Promoter hypermethylation of the potential tumor suppressor DAL-1/4.1B gene in renal clear cell carcinoma

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Renal clear cell carcinoma (RCCC) is a malignant tumor with poor prognosis caused by the high incidence of metastasis to distal organs. Although metastatic RCCC cells frequently show aberrant cytoskeletal organization, the underlying mechanism has not been elucidated. DAL-1/4.1B is an actin-binding protein implicated in the cytoskeleton-associated processes, while its inactivation is frequently observed in lung and breast cancers and meningiomas, suggesting that 4.1B is a potential tumor suppressor. We studied a possible involvement of 4.1B in RCCCs and evaluated it as a clinical indicator. 4.1B protein was detected in the proximal convoluted tubules of human kidney, the presumed cell of origin of RCC. On the other hand, loss or marked reduction of its expression was observed in 10 of 19 (53%) renal cell carcinoma (RCC) cells and 12 of 19 (63%) surgically resected RCC by reverse transcription-PCR. Bisulfite sequencing or bisulfite SSCT analyses revealed that the 4.1B promoter was methylated in 9 of 19 (47%) RCC cells and 25 of 55 (45%) surgically resected RCC, and inversely correlated with 4.1B expression (p < 0.0001). Aberrant methylation appeared to be a relatively early event because more than 40% of the tumors with pT1a showed hypermethylation. Furthermore, 4.1B methylation correlated with a nuclear grade (p = 0.017) and a recurrence-free survival (p = 0.0036) and provided an independent prognostic factor (p = 0.038, relative risk 10.5). These results indicate that the promoter methylation of the 4.1B is one of the most frequent epigenetic alterations in RCCC and could predict the metastatic recurrence of the surgically resected RCC.

Key words: tumor suppressor gene; bi-sulfite sequencing; two-hit inactivation; recurrence-free survival rate; independent prognostic factor

Renal cell carcinoma (RCC) accounts for about 2% of human cancers worldwide, with an incidence of 189,000 and a mortality of 91,000 reported in the year of 2000.1 Renal clear cell carcinoma (RCCC), which represents 75% of all RCC, exhibits frequent metastasis to distant organs without any clinical symptoms. Furthermore, 40–60% of RCCC tumors without metastasis at first presentation eventually develop metastasis as they progress.2 Finally, metastatic RCCC becomes refractory to any therapeutic approaches, including chemotherapies, radio- and hormonal therapies, resulting in a poor prognosis of patients, with a 5-year survival of less than 10%.3 Thus, understanding the molecular mechanisms of the development and progression of RCCC is a critical issue for controlling this refractory cancer.

Several genetic and epigenetic alterations have been reported in RCCC. The mutation of the VHL gene, associated with loss of heterozygosity (LOH) at the gene locus on chromosomal fragment 3p25–p26, was observed in ~50% of sporadic RCCC.4 Since the VHL encodes a component of an E3 ubiquitin ligase that promotes the degradation of hypoxia-inducible factors, loss of VHL function could be involved in angiogenesis, one of the most characteristic features of RCCC.5 Epigenetic inactivation of the RASSF1A gene is also reported frequently in RCCC.6–8 In addition, promoter methylation and/or aberrant expression of the E-cadherin and beta-catenin genes are also found at a high incidence in RCCC, suggesting that disruption of cell adhesion and cytoskeleton organization is also involved in RCCC.9,10 On the other hand, mutation of the H-, K-, N-ras and inactivation of the TP53 and RB1 genes are relatively rare events,11 while inactivation of the p16/CDKN2A gene is involved in a small subset of advanced RCCC.12

We have reported that the loss of function of the tumor suppressor in lung cancer 1 (TSLC1) protein, an immunoglobulin superfamily cell adhesion molecule, is implicated in a variety of human cancers in their advanced stages.13–17 In addition, we have demonstrated that TSLC1 directly binds to DAL-1/4.1B, an actin-binding protein, through its 4.1-binding motif. DAL-1 was originally isolated as an expressed fragment of the 4.1B gene, whose expression was down regulated in adenocarcinoma of the lung.18 Restoration of DAL-1 expression in nonsmall-cell lung cancer or breast cancer cell lines significantly suppressed cell growth in vitro.19 Moreover, loss of 4.1B expression was observed in human breast cancers and meningiomas, suggesting that the 4.1B gene is an additional target for inactivation in human cancers.1–21 Interestingly, 4.1B/DAL-1 interacts with spectrin, an actin-binding protein, and over expression results in altered cytoskeleton-associated properties, including cell adhesion and motility.20

To analyze the role of TSLC1 and 4.1B in RCCC, we analyzed 55 surgically resected RCCC and 19 cell lines in the present study. While we could not detect loss of TSLC1 expression, we did find significant alterations in 4.1B gene expression in these tumors. Herein, we demonstrated that hypermethylation of the 4.1B gene was a frequent event and could provide an independent prognostic factor for metastatic recurrence after completely resected RCCC.

Material and methods

Cell lines

RCC cell lines, Caki-2, SW839, ACHN, 786-O, 769-P, A-704, A-498 and Hs891.T, were obtained from the American Type

Abbreviations: LOH, loss of heterozygosity; NDS, normal donkey serum; PCR, polymerase chain reaction; RCC, renal cell carcinoma; RCCC, renal clear cell carcinoma; RT-PCR, reverse transcription-polymerase chain reaction; SNP, single nucleotide polymorphism; SSCT, single-strand conformation polymorphism; TNM, tumor-node-metastasis.

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Culture Collection (Rockville, MD); KMRC-1, KMRC-2, KMRC-3, VMRC-RCW, VMRC-RCZ and Caki-1 cells were from the Japanese Collection of Research Bio-resources (Tokyo, Japan); OS-RC-2, RCC10RGB, TUHR4TKB, TUHR10TKB and TUHR14TKB cells were from the Riken Cell Bank (Tsukuba, Japan). Cells were cultured according to the supplier’s recommendations.

Surgical specimens
Fifty-five pairs of cancerous and adjacent noncancerous tissues of RCCC were surgically resected at the National Cancer Center Hospital or the Hospital of the University of Tokyo, after obtaining written informed consent from each patient. Pathological diagnosis was performed or confirmed at Pathology Division, National Cancer Center Research Institute, and the clinicopathological features were determined according to the 1997 Union Internationale Contre le Cancer.22 Analyses of human materials were carried out according to the institutional guidelines.

Reverse transcriptase-polymerase chain reaction (RT-PCR)
Total cellular RNA was extracted using the RNeasy Mini Kit (QiAGEN, Valencia, CA). By using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA), 1 µg of total cellular RNA was reverse-transcribed, and an aliquot was amplified by polymerase chain reaction (PCR), using TITANIUM Taq DNA polymerase (BD Biosciences Clontech, Palo Alto, CA) to obtain a 572-bp fragment of DAL-1 cDNA and a 646-bp fragment of human β-actin cDNA in the same reaction. The primers used for PCR were 5’-GGTGCCGAGGAGGTCACTGACAAGGAACA G3’ and 5’-CGCTCCCACATTCATCTGGGTCAGTCTCCCG AG3’ for DAL-1 (1.0 µM, each) and 5’-GGTGCCGAGGAGGAAGAGGCACAAGGAACG-3’ and 5’-CGCTCCCACATTCATCTGGG TCAGTCTCCCGAG3’ for β-actin (0.2 µM, each).

Restoration of DAL-1 expression by 5-aza-2’-deoxycytidine
At day 0, 1 × 10^5 cells were seeded, treated with 5-aza-2’-deoxycytidine (10 µM; Sigma-Aldrich, St. Louis, MO) or PBS for 24 hr on days 2 and 5 and collected on day 8, as reported previously.23

Loss of heterozygosity (LOH) analysis
Five DNA fragments containing single nucleotide polymorphisms (SNPs) on 18p11.3, namely IMS-JST067229, IMS-JST031621, IMS-JST082513, IMS-JST143134 and IMS-JST119847, were analyzed for LOH as described previously.24

Bisulfite sequencing
Bisulfite sequencing was performed as described previously.25 Briefly, genomic DNA was denatured with NaOH (0.3 M) and incubated with sodium bisulfite (3.1 M; Sigma) and hydroquinone (0.8 mM; Sigma), pH 5.0, at 55°C for 20 hr, followed by purification and treatment of DNA with NaOH (0.2 M) for 10 min at 37°C. Modified DNA (100 ng) was subjected to PCR to amplify a 92-bp DNA fragment, using a pair of primers (DAL-1 PR2F: 5’-CGGAGTTTCCGTGTTTTTGTAAAGG-3’ and DAL-1 PR2R: 5’-GGCAGGCGTAGATAAAACCTAAACG-3’). The PCR products were subcloned to confirm the sequence of at least 4 clones for each sample.

Promoter hypermethylation of the 4.1B gene in RCCC
The 4.1B gene harbors a typical DNA sequence matching the criteria of a CpG island in its upstream region, exon 1, and the beginning of intron 1. To elucidate the molecular mechanisms underlying the loss of 4.1B expression, we examined the methylation status of the 4.1B promoter in RCCC cells. By using bisulfite sequencing, we had previously determined that hypermethylation of the 14 CpG sites within the 92-bp fragment around the 4.1B promoter strongly correlates with loss of expression in nonsmall-cell lung cancer cell lines. Bisulfite sequencing of the same fragment revealed that these CpG sites were highly methylated in TUHR10TKB and A704 cells lacking 4.1B expression, whereas they were not methylated in KMRC1 cell expressing a significant amount of 4.1B transcript (Figs. 2a and 2b). A similar analysis showed that hypermethylation was observed in 9 of 19 (47%) RCCC cell lines, where hypermethylation strongly correlated with loss of 4.1B expression (p = 0.0004, Fig. 1a). To examine the methylation status of the promoter quantitatively, we analyzed the promoter fragments by SSCP after PCR amplification of the bisul-
fite-treated DNA. As shown in Figures 2a and 2c, clones with known sequences in terms of CpG methylation showed distinct mobility in SSCP analysis, where clone I with no methylation and clone VI with complete methylation showed the slowest and the fastest mobility, respectively. Bisulfite SSCP of RCC cells revealed that TUHR10TKB and A704 cells showed a pattern of hypermethylation, while KMRC1 cell showed a pattern of no methylation, in agreement with the results obtained using bisulfite sequencing (Figs. 2a and 2d). Next, we examined the methylation status of the 4.1B in surgically resected RCCC. As shown in Figure 2e, DNA from tumors 4C, 5C and 6C showed no methylation, while that from 13C, 14C and 15C showed hypermethylation. DNA from noncancerous renal tissues 4N and 13N showed no methylation. A similar analysis revealed that 25 of 55 (45%) surgically resected RCCC showed hypermethylation.

**FIGURE 2** – Methylation analysis of the 4.1B promoter. (a): Schematic representation of the methylation status of the 4.1B promoter. A hatched box and an open box indicate a CpG island and exon 1 of the 4.1B. Vertical bars indicate CpG sites numbered 1–40. Black and white circles represent methylated and unmethylated CpG, respectively. Rows 1–4 indicate the results of independent clones. (b): Bisulfate sequencing of the 4.1B promoter in 3 RCC cells. Sequence traces in each sample correspond to the genomic sequence (~65 bp to ~23 bp from the transcription initiation site) shown in the top line. CpG sites, numbered 19–22, are underlined. Asterisks indicate the nucleotides corresponding to methylated cytosine residues at the CpG sites. (c)–(e): Bisulfate SSCP analyses of the cloned DNA fragments of known sequences (c), RCC cells (d), and surgically resected RCCC and corresponding noncancerous kidney (e). C and N in (e) indicate DNA from a cancerous and noncancerous portion of the kidney, respectively. Presence or absence of 4.1B expression determined in Figure 1 is shown as (+) or (−), respectively (d) (e).
FIGURE 2. METHYLATION OF 4.1B IN RENAL CLEAR CELL CARCINOMA

A. 4.1B CpG island

B. 5' - GGGGCGGGGGAGAAGCGCCAGCCCTGCGGCCTCC - 3'

C. Cloned DNA fragments

D. RCC cells

E. Surgically resected RCCCs

Figure 2.
We then examined the role of promoter methylation in gene silencing of the 4.1B gene by treating RCC cells with the de-methylating agent 5-aza-2′-deoxycytidine. Semi-quantitative RT-PCR analysis revealed that the expression of 4.1B mRNA following 5-aza-2′-deoxycytidine treatment was only observed in the Caki-2 and KMRC-3 cell lines harboring the hypermethylated 4.1B promoter, but not in the Caki-1 cell line lacking 4.1B promoter methylation. These results suggest that 4.1B promoter methylation is causally related to loss of 4.1B expression (Fig. 1c).

We next analyzed the allelic status of the chromosomal fragment, 18p11.3, around the 4.1B locus in RCC cells, using 5 highly polymorphic SNP markers. Ten of 19 RCC cell lines showed retention of heterozygosity in at least 1 locus per tumor. Five of these RCC cell lines (A704, TUHR4TKB, TUHR10TKB, KMRC3 and 769-P) harbored a hypermethylated 4.1B promoter and lacked 4.1B expression. These findings suggest that the 4.1B gene is inactivated by bi-allelic methylation in some RCC cell lines. In contrast, 9 RCC cell lines did not show heterozygosity at any loci examined, strongly suggesting that one allele of the 4.1B gene was deleted. Four of these RCC cell lines (ACHN, Caki-2, OS-RC-2, and 786-O) showed promoter hypermethylation with loss of 4.1B expression, suggesting that the 4.1B gene was inactivated by 2 hits involving both promoter methylation and LOH. Last, LOH was only observed in 4 of 54 (7.4%) informative cases in surgically resected RCCC, suggesting that bi-allelic methylation may represent the major mechanism to suppress 4.1B expression in primary RCCC.

Aberrant expression of 4.1B protein in surgically resected RCCC

We then examined 4.1B protein expression in human normal kidney as well as primary RCCC, using a polyclonal antibody against U2 domain of human 4.1B.13 As shown in Figure 3a, 4.1B protein was expressed in the baso-lateral membrane of the proximal convoluted tubules, from which RCCC arises. 4.1B protein expression was also found in the basement membrane of the glomeruli, but not in the distal convoluted tubules, Henle’s loops or collecting ducts in normal human kidney. An immunohistochemical study of 20 surgically resected RCCC revealed that 9 tumors (45%) demonstrated significant expression of 4.1B protein along the cell membrane, 8 of which (89%) carried the unmethylated 4.1B promoter (Fig. 3b). On the other hand, 6 tumors (30%), all of which (100%) harbored the hypermethylated 4.1B promoter, showed absence of 4.1B protein expression (Fig. 3c). In this regard, loss of 4.1B protein expression significantly correlated with 4.1B promoter hypermethylation (p = 0.0040, Table I). In addition, 5 tumors (25%) showed an aberrant pattern of 4.1B expression, in which weak signals of 4.1B protein were detected

| LOH analysis of the 4.1B gene |
|-----------------------------|
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| Table I – Methylation and Expression Status of 4.1B and Clinicopathological Characteristics in RCCC |
|-----------------------------------------------|
| 4.1B promoter expression analysis |
| Number of cases | Hypermethylation (%) | No methylation (%) | p-value |
|-----------------|----------------------|---------------------|--------|
| RT-PCR |
| Analyzed | 19 | 9 (47) | 10 (53) |
| Positive | 7 | 1 (14) | 6 (86) |
| Reduced | 2 | 0 (0) | 2 (100) |
| Negative | 10 | 8 (80) | 2 (20) | 0.0061 |
| Immunohistochemistry |
| Analyzed | 20 | 10 (50) | 10 (50) |
| Membrane | 9 | 1 (11) | 8 (89) |
| Aberrant | 5 | 3 (60) | 2 (40) |
| Negative | 6 | 6 (100) | 0 (0) | 0.0042 |
| Clinicopathological characteristics |
| Analyzed | 55 | 25 (45) | 30 (55) |
| Age (years) |
| 60 and older | 32 | 15 (47) | 17 (53) |
| Under 60 | 23 | 10 (43) | 13 (57) | NS1 |
| Gender |
| Male | 37 | 17 (46) | 20 (54) |
| Female | 18 | 8 (44) | 10 (56) | NS1 |
| Pathological stage |
| I | 36 | 15 (42) | 21 (58) |
| II | 8 | 4 (50) | 4 (50) |
| III | 8 | 4 (50) | 4 (50) |
| IV | 3 | 2 (67) | 1 (33) | NS1 |
| TNM classification |
| pT1a | 17 | 8 (47) | 9 (53) |
| pT1b | 21 | 8 (38) | 13 (62) |
| pT2 | 8 | 4 (50) | 4 (50) |
| pT3a | 2 | 1 (50) | 1 (50) |
| pT3b | 5 | 3 (60) | 2 (40) |
| pT3c | 2 | 1 (50) | 1 (50) | NS1 |
| pT4pT4 | 0 | 0 (0) | 0 (0) |
| pN0 | 54 | 25 (46) | 29 (54) |
| pN1pN2 | 1 | 0 (0) | 1 (100) | NS1 |
| pM0 | 53 | 23 (43) | 30 (57) |
| pM1 | 2 | 2 (100) | 0 (0) | NS1 |
| Nuclear grade |
| G1 | 22 | 5 (23) | 17 (77) |
| G2 | 27 | 17 (63) | 10 (37) |
| G3 | 6 | 3 (50) | 3 (50) | 0.0171 |

NS, not significant.
1Mann–Whitney U-test. 2Kruskal–Wallis test.
Methylation of 4.1B in Renal Clear Cell Carcinoma

Clinicopathological features of RCCC with hypermethylation of the 4.1B gene

To understand the clinicopathological significance of the promoter methylation of the 4.1B gene in surgically resected RCCC, we examined the pathological stage, tumor-node-metastasis (TNM) classification and nuclear grade of the tumors as well as the age and gender of the 55 patients. As shown in Table I, 4.1B hypermethylation was observed in 15 of 36 (42%) tumors representing stage I and in 8 of 17 (47%) tumors with pT1a, whereas the incidence of hypermethylation did not increase significantly in tumors in more advanced stages. These results suggest that 4.1B hypermethylation occurs in a subset of tumors as a relatively early event in multi-stage renal carcinogenesis. Correlation of the 4.1B hypermethylation with lymph node metastasis (pN) or distant metastasis (pM) could not be determined because the great majority of tumors examined were pN0 and pM0 at the time of resection. Interestingly, 4.1B hypermethylation was preferentially observed in tumors with higher nuclear grade (p = 0.017). On the other hand, the age and gender of the patients were not correlated with 4.1B hypermethylation.

Hypermethylation of the 4.1B gene correlates with the recurrence-free survival of the RCCC patients

Finally, we examined the significance of 4.1B methylation as a prognostic factor of metastatic recurrence for RCCC patients. Of 55 patients examined for 4.1B methylation, 53 patients who received complete surgical resection of RCCC were examined for their prognosis, whereas the other two patients were excluded from the analyses because they harbored metastasis at the time of resection. Kaplan-Meier analysis revealed that the recurrence-free survival of patients with tumors of 4.1B methylation was significantly shorter than that observed in patients with the unmethylated 4.1B promoter (p = 0.0036, Fig. 4). Furthermore, the multivariate analysis by the Cox hazard model indicated that 4.1B methylation was an independent prognostic factor, as shown in Table II (p = 0.038; relative risk, 10.5).

Discussion

The present study demonstrates that the epigenetic inactivation of the 4.1B gene is involved in primary RCCC and represents an independent prognostic factor for RCCC patients. Analysis of the expression, methylation and allelic status of the 4.1B gene revealed that hypermethylation and loss of expression were strongly correlated with each other in both the cell lines and surgically resected RCCC (p < 0.0001), as observed in other tumor suppressor genes. The 92-bp fragment including 14 CpG sites that we examined in this study contained a putative transcription start site of 4.1B gene and a Sp1-binding sequence, which suggests that some methyl-CpG binding proteins might suppress the transcription through interaction with this regulatory motif. While LOH at the 4.1B locus on 18p11.3 was not frequently observed in surgically resected RCCC, we demonstrated a two-hit inactivation of the 4.1B in a subset of cell lines by the promoter hypermethylation associated with LOH as well as through bi-allelic hypermethylation. These findings suggest that 4.1B may act as a potential tumor suppressor in human RCCC. It is worth noting that loss of 4.1B expression was also observed in Caki-1 cells and several tumors without 4.1B methylation (Figs. 1a and 1b). In this regard, treatment of Caki-1 cells with 5-aza-2'-deoxycytidine did not restore 4.1B expression (Fig. 1c). These results suggest that some mechanisms other than promoter methylation, such as histone deacetylation,
tion and deficiency of transcription factors, might be involved in the regulation of 4.1B expression in additional populations of RCC.

Immunohistochemical studies using anti-4.1B antibody provided information about 4.1B expression, but also 4.1B subcellular localization in primary RCC. In this study, we found a group of tumors with 4.1B mislocalization, in addition to RCC tumors lacking 4.1B expression due to promoter hypermethylation. In the tumors with abnormal 4.1B subcellular localization, 4.1B protein was expressed diffusely within the cytoplasm, but not along the cell membrane. Some membrane proteins anchoring DAL-1 to the cell membrane might be inactivated in these cases. This mislocalization might impair the ability of 4.1B to function as a potential tumor suppressor. In this regard, Robb et al. have recently shown that growth suppression of meningioma cells by 4.1B/DAL-1 requires proper membrane localization.26 This aberrant pattern of subcellular distribution in RCC tumors would be associated with impaired 4.1B function.

By using bisulfite-SSCP, a sensitive and highly quantitative method to detect the methylation status, we found 4.1B promoter hypermethylation in 25 of 55 (45%) surgically resected RCC. It has been speculated that the DNA methylation changes are rather rare events in RCC in comparison with other major malignancies.27,28 In fact, previous studies have reported that the incidences of promoter methylation in representative tumor suppressor genes, including the VHL, p16/CDKN2A, p14/ARF and APC genes, are less than 16% in RCC.8,28 However, the extensive analyses have demonstrated that the promoters of the Timp-3 and RASSF1A genes are methylated in 60% and 23–91% of primary RCC, respectively, suggesting that several critical genes are inactivated frequently by methylation in RCC as are in many other tumors.6-8 The incidence of promoter methylation of the 4.1B (45%) that we have observed in this study is comparable to that of the Timp-3 and RASSF1A genes. Therefore, loss of 4.1B function appears to be strongly selected for the malignant growth of RCC cells.

It is interesting that the incidence of 4.1B methylation is more than 40% in tumors with pT1a but does not increase as the T classification advances. The T classification of RCC is determined by the tumor size and the degree of invasion into the renal capsule or vein. In this regard, our findings suggest that 4.1B promoter hypermethylation is involved in a subset of tumors in a relatively early stage, and is not significantly associated with the tumor size or the degree of invasion at the time of surgical resection. Another interesting result is the significant correlation of 4.1B promoter hypermethylation with the nuclear grade, which is an indicator of nuclear abnormality of cancer cells (p = 0.017). It is worth noting that 4.1B interacts with 14-3-3, a crucial modifier of the G2 checkpoint, by sequestering Cdc2-cyclin B1 complex in the cytoplasm.29,30 While Robb et al. recently suggest that 14-3-3 might not represent the critical 4.1B effector protein,31 there is emerging data to support a role for 4.1B in the regulation of apoptosis.19,26

One of the most serious clinical problems of RCC is a frequent metastatic recurrence that occurs even after the tumors are completely resected in their early stages. 4.1B is an actin-binding protein involved in actin cytoskeleton organization and actin-mediated processes, including cell motility and adhesion.19,20 It is possible, therefore, to hypothesize that loss of 4.1B function might be involved in metastasis of RCC cells to distant organs. Our

![FIGURE 4 – Recurrence-free survival of the patients who received complete resection of RCC with hypermethylated and unmethylated 4.1B promoters. Intervals between the primary surgical resection and the metastatic recurrence at the lung, bone, liver, or pancreas are plotted in the Kaplan–Meier analysis. Log-rank P is included. N indicates number of cases.](image)

| Variable | Kaplan–Meier analysis p-value | Relative risk | 95% confidence interval | Multivariate proportional hazard analysis p-value |
|----------|-------------------------------|--------------|------------------------|-----------------------------------------------|
| 4.1B methylation status (U vs. M) | 0.0036 | 10.5 | 1.1–97.4 | 0.038 |
| Pathological stage (I, II vs. III, IV) | 0.039 | 4.0 | 0.83–19.6 | 0.083 |
| Nuclear grade (1 vs. 2, 3) | 0.059 | 1.8 | 0.18–18.1 | 0.62 |

1U, no methylation; M, hypermethylation.
findings that 4.1B promoter methylation is an independent prognostic factor of metastatic recurrence for RCCC patients would support this hypothesis. Furthermore, the observation that the recurrence-free survival of patients with tumors of 4.1B promoter hypermethylation was significantly shorter than that in patients without 4.1B promoter hypermethylation \((p = 0.0036)\) suggests that 4.1B expression might represent a surrogate marker for this metastatic feature. It should be noted that 2 patients with metastasis at the time of resection, who were excluded from this analysis, also showed 4.1B promoter hypermethylation in the primary RCCC. In conclusion, our results provide the first demonstration that 4.1B promoter hypermethylation was involved in the development and/or progression of RCCC and may represent an independent and novel prognostic factor of the metastatic recurrence for RCCC patients.

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