Methylation of the \(\gamma\)-Catenin Gene Is Associated With Poor Prognosis of Renal Cell Carcinoma

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ABSTRACT

Purpose: \(\gamma\)-Catenin is a cell adhesion protein, and its functional loss is associated with tumor invasion and metastasis. We hypothesize that (1) promoter CpG methylation regulates the expression and function of the \(\gamma\)-catenin gene in renal cell carcinoma (RCC) and (2) methylation of the \(\gamma\)-catenin gene is associated with poor prognosis of RCC. To test these hypotheses, we analyzed the CpG methylation status of the \(\gamma\)-catenin gene and its correlation with clinical outcome in RCC.

Experimental Design: Genomic DNA and total RNA were extracted from three renal cancer cell lines (A498, Caki-1, and Caki-2) and 54 RCC tissue samples with their corresponding normal kidney tissue samples. Expression of \(\gamma\)-catenin gene was analyzed by reverse transcription-PCR and immunostaining. Promoter methylation was analyzed by two different methylation-specific PCR (MSP-A and MSP-B), and the results were verified by DNA sequencing.

Results: The demethylating agent (5-aza-2'-deoxycytidine) increased levels of mRNA transcript of the \(\gamma\)-catenin gene in three renal cancer cell lines. \(\gamma\)-Catenin mRNA and protein expression were significantly reduced in RCC samples compared with normal kidney samples, respectively \((P < 0.05)\). MSP-A and MSP-B bands were detected in 45 of 54 (83.3%) and 49 of 54 (90.7%) RCC samples, respectively. In normal kidney, weak products of MSP-A and MSP-B were detected in 5 of 54 (9.3%) and 6 of 54 (11.1%) samples, respectively. Likewise, both MSP-A and MSP-B ratios were significantly higher in RCC samples compared with normal kidney samples, respectively \((P < 0.01)\). Multivariate analysis revealed that the MSP-B ratio was a powerful and independent predictor superior to nuclear grade and Robson stage with respect to survival and disease progression \((P = 0.029\) and 0.0071, respectively). No mutations in the NH2-terminal region of \(\gamma\)-catenin were found in this study.

Conclusion: Expression of \(\gamma\)-catenin is regulated by promoter CpG methylation, and the balance between methylated and unmethylated RCC cell populations could determine its functional role. Because the conventional nuclear grade and/or staging system have some limitations to predict precise clinical outcome, this is the first report demonstrating that promoter CpG methylation of \(\gamma\)-catenin can be an independent and superior predictor for survival and disease progression.

INTRODUCTION

The catenins are a family of cytoplasmic proteins that were originally identified by their association with the cell adhesion molecule E-cadherin (1). The \(\gamma\)-catenin gene, mapped to the long arm of human chromosome 17 near the BRCA1 gene (2), is one of the components of the submembranous plaque of adhesion junctions and desmosomes in mammalian cells. Like its structural and functional homologue, \(\beta\)-catenin and \(\gamma\)-catenin contain a highly conserved central region of 12 armadillo repeats, which facilitate binding to various proteins, including E-cadherin, \(\alpha\)-catenin, and adenomatous polyposis coli tumor suppressor (3). Both \(\beta\)-catenin and \(\gamma\)-catenin play a pivotal role in maintaining the cellular polarity and serve as cell-to-cell attachment molecules through direct interaction with the cytoplasmic domain of E-cadherin (4, 5). In cancer tissue, escape from the cell adhesive system is an important mechanism whereby tumor cells gain the ability to invade and/or metastasize. Functional loss of \(\gamma\)-catenin results in the disruption of intercellular interaction among tumor cells and allows them to invade into surrounding tissues, thus representing worse biological potential (6, 7).

Renal cell carcinoma (RCC) accounts for \(\sim 2\%\) of visceral malignancies worldwide (8), and the incidence in North America and northern Europe has been increasing at \(\sim 3\%\) per year (8). This increased incidence is in part due to the widespread usage of noninvasive imaging such as ultrasound for the detection of asymptomatic early-stage tumors (9). Although tumor stage is significantly associated with clinical outcome, one study showed that 11% of patients with locally confined tumors die of disease progression in \(<5\) years after radical nephrectomy (10). Although several prognostic variables for renal carcinoma and its various subtypes have been proposed, tumor outcome prediction for this form of malignancy is the subject of much debate (11, 12). Tumor heterogeneity in RCC leaves the tumor-node-metastasis staging system inadequate for accurate prediction of prognosis. Current prognostic research focuses on...
the molecular pathology of renal epithelial tumors and the potential identification of molecular mechanisms that contribute to differences in tumor aggressiveness. Because of its role in intracellular adhesion and the maintenance of normal structure and function, studies on cadherin/catenin cell adhesion complex warrants further investigation.

Recent studies have shown that hypermethylation of normally unmethylated CpG dinucleotides (CpG islands) in the promoter region of genes are involved in transcriptional silencing (13, 14). In subsets of thyroid cancer (15) and lung cancer (16), expression level of g-catenin was decreased or lost. In these cancers, one of the mechanisms underlying down-regulation of g-catenin is through CpG hypermethylation of the g-catenin promoter (17). However, such studies are limited in human RCC. In this study, we hypothesized that the mechanism of g-catenin down-regulation in renal cancer is through epigenetic alteration resulting from hypermethylation of CpG islands within its promoter region. We also hypothesize that methylation of the g-catenin gene is associated with poor prognosis of RCC. To test these hypotheses, the expression and methylation status of the functional promoter of g-catenin gene were analyzed in normal kidney and RCC samples with special reference to the clinical outcome of RCC patients.

MATERIALS AND METHODS

Renal Cancer Samples. Tissue specimens were obtained from 54 patients (42 males and 12 females) with primary RCC who underwent radical nephrectomy. The mean age of these patients was 58.3 years (range, 37-82 years). Half of the surgical specimens was fixed in 10% buffered formalin (pH 7.0) and embedded in paraffin wax, and 5-μm sections were used for histologic evaluation. The remaining half was stored in aliquots at −80°C until processed for nucleic acid extraction. All tumors were classified based on the tumor-node-metastasis classification of malignant tumors by the Union International Contra Cancer (18). The staging was as follows: pT1 in 26, pT2 in 10, pT3 in 16, and pT4 in 2 cases; grade 1 in 17, grade 2 in 29, and grade 3 in 8 cases; nonpapillary in 53 cases and papillary in 1 case. In addition, the Robson staging system was also employed (ref. 19; stage I/II in 36 and stage III/IV in 18 cases).

Cell Culture. The human RCC cell lines A498, Caki-1, and Caki-2 were obtained from the American Type Culture Collection (Manassas, VA). The A498 and Caki-1 cell lines were maintained in RPMI 1640 with L-glutamine and sodium pyruvate. The Caki-2 cell line was incubated in McCoy’s 5A medium with L-glutamine, and 10% FCS were added to all media. The cells were maintained in a humidified atmosphere of 5% CO2, 95% air at 37°C.

Nucleic Acid Extraction. Genomic DNA and total RNA were extracted from each freshly frozen sample or cultured cells using Tri-Reagent (Molecular Research Center, Cincinnati, OH). To avoid contamination of normal tissues, the samples were microdissected under high-power microscope. The concentrations of DNA and RNA were determined spectrophotometrically, and its integrity was checked by 1.5% gel electrophoresis.

cDNA Preparation and Reverse Transcription-PCR Analysis. The cDNA was constructed by reverse transcription (Promega Corp., Madison, WI) using RNA as a template. Samples were stored at −20°C until further used. For differential reverse transcription-PCR (RT-PCR), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer (100 nmol/L each) and g-catenin primer (200 nmol/L each) or E-cadherin primer (150 nmol/L each) were used. The annealing temperature was 55°C for differential RT-PCR reactions, with 26 cycles for cell line samples and 32 cycles for clinical samples. The primer sequences are shown in

![Fig 1](expression_of_g-catenin_in_RCC_cell_lines.jpg)
The PCR products were electrophoresed on 2.0% agarose gel, and the expression levels were evaluated by ImageJ software (http://rsb.info.nih.gov/ij), and the areas under the curves were calculated and analyzed. The expression level of γ-catenin was quantified relative to GAPDH expression and expressed as arbitrary units. For semiquantitative analysis of the amplified products, a suitable number of PCR cycles for γ-catenin and GAPDH were determined for the exponential phase. For this purpose, we performed RT-PCR using five different diluted specimens from the same sample (1:1, 1:2, 1:4, 1:8, and 1:16) and calculated the absorbance of each band. At 32 cycles, the logistically converted densities of each band from the same sample were on the same regression line. Thus, the PCR amplification was considered to be within the exponential phase.

V-Aza-2-Deoxycytidine Treatment. A498, Caki-1, and Caki-2 cell lines were treated with 5-aza-2′-deoxycytidine (5-Aza-dC) to screen for epigenetic alterations. 5-Aza-dC was added to fresh medium at a concentration of 5 μM/L in duplicate. The cultured cells were harvested after 4 days of 5-Aza-dC treatment. Using both protein and cDNA obtained from renal cancer cell lines, γ-catenin gene expression before and after 5-Aza-dC treatment was analyzed by Western blotting and RT-PCR.

Immunohistochemistry. Immunostaining was done on formalin-fixed, paraffin-embedded sections. Antigen retrieval was carried out by microwaving in 10 mmol/L sodium citrate buffer. Slides were incubated overnight with a 1:400 dilution of monoclonal antibody (Transduction Laboratories, Lexington, KY). The remainder of the staining process was done using a commercial kit (Santa Cruz Biotechnology, Santa Cruz, CA). 3,3′-Diaminobenzidine (Sigma, St. Louis, MO) was used as chromogen, and slides were counter stained with hematoxylin.

Methylation Analysis. Genomic DNA (100 ng) was subjected to sodium bisulfite modification using a commercial kit (Invitrogen Life Technologies, San Diego, CA). Complete conversion of “C” to “T” by sodium bisulfite modification was verified using the method developed in our laboratory (20). Based on the functional promoter sequence (21), methylation-specific and unmethylation-specific primers were designed using MethPrimer as described by our laboratory (ref. 22; http://itsa.ucsf.edu/~urolab/methprimer). The regions amplified by these primers, in which 16 CpG sites are present, and their relationship to the CpG sites are shown in Fig. 2. Methylation-specific PCR (MSP) was carried out by a second round of nested PCR (MSP-A, MSP-B, and USP) using the universal PCR product amplified by Pan-S and Pan-AS primers as a template.

![Diagram showing the location of CpG sites and primers within the functional promoter of the γ-catenin gene.](image)

- **Methylation analysis**
  - Universal primer
    - Pan-S: 5′-GGTTTTGGTGTAGTTGTTTTT-3′
    - Pan-AS: 5′-ACCTCAATCACCTTTAATAAACTTACCTC-3′
  - MSP primer
    - GM1-S: 5′-GGTTTTGGTGTAGTTGTTTTT-3′
    - GM1-AS: 5′-ACCTCAATCACCTTTAATAAACTTACCTC-3′
  - GMS: 5′-TAACCTACTAAATAAAACCTAAGCG-3′
  - gamma-MAS: 5′-GACGAAACGCCCTAA-3′
  - gamma-M-S: 5′-ACCGAAATCAGAGTCGTCG-3′
  - gamma-M-AS: 5′-ACCGAAATCAGAGTCGTCG-3′
  - USP primer
    - gamma-U-S: 5′-GGTTTTGGTGTAGTTGTTTTT-3′
    - gamma-U-AS: 5′-ACCTCAATCACCTTTAATAAACTTACCTC-3′

- **Bisulfite DNA sequencing primer**
  - BD-S: 5′-TTCAGGTGGGGTTTTGTTAGT-3′
  - BD-AS: 5′-TAACCTACTAAATAAAACCTAAGCG-3′
  - SSCP primer
    - G-S: 5′-CTCAGTACGCTTACGATGAAAGTG-3′
    - G-AS: 5′-GCCCGTACGCTTACGATGAAAGTG-3′
    - G-AAS: 5′-CGGAGGTGATGAAACCTGAGT-3′
    - G-AS: 5′-CTCAGTACGCTTACGATGAAAGTG-3′
    - G-AAS: 5′-CGGAGGTGATGAAACCTGAGT-3′

Fig. 2. Schematic representation of the location of CpG sites and primers within the functional promoter of the γ-catenin gene. The first universal PCR was done using the Pan-S and Pan-AS primers. These universal primers do not cover any CpG sites within the primer sequences. MSP-A and MSP-B methylation was determined by the primer pairs GM1-S and γM-AS and γM-S and GM1-AS, respectively, using the first universal PCR product as a template. USP was determined by the primer pairs γU-S and γU-AS. PCR-SSCP was done using the primer pairs γG-S and γG-AS or G-S and G-AS to confirm whether mutation or single nucleotide polymorphism was present within the functional promoter of γ-catenin gene. The location of G-AS was not provided in this schema. Either primers BD-S or BD-AS were used for bisulfite DNA sequencing. Numbers on individual CpG sites, nucleotide number from the transcription start site based on the sequence (21) and Genbank data (accession no. AF233882). Lower half, primer sequence.
The first universal primer sets have no CpG sites in either forward or reverse primer. The PCR products MSP-A, MSP-B, and USP correspond to those obtained using the primer pairs GM1-S and γM-AS, γM-S and GM1-AS, and γU-S and γU-AS, respectively. For semiquantitative analysis, a suitable number of PCR cycles for MSP-A, MSP-B, and USP were determined within the linear range of the reaction. To check this, at least one initial PCR was done using 32 cycles each for MSP-A and MSP-B and 30 cycles for USP. Products were electrophoresed and stained with ethidium bromide, and a suitable PCR cycle was chosen for each sample. In each assay, absence of DNA template served as negative control. The primer sequences and PCR conditions are shown in Fig. 2. The products of MSP-A, MSP-B, and USP primers were analyzed on a nondenaturing 12% polyacrylamide gel. The area under the curve corresponding to each band was calculated using ImageJ software (http://rsb.info.nih.gov/ij), and the relative MSP-A and MSP-B levels were determined using the formula: A/A + U × 100%, where A and U are the areas under the curve corresponding to the MSP-A and USP band intensity. MSP-B ratios were also determined by the same procedure. B, MSP-A, MSP-B, and USP bands were present in all the normal kidney samples. MSP-A or MSP-B bands were lacking in most of the normal kidney samples.

**Statistical Analysis.** The differences in γ-catenin mRNA expression between normal kidney and RCC samples or among RCC samples with lower and higher MSP-B ratios were analyzed by the Mann-Whitney’s U test. Relationship of γ-catenin immunostaining between normal kidney and RCC samples was analyzed by a χ² test. To evaluate the prognostic relevance of the variables, the probability was computed from the day of surgery until occurrence of disease progression, death, or the last follow-up. Curves were generated using the Kaplan-Meier method (19), and the difference between curves was analyzed using a log-rank test. Multivariate survival analysis was carried out using a logistic regression and its stepwise variant. P < 0.05 was regarded as statistically significant.

**RESULTS**

**Expression Level of γ-Catenin in Human Kidney Samples.** Typical immunostaining and RT-PCR of γ-catenin with the corresponding densitometry graph are shown in Fig. 4. In all normal kidney samples, γ-catenin expression was strong, whereas in RCC tissues the majority of samples showed weak immunostaining. No nuclear accumulation of γ-catenin protein was observed in either renal cancer cells or normal kidney cells (data not shown). The difference in γ-catenin immunoreactivity between normal kidney and RCC samples reached a statistical significance (P < 0.01). Similarly, the expression level of γ-catenin mRNA transcripts was significantly lower in RCC samples than in normal kidney samples (P < 0.05).

**γ-Catenin Expression in RCC Cell Lines before and after Treatment with Demethylating Agent (5-aza-dC).** Expression level of γ-catenin mRNA transcript was significantly increased in Caki-1, Caki-2, and A498 cell lines after 5-Aza-dC treatment. The difference in the same region of methylation analysis, genomic DNA from each clinical sample was amplified using the primer pairs G-S and G-AS (Fig. 2). Mutation screening by SSCP was done using a nonradioactive procedure (23).
treatment, whereas low levels of mRNA transcript were observed in untreated controls (Fig. 1A). Similarly, the mRNA transcript of E-cadherin was very low or absent before 5-Aza-dC treatment, whereas it was significantly increased in these three cell lines with treatment with 5-Aza-dC (Fig. 1B).

**Methylation Status of γ-Catenin Promoter in Clinical Samples.** Typical MSP-A, MSP-B, and USP bands in RCC samples and in corresponding normal kidney samples are shown in Fig. 3B and C. Forty-five of 54 (83.3%) and 49 of 54 (90.7%) cancer samples showed positive MSP-A and MSP-B bands, respectively, whereas in normal kidney samples 5 of 54 (9.3%) and 6 of 54 (11.1%) had only weakly positive MSP-A and MSP-B bands, respectively. Surprisingly, 52 of 54 RCC samples (96.3%) also showed USP bands (Fig. 3B). Because either MSP-A or MSP-B ratios did not follow normal distribution, the RCCs were divided into two groups based on the median value of each ratio. In RCC with a higher MSP-B ratio, there was a significantly lower level of γ-catenin mRNA expression compared with those RCC with lower MSP-B ratio \((P < 0.05; \text{Fig. 3D})\), indicating an inverse correlation between MSP-B status and γ-catenin mRNA expression. On the other hand, there was no significant correlation between MSP-A status and γ-catenin expression (data not shown).

Bisulfite DNA sequencing of RCC samples (Fig. 5) showed coexistence of completely methylated CpG sites (as is evident by only “C” peak) along with partially methylated (“C” peak covered with “T” peak) and unmethylated (only “T” peak) CpG sites. Both MSP-A and MSP-B ratios were significantly higher in RCC samples than in normal kidney samples \((P < 0.01, \text{respectively})\). In RCC samples, the MSP-A ratio \((0.21 \pm 0.14)\) was significantly lower than the MSP-B ratio \((0.46 \pm 0.16; P < 0.001)\). However, both ratios did not correlate with nuclear grade and Robson stage (data not shown).

**Prognostic Correlation of γ-Catenin Methylation Status.** As shown in Fig. 6, univariate analysis showed that 27 patients with higher MSP-B ratios were associated with both worse progression-free and overall survival in comparison with 27 with lower MSP ratio \((P < 0.01)\). On the other hand, the correlation of higher MSP-A ratios with prognosis failed to reach statistical significance (data not shown). Because the variables used in this study might be interrelated with each other, multivariate analysis was done using logistic regression with a stepwise variant. The variables applied to multivariate analysis were age (<60 versus >60 years), sex (male versus female), grade (grade 1 versus grade 2/3), Robson stage (stage I/II versus stage III/IV), and MSP-B ratio (lower versus higher). As shown in Fig. 6, multivariate analysis revealed that MSP-B ratio in γ-catenin promoter was a powerful and independent predictor factor superior to nuclear grade and Robson stage in the light of disease progression and survival \((P = 0.012 \text{ and } 0.039, \text{respectively})\).

**Mutational Analysis of γ-Catenin Gene.** Among 54 RCC samples, no mutations in the NH2-terminal region of γ-catenin were observed.

**DISCUSSION**

Down-regulation of γ-catenin is frequently observed in thyroid, lung, and bladder cancers \((6, 7, 15–17)\). Such studies are limited in kidney cancer, and only one article reported that, using immunohistochemistry, down-regulation of γ-catenin was associated with poor prognosis in patients with kidney...
cancer (24). However, the mechanisms that contributed to the inactivation of γ-catenin expression in malignant cells has not been fully investigated. Recent publications on lung cancer suggest that CpG island methylation is one of the mechanisms underlying the loss of γ-catenin expression (17). In the present study, Western analysis of γ-catenin in A498, Caki-1, and Caki-2 cells showed that γ-catenin expression was increased after treatment with demethylating agent (5-aza-dC) compared with nontreated cells (data not shown). Likewise, expression levels of mRNA transcripts of γ-catenin gene were significantly higher after 5-aza-dC treatment. There was weak expression of γ-catenin gene even before 5-aza-dC treatment (Fig. 1A), suggesting that γ-catenin gene promoter is partially methylated. Similarly, at both protein and mRNA levels, γ-catenin expression was significantly lower in renal cancer samples than in normal kidney samples. These finding indicated that CpG hypermethylation may be one of the mechanisms underlying down-regulation of γ-catenin in RCC tissues. We also hypothesize that the functional role of γ-catenin as a cell adhesion molecule is regulated by the balance between methylated and unmethylated CpG sites in the γ-catenin gene promoter, which may define the biological aggressiveness of individual renal cancer tissues.

E-cadherin, another important molecule involved in cell adhesion signaling, is frequently affected by epigenetic CpG hypermethylation in human prostate and renal cancer tissues (22, 25). As shown in Fig. 1B, mRNA transcripts for E-cadherin were lacking in all three RCC cell lines before 5-aza-dC treatment, although they were significantly increased after 5-aza-dC treatment.

Based on the structure of the minimum functional promoter of the γ-catenin gene (20), we designed universal primers, methylation-specific primers and unmethylation-specific primers (Fig. 2). For this study, the PCR products of the first universal primers were used as template for second nested PCR using methylation-specific (MSP-A and MSP-B) and unmethylation-specific primers (USP). As shown in Fig. 3, the vast majority of RCC samples showed either MSP-A or MSP-B band, whereas in normal kidney samples frequency of MSP-A and MSP-B bands were 9.3% and 11.1%, respectively. The primer sequence in forward and reverse directions used to detect USP bands covers 5 and 4 CpG sites, respectively. This in turn indicates that these primers are sufficient enough to distinguish between unmethylated and methylated alleles. Interestingly, despite using these specific primers, the majority of our RCC samples also had both USP and MSP-B bands (Fig. 3B). Taken together with the Western blotting and RT-PCR results, we found that γ-catenin CpG sites are partially but not completely methylated in the majority of RCC samples. To confirm these observations, direct bisulfite sequencing was done. As shown in Fig. 5, bisulfite DNA sequencing of surgically obtained RCC tissues showed that several CpG sites were completely methylated (“C” peak only), partially methylated (“C” peak covered with “T” peak), or unmethylated (“T” peak only). When dealing with surgically obtained RCC samples, major concern is the influence of contaminated unmethylated “normal” cells on the results. As shown in the bisulfite DNA sequencing, because every CpG site was not always evenly methylated with the same percentage between “C” and “T” peaks, these partially methylated CpG sites were probably not caused by the contamination of normal unmethylated cells in the cancer cell population. Therefore, bisulfite DNA sequencing in clinical RCC tissues may be one of the mechanisms underlying the loss of γ-catenin and E-cadherin expression in malignant cells.
be an epigenetic characteristic in RCC tissues and that levels of γ-catenin may be regulated by the balance between methylated and unmethylated cancer cell populations.

Based on the heterogeneous renal cancer cell populations with respect to γ-catenin methylation, the balance between methylated and unmethylated alleles of γ-catenin seems to be more relevant as determined by MSP-A and MSP-B ratios. Although both MSP-A and MSP-B ratios are correlated with each other, only the MSP-B ratio showed significant correlation with mRNA transcript levels of γ-catenin. In comparison with normal kidney samples, the MSP-A or MSP-B ratio was significantly higher in RCC specimens; however, none of the MSP-A and MSP-B ratios showed any significant correlation with either higher nuclear grade or Robson staging. Taken together, these findings suggest that methylation of the functional promoter of γ-catenin gene might be an early event in the pathogenesis of renal cancer. As shown in Fig. 6, 25 patients with elevated MSP-B ratio were strongly associated with worse overall and progression-free survival. This is in accordance with the functional loss of catenin-dependent intracellular adhesion system representing the disintegration of the barrier that prevents tumor progression and metastasis (7, 16). Multivariate analysis also revealed that the MSP-B ratio of γ-catenin gene is a powerful and independent predictor of both survival and disease progression. In several cancer cell lines, reduced expression of γ-catenin correlates with neoplastic transformation (17), and its overexpression can inhibit the tumorigenicity and cell growth of SV40-transformed 3T3 cells (26), confirming its functional role as a tumor suppressor. Thus, it is possible that the epigenetic dysregulation of γ-catenin gene by promoter hypermethylation in renal cancer tissue could provide an extraordinary opportunity to alter tumor suppressive potential in addition to metastasis and/or invasion potential at an early phase of renal cancer pathogenesis.

γ-Catenin is a very closely related to β-catenin in amino acid sequences (5) and has a GSK-3β phosphorylation consensus motif at the NH2 terminus (27). Overexpression of β-catenin in cells is generated by mutations within the consensus motif of the GSK-3β phosphorylation site or by an adenomatous polyposis coli mutation. β-Catenin serves as a mediator of signal transduction by the Wnt signaling pathway (27). Because mutation of the GSK-3β phosphorylation site is associated with intracellular accumulation of γ-catenin (27, 28), the presence of both methylated and mutant alleles could reduce the influence of methylation on γ-catenin expression. In this study, mutational analysis using SSCP failed to show any mutations within the region encompassing the consensus motif of GSK-3β phosphorylation site. In addition, overexpression and/or nuclear

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**Fig. 6** Relationship of MSP-B ratio with progression-free and survival probability. A, prognostic relevance of MSP-B ratio to disease progression. Multivariate analysis shows that higher MSP-B ratio is powerful predictor for early disease progression. B, RCC tissues with higher MSP-B ratio are significantly associated with worse survival probability. Prognostic relevance of MSP-B ratio survival is superior to nuclear grade and Robson stage.

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accumulation of γ-catenin were not observed in RCC tissues. Therefore, these findings suggest that coexistence of methylated and mutant alleles, which might alternatively explain the rare loss of γ-catenin expression in RCC tissues, is less plausible.

In conclusion, we have shown that partial methylation of γ-catenin is an epigenetic characteristic in RCC tissues and that the functional role of γ-catenin might be regulated by the balance between methylated and unmethylated cancer cell populations. Because the conventional nuclear grade and/or staging system have limitations in their ability to predict clinical outcome, CpG methylation of γ-catenin gene promoter could provide better information to help manage RCC patients postoperatively.

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