,* sFRP-1 (/MYOD1) methylation ratio using quantitative real-time MSP in RCC and corresponding NRT tissues. sFRP-1 methylation ratio in representative 10 RCC samples ($2,443.1 \times 10^3 \pm 1,935.0 \times 10^3$) was significantly increased compared with that in corresponding NRT samples ($2.4 \times 10^3 \pm 1.3 \times 10^3$; P = 0.0002). *C,* reverse transcription-PCR of Wnt antagonist genes in two RCC cell lines (Caki-1 and Caki-2). Glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) was used as a control. The expression of Wnt antagonist gene mRNAs was increased after 5-Aza-dC treatment compared with control.

when compared with those before treatment. In addition, we confirmed demethylation of the Wnt antagonist genes in these cell lines after 5-Aza-dC treatment by bisulfite DNA sequencing.

Evaluation of M score for distinguishing RCC from NRT (multigene methylation analysis using Wnt antagonists). The optimal cutoff point (MSP negative versus MSP positive) of each relative methylation level was established using ROC curve analysis to distinguish RCC from NRT. However, using these optimal cutoff values, none of the Wnt antagonist genes could be used as a single reliable biomarker for RCC because of their limited sensitivity/specificity and AUC (Table 3A). Next, we did multivariate logistic analysis to determine which genes are independent predictors of pathology comparing RCC and normal NRT using multigene methylation analysis. As shown in Table 3B, multivariate logistic regression analysis revealed that the relative methylation level of sFRP-1 (P < 0.01) was a significant independent predictor of RCC. Although sFRP-2 and sFRP-4 were not significant, they showed trends toward being independent predictors of RCC (P < 0.1, both). The individual gene HRs for pathology (RCC versus NRT) were different from one another. In overall patients, the M score, determined by the sum of the corresponding log HR of the six Wnt antagonist genes, was significantly higher in RCCs than in normal NRTs (P < 0.0001; Fig. 2A). The M score had a sensitivity of 79.0% and a specificity of 75.8% (AUC, 0.808) using the optimal cutoff point (2.99) by ROC curve analysis (Fig. 2B; Table 3C). When used as a single variable, the methylation levels of Wnt antagonist genes were not significantly different from one another using a pair-wise comparison test for AUC (Table 3A). However, the M score was an excellent biomarker even compared with single predictors of RCC, such as *sFRP-1* (P = 0.002), *sFRP-2* (P < 0.05), *sFRP-4* (P < 0.0001), *sFRP-5* (P = 0.001), *Dkk-3* (P = 0.003), and *Wif-1* (P = 0.001), using a pair-wise comparison test (Table 3A and C). In short, the M score was the best variable compared with other single variables.

Correlation of the M score with clinicopathologic findings. The M score was significantly higher in higher-grade, higher pT or metastatic RCCs than in lower-grade, lower pT or

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nonmetastatic RCCs ($P < 0.002$, 0.005, and 0.02, respectively) as shown in Fig. 2C-E. In addition, the M score showed a high AUC by ROC curve analyses in predicting grade (AUC, 0.787), pT category (AUC, 0.718), and M category (AUC, 0.868), except for pN category (AUC, 0.488), of RCCs as shown in Table 3C. However, the M score was not a significantly superior biomarker when compared with other single significant predictors for distinguishing grade, pT category, pN category, and M category using a pair-wise comparison test for AUC (data not shown; Table 3C).

Correlation of the M score with prognosis. The cutoff point of the M score for distinguishing outcomes of RCCs was determined as 5.91, the median level of the M score in RCC. As shown in Fig. 2F, a log-rank test showed that RCC patients with higher M score were associated with worse overall survival compared with lower M score ($P < 0.05$). On the other hand, the correlation of higher M score with progression-free survival failed to reach statistical significance (data not shown). Because the variables, such as age and gender, might be interrelated with each other, multivariate analysis was done using the Cox proportional hazards regression model. The variables applied to multivariate analysis were age, sex (male versus female), and M score (lower versus higher). As shown in Table 3D, the multivariate analysis model revealed that the M score was an independent predictor (HR, 3.253) in overall survival ($P < 0.05$).

Detection of methylation of Wnt antagonist genes in serum. Typical MSP results for Wnt antagonist genes in matched RCC and NRT samples and serum from RCC patients (numbers 1 and 2) are shown in Fig. 1A. Representative MSP results of serum DNA from normal controls are also shown in Fig. 1A. These MSP results of serum DNA are summarized in Table 4A. An identical methylation status was found in 22 RCC and matched serum samples in 96 (72.7%) of 132 MSP results. The prevalence of methylation in tissue DNA from RCC and matched NRT was 55.4% and 14.2%, respectively, as shown in Table 4B. On the other hand, it was 33.3% and 0% in serum DNA from RCC patients and normal controls, and the difference between them was statistically significant ($P < 0.0001$). All RCC samples that were methylation negative were also negative in the matched serum DNA (Table 4A). Overall, the frequency of methylation in serum DNA from RCC patients was higher in higher-grade and higher-stage RCCs when compared with lower-grade and lower-stage RCCs (grade, $P < 0.01$; pT, $P < 0.003$; M, $P < 0.02$; Table 4B). The difference in pN category was not significant.

Discussion

The Wnt antagonist genes function as tumor suppressors and have been implicated in the pathogenesis or progression of several human malignancies (10). Recently, gene silencing in *sFRP-1*, *sFRP-2*, *sFRP-4*, *sFRP-5*, *Dkk-3*, and *Wif-1* genes due to their promoter CpG hypermethylation has been identified in several human malignancies (11, 22, 23). Although promoter CpG methylation of the Wnt antagonist genes has not been analyzed in RCC, Dkk-3 mRNA has been reported to be down-regulated in human RCC samples (13). Therefore, we hypothesized that down-regulation of Wnt antagonist genes caused by promoter hypermethylation may contribute to the pathogenesis and progression of RCC. In the current study, all Wnt antagonist genes had higher methylation levels in RCC compared with NRT. Furthermore, Wnt antagonist gene mRNA expression was increased in two RCC cell lines after treatment with 5-Aza-dC and confirmed to be demethylated by DNA sequencing. These results suggest that functional impairment of Wnt antagonists caused by promoter hypermethylation may be involved in the pathogenesis of RCC.

ROC curve analysis showed that none of the Wnt antagonist genes as a single variable were reliable biomarkers for RCC

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Table 2. Univariate analysis of relative methylation level in Wnt antagonist genes

A. Univariate analysis of relative methylation levels in Wnt antagonist genes (1)

Variables	Relative methylation level (mean ± SE)					
	Normal vs cancer			Grade		
	NRT (%)	RCC (%)	*P*	Grade 1-2 (%)	Grade 3 (%)	*P*
sFRP-1	1.5 ± 0.6	12.9 ± 2.1	0.0002	10.0 ± 2.2	22.7 ± 4.6	<0.02
sFRP-2	25.5 ± 2.4	39.6 ± 2.8	0.0001	40.9 ± 2.9	35.5 ± 7.9	>0.05
sFRP-4	5.7 ± 1.4	13.6 ± 2.4	<0.02	11.9 ± 2.5	19.6 ± 6.0	<0.1
sFRP-5	31.5 ± 2.9	41.1 ± 3.5	<0.05	39.8 ± 4.1	45.4 ± 7.1	>0.05
Dkk-3	9.1 ± 2.6	24.6 ± 3.8	<0.002	18.1 ± 3.8	46.9 ± 8.0	0.0005
Wif-1	25.5 ± 3.2	36.8 ± 3.2	<0.002	39.2 ± 3.6	28.4 ± 6.6	>0.05

B. Univariate analysis of relative methylation levels in Wnt antagonist genes (2)

Variables	Relative methylation level (mean ± SE)								
	pT category			pN category			M category		
	pT1 (%)	pT2-4 (%)	*P*	pN0 (%)	pN1-2 (%)	*P*	pM0 (%)	pM1 (%)	*P*
sFRP-1	9.2 ± 2.5	18.4 ± 3.5	<0.02	13.0 ± 2.2	11.6 ± 8.4	>0.05	10.1 ± 2.0	34.5 ± 5.7	<0.001
sFRP-2	40.9 ± 3.5	37.8 ± 4.9	>0.05	41.1 ± 2.9	23.2 ± 9.8	>0.05	39.0 ± 2.8	44.8 ± 13.4	>0.05
sFRP-4	12.7 ± 3.1	15.0 ± 3.8	>0.05	13.7 ± 2.6	13.1 ± 5.3	>0.05	12.4 ± 2.4	22.9 ± 10.3	>0.05
sFRP-5	44.5 ± 4.6	36.0 ± 5.4	>0.05	41.5 ± 3.7	36.0 ± 9.3	>0.05	38.6 ± 3.7	60.9 ± 8.9	<0.05
Dkk-3	16.4 ± 3.9	36.8 ± 6.7	<0.01	24.6 ± 3.8	24.9 ± 17.9	>0.05	23.6 ± 4.0	32.6 ± 10.9	>0.05
Wif-1	41.1 ± 4.0	30.3 ± 5.1	>0.05	37.0 ± 3.4	34.2 ± 9.1	>0.05	35.4 ± 3.5	47.6 ± 5.6	0.05

Table 3. Wnt antagonist genes and M score

A. ROC curve analyses of relative methylation levels in Wnt antagonist genes

Variables	AUC	Optimal cutoff point of relative methylation level (%)	Sensitivity, % (95% CI)	Specificity, % (95% CI)
sFRP-1	0.692	0	46.8 (34.0-59.9)	88.7 (78.1-95.3)
sFRP-2	0.702	42.1	53.2 (40.1-66.0)	57.4 (76.1-94.2)
sFRP-4	0.633	0	53.2 (40.1-66.0)	71.0 (58.0-81.8)
sFRP-5	0.610	41.1	56.5 (43.3-69.0)	71.0 (58.0-81.8)
Dkk-3	0.668	12.0	50.0 (37.0-63.0)	82.3 (70.5-90.8)
Wif-1	0.629	16.8	72.6 (59.8-83.1)	50.0 (37.0-63.0)

B. Multiple logistic regression analysis of methylation status in Wnt antagonist genes

Variables	Log HR	SE	χ^2	*P*	HR	95% CI
sFRP-1	7.310	2.785	6.888	<0.01	1,494.639	6.361-351,197.500
sFRP-2	1.802	1.145	2.478	<0.1	6.064	0.643-57.210
sFRP-4	2.987	1.629	3.364	<0.1	19.830	0.814-482.781
sFRP-5	0.131	0.959	0.019	>0.5	1.139	0.174-7.468
Dkk-3	1.272	0.870	2.136	>0.5	3.567	0.648-19.641
Wif-1	0.716	0.939	0.581	>0.5	2.046	0.325-12.886

C. ROC curve analyses of the M score in clinicopathologic findings

Clinicopathologic findings	AUC	Optimal cutoff point of relative methylation level (%)	Sensitivity, % (95% CI)	Specificity, % (95% CI)
Normal vs cancer*	0.802	2.99	79.0 (66.8-88.3)	75.8 (63.3-85.8)
Grade	0.787	10.30	71.4 (41.9-91.4)	81.2 (67.4-91.0)
pT category	0.718	6.19	72.0 (50.6-87.9)	67.6 (50.2-82.0)
pN category	0.488	3.83	60.0 (15.4-93.5)	71.9 (58.5-83.0)
M category	0.868	10.30	85.7 (42.2-97.6)	76.4 (63.0-86.8)

D. Multivariable Cox proportional hazards model for overall survival

Variables	χ^2	HR	95% CI	*P*
Gender (female vs male)	0.567	0.610	0.169-2.205	0.451
Age	0.690	1.019	0.974-1.066	0.406
M score (higher vs lower)	3.823	3.253	1.015-10.614	<0.05

NOTE: When used as a single variable, the relative methylation levels of Wnt antagonist genes were not significantly different from one another using a pair-wise comparison test for AUC. The optimal cutoff values of relative methylation level using ROC curve analysis were used to assess whether samples were relative methylation level negative (MSP negative) or positive (MSP positive).

*The M score for distinguishing cancer from normal was an excellent biomarker even compared with single predictors of RCC, such as sFRP-1 ($P = 0.002$), sFRP-2 ($P < 0.05$), sFRP-4 ($P < 0.0001$), sFRP-5 ($P = 0.001$), Dkk-3 ($P = 0.003$), and Wif-1 ($P = 0.001$), using a pair-wise comparison test (Table 3A). However, the M score was not a significantly superior biomarker when compared with other single significant predictors for distinguishing grade, pT category, pN category, and M category (data not shown).

because of their limited sensitivity/specificity and AUC. To date, many previous studies have examined the methylation status of only single genes in RCC (24) and there had been no appropriate method for combination analysis of multiple hypermethylated genes. Very recently, we have reported results showing that the M score is a new method for multigene methylation analysis in prostate and bladder cancer (11, 20, 21). In this study, we showed that using the M score of Wnt antagonist genes provides high sensitivity and specificity (AUC, 0.808) for RCC detection. It serves as an excellent biomarker when compared with the *sFRP-1* gene, which is a significant independent predictor of RCC in multivariate analysis. In a review of the literature, the M score of Wnt antagonist genes has a higher sensitivity and specificity than other tumor markers, such as ferritin and interleukin-2, in RCC (25). Therefore, the M score of Wnt antagonist genes can be a useful molecular marker for RCC detection. In addition, we confirmed our conventional semiquantitative MSP data with quantitative real-time MSP. Therefore, the M score results based on conventional MSP were validated.

Recently, elective nephron-sparing surgery has been considered appropriate and popular for small RCC. However, it is difficult to detect aggressive tumor features, such as in high-grade RCC. Consequently, RCC recurs after nephron-sparing surgery in ~10% of cases (26). Furthermore, treatment for high-stage RCC by immunotherapy or gene therapy is hampered by the lack of reliable tumor markers for monitoring the response to treatment. Therefore, it is important to identify tumor markers for prognostic prediction and monitoring of RCC. In our study, the methylation status of several Wnt antagonist genes was significantly related to tumor grade or stage. In addition, a comparison of the clinicopathologic data and the M score of the Wnt antagonist genes revealed a significant association between the M score and pathologic

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grade, stage, and overall survival. RCC is more common in men than in women, and it most often occurs in patients between the ages of 50 and 70 years (25). Furthermore, aging has an effect on DNA methylation with higher levels found in older people (27). Therefore, we did multivariate analysis of overall survival to eliminate possible interrelation of gender and age. Consequently, the M score was a good predictor independent of age and sex in overall survival. Wnt antagonist genes have been implicated in apoptosis, immortalization, proliferation, invasiveness, and osteoorgan differentiation (10). The loss of function of Wnt antagonist family genes by their hypermethylation may contribute to aggressive tumor characteristics in RCC as observed by a high M score. Therefore, we believe that an optimal panel of hypermethylated Wnt antagonist genes can contribute to detection or monitoring of RCC and predict RCC aggressiveness. Furthermore, considering the pathogenesis and progression in RCC owing to functional loss of Wnt antagonist family genes by their hypermethylation, block of activated Wnt signaling pathway, such as negative conversion or suppression of methylation in Wnt antagonist genes using demethylating agents or methylation inhibitors, may provide a basis for developing novel therapies in inoperable metastatic RCCs resistant to radiotherapy, immunotherapy, and chemotherapy. In this regard, prior studies have shown that hypermethylation of Wnt antagonist genes plays an important role in the pathogenesis or progression of several cancers through aberrant activation of canonical Wnt/β-catenin signaling pathway (11–15, 28). The restoration of Wnt antagonist gene expression by demethylation reagents or transfection induced down-regulation of downstream-activated genes of canonical Wnt pathway and inhibition of tumor growth rate. In addition, the methylation status of several types of Wnt antagonist family genes has been reported to be associated with clinical prognosis. For example, loss of sFRP-1 is an independent indicator of poor survival in papillary bladder cancer and breast cancer (29, 30). Dkk-3 methylation is an independent prognostic factor predicting disease-free survival in acute lymphoblastic leukemia (31). The epigenetic inactivation of Wnt antagonist genes can be generally implicated in aggressiveness features of many human malignancies. Therefore, the specific restoration of Wnt antagonist genes may be extensively applied as a novel treatment to improve prognosis not only in RCCs but also in many types of cancer.

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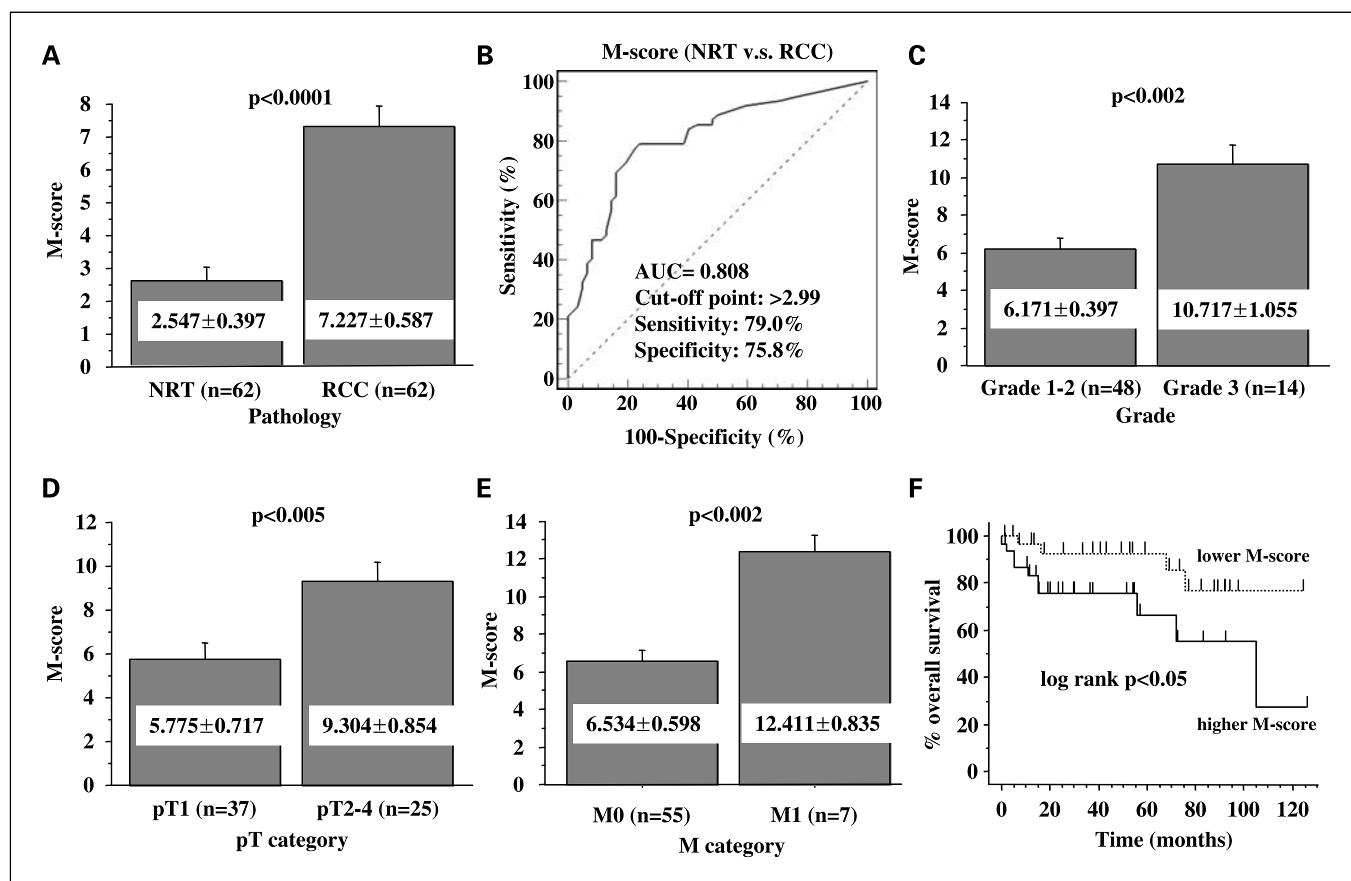

Fig. 2. *A,* the M score, determined as the sum of the corresponding log HRs for pathology (NRT versus RCC; Table 3B), was significantly higher in RCC samples than in NRT samples ($P < 0.0001$). *B,* ROC curve analysis of the M score. In overall patients, the optimal cutoff point of the M score for distinguishing RCC from NRT was determined as 2.99 using ROC curve. The M score had a sensitivity of 79.0% [95% confidence interval (95% CI), 66.8-88.3] and a specificity of 75.8% (95% CI, 63.3-85.8; AUC, 0.808). *C,* relationship of the M score with pathologic grade in RCC. There was a significant difference in M score between lower-grade (grades 1-2) and higher-grade RCC (grade 3; $P < 0.002$). *D,* relationship of the M score with the pT category of RCC. The M score was significantly higher in higher pT RCC (*pT2-4*) than in lower pT RCC (*pT1*) ($P < 0.005$). *E,* relationship of the M score with M category of RCC. The M score was significantly higher in metastatic RCC (*M1*) than in nonmetastatic RCC (*M0*; $P < 0.002$). *F,* relationship of the M score with overall survival in RCC patients. Kaplan-Meier survival analysis and a log-rank test showed that RCC patients with higher M score are significantly associated with worse overall survival probability than RCC patients with lower M score ($P < 0.05$).

Table 4. Wnt antagonist genes in tissue and serum DNA from RCC

A. Methylation status of Wnt antagonist genes in tumor tissue and corresponding serum DNA from RCC patients (n = 33)

Case no.	Sex	Age	Grade	pTNM	Tumor DNA/serum DNA						Identical results 96/132 (72.7%)
					sFRP-1	*sFRP-2*	*sFRP-4*	*sFRP-5*	*Dkk-3*	*Wif-1*	
1	M	63	3	T2N0M1	M/M	U/U	M/U	M/U	M/M	M/U	3/6
2	M	71	2	T2N0M1	M/M	M/U	M/M	M/U	M/U	M/U	2/6
3	F	58	2	T1aN0M0	M/U	U/U	U/U	M/U	U/U	M/U	3/6
4	M	44	1	T1aN0M0	U/U	M/M	M/U	M/M	U/U	M/U	3/6
5	F	74	2	T1aN0M0	U/U	M/M	U/U	U/U	U/U	M/M	6/6
6	M	64	2	T3bN2M0	U/U	M/M	M/U	M/U	U/U	M/M	4/6
7	M	65	2	T1bN0M0	U/U	M/M	M/M	U/U	U/U	U/U	6/6
8	M	74	2	T1aN0M0	U/U	M/M	M/U	U/U	U/U	U/U	5/6
9	M	70	2	T1bN2M0	U/U	U/U	M/U	U/U	U/U	M/U	4/6
10	F	69	1	T1bN0M0	U/U	M/M	M/M	U/U	U/U	M/M	6/6
11	M	68	3	T3aN0M1	M/M	M/M	M/U	M/U	M/M	M/U	3/6
12	M	61	3	T2N0M0	M/U	U/U	M/U	M/U	M/M	M/M	3/6
13	F	41	2	T1aN0M0	U/U	M/M	M/U	M/M	U/U	M/U	4/6
14	M	48	2	T1aN0M0	U/U	U/U	U/U	M/U	U/U	U/U	5/6
15	F	43	2	T2N0M0	U/U	M/M	U/U	M/M	M/U	U/U	5/6
16	M	42	3	T2N0M0	M/U	M/M	U/U	U/U	M/M	U/U	5/6
17	M	40	2	T1bN0M0	U/U	M/M	M/M	M/M	M/U	U/U	5/6
18	M	55	3	T3aN0M0	M/M	M/M	U/U	M/M	M/M	M/M	6/6
19	F	77	2	T1bN0M0	U/U	U/U	U/U	M/M	U/U	U/U	4/6
20	M	84	2	T1aN0M0	U/U	U/U	M/U	M/M	M/U	U/U	4/6
21	F	71	3	T3bN2M0	U/U	U/U	M/U	M/M	U/U	M/U	4/6
22	F	72	3	T4N2M0	M/M	U/U	M/M	M/M	M/M	U/U	6/6
23	M	66	2	T3aN0M1	ND/U	ND/M	ND/M	ND/M	ND/M	ND/M	NA
24	M	46	2	T1aN0M0	ND/U	ND/U	ND/U	ND/M	ND/U	ND/M	NA
25	M	67	2	T3aN2M0	ND/M	ND/M	ND/M	ND/M	ND/U	ND/U	NA
26	M	71	2	T3aN0M0	ND/M	ND/M	ND/U	ND/M	ND/M	ND/U	NA
27	F	42	2	T2N0M0	ND/U	ND/U	ND/M	ND/U	ND/U	ND/U	NA
28	M	75	2	T1bN0M0	ND/M	ND/U	ND/U	ND/U	ND/U	ND/U	NA
29	M	39	2	T1aN0M0	ND/U	ND/U	ND/U	ND/U	ND/U	ND/U	NA
30	M	76	2	T2N0M0	ND/U	ND/U	ND/U	ND/M	ND/U	ND/M	NA
31	M	65	3	T2N0M0	ND/M	ND/M	ND/U	ND/M	ND/M	ND/M	NA
32	M	62	1	T2N0M0	ND/U	ND/U	ND/U	ND/U	ND/U	ND/U	NA
33	F	60	2	T2N0M0	ND/U	ND/U	ND/U	ND/U	ND/U	ND/U	NA

B. Summary of Wnt antagonist genes (MSP-positive cases) in tissue and corresponding serum DNA from RCC patients

	sFRP-1, n (%)	*sFRP-2*, n (%)	*sFRP-4*, n (%)	*sFRP-5*, n (%)	*Dkk-3*, n (%)	*Wif-1*, n (%)	Total, n/total (%)
Tissue of RCC patients							
Normal tissue (n = 62)	5 (8.1)	6 (9.7)	9 (14.5)	9 (14.5)	10 (16.1)	14 (22.6)	53/372 (14.2)
Tumor-tissues (n = 62)	29 (46.8)	33 (53.2)	33 (53.2)	35 (56.5)	31 (50.0)	45 (72.6)	206/372 (55.4)
Serum							
Control-serum (n = 20)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0/120 (0.0)*
Patient-serum (n = 33)	9 (27.3)	16 (48.5)	8 (24.2)	15 (45.5)	9 (27.3)	9 (27.3)	66/198 (33.3)
Grade 1-2 (n = 25)	4 (16.0)	12 (48.0)	7 (28.0)	11 (44.0)	2 (8.0)	6 (24.0)	42/150 (28.0)†
Grade 3 (n = 8)	5 (62.5)	4 (50.0)	1 (12.5)	4 (50.0)	7 (87.5)	3 (37.5)	24/48 (50.0)
pT1 (n = 15)	1 (6.7)	7 (46.7)	3 (20.0)	6 (40.0)	0 (0.0)	3 (20.0)	20/90 (22.2)‡
pT2-4 (n = 18)	8 (44.4)	9 (50.0)	5 (27.8)	9 (50.0)	9 (50.0)	6 (33.3)	46/108 (42.6)
pN0 (n = 28)	7 (25.0)	14 (50.0)	6 (21.4)	12 (42.9)	6 (21.4)	8 (28.6)	53/168 (31.5)
pN1-2 (n = 5)	2 (40.0)	2 (40.0)	2 (40.0)	3 (60.0)	3 (60.0)	1 (20.0)	13/30 (43.3)
M0 (n = 29)	6 (20.7)	14 (48.3)	6 (20.7)	14 (48.3)	6 (20.7)	8 (27.5)	54/174 (31.0)§
M1 (n = 4)	3 (75.0)	2 (50.0)	2 (50.0)	1 (25.0)	3 (75.0)	1 (25.0)	14/24 (58.3)

NOTE: The optimal cutoff values of relative methylation level using ROC curve analysis were used to assess whether samples were MSP positive or negative.
Abbreviations: M, methylation; U, unmethylation; ND, not done; NA, not available.
*$P < 0.0001$, Fisher's exact test.
†$P < 0.0079$, Fisher's exact test.
‡$P < 0.0024$, Fisher's exact test.
§$P < 0.0105$, Fisher's exact test.

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At the present time, relatively few preoperative or monitoring RCC tumor markers exist in clinical practice. Previous studies have shown that DNA methylation of various genes in serum and plasma can be highly specific biomarkers for several human cancers (32). Although the true origins of cell-free DNA in serum related to this phenomenon remain unknown, possible sources include the release of DNA by normal cells undergoing apoptosis and the release of nucleic acids by tumor cells undergoing necrosis (33). In the present study, methylation of Wnt antagonist genes could be detected in serum cell-free DNA of RCC patients by conventional MSP analysis with methylation patterns identical to that in tumor tissue DNA. Conversely, no serum DNA from normal controls had aberrant methylation. In addition, hypermethylation of Wnt antagonist genes in serum DNA was significantly correlated with tumor grade and stage as was tissue DNA. These findings clearly suggest that (*a*) detection of methylated Wnt antagonist genes is feasible and reliable and (*b*) the serum M score of Wnt antagonist genes can be used as an excellent noninvasive biomarker for preoperative RCC detection, staging, and prognosis. In fact, some reports have found blood-borne hypermethylated DNA in patients with advanced-stage cancer and have noted a correlation with diminished prognosis (34, 35). Clinically, the serum M score of Wnt antagonist genes can be used for RCC detection, staging, and prognosis. Therefore, we think that this study can advance our knowledge on the role of Wnt antagonist family genes in clinical diagnosis and treatment. In addition, we hypothesize that this can be used as a surrogate marker for monitoring response to RCC therapy.

In conclusion, hypermethylation of the Wnt antagonist genes plays an important role in the pathogenesis and progression of RCC and can be readily detected in the serum of RCC patients. We have shown that a novel M score analysis using methylated Wnt antagonist genes as molecular markers can contribute to RCC detection, staging, and prognosis. To the best of our knowledge, this is the first report showing that M score analysis of Wnt antagonist genes can serve as a valuable new biomarker blood test for RCC.

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