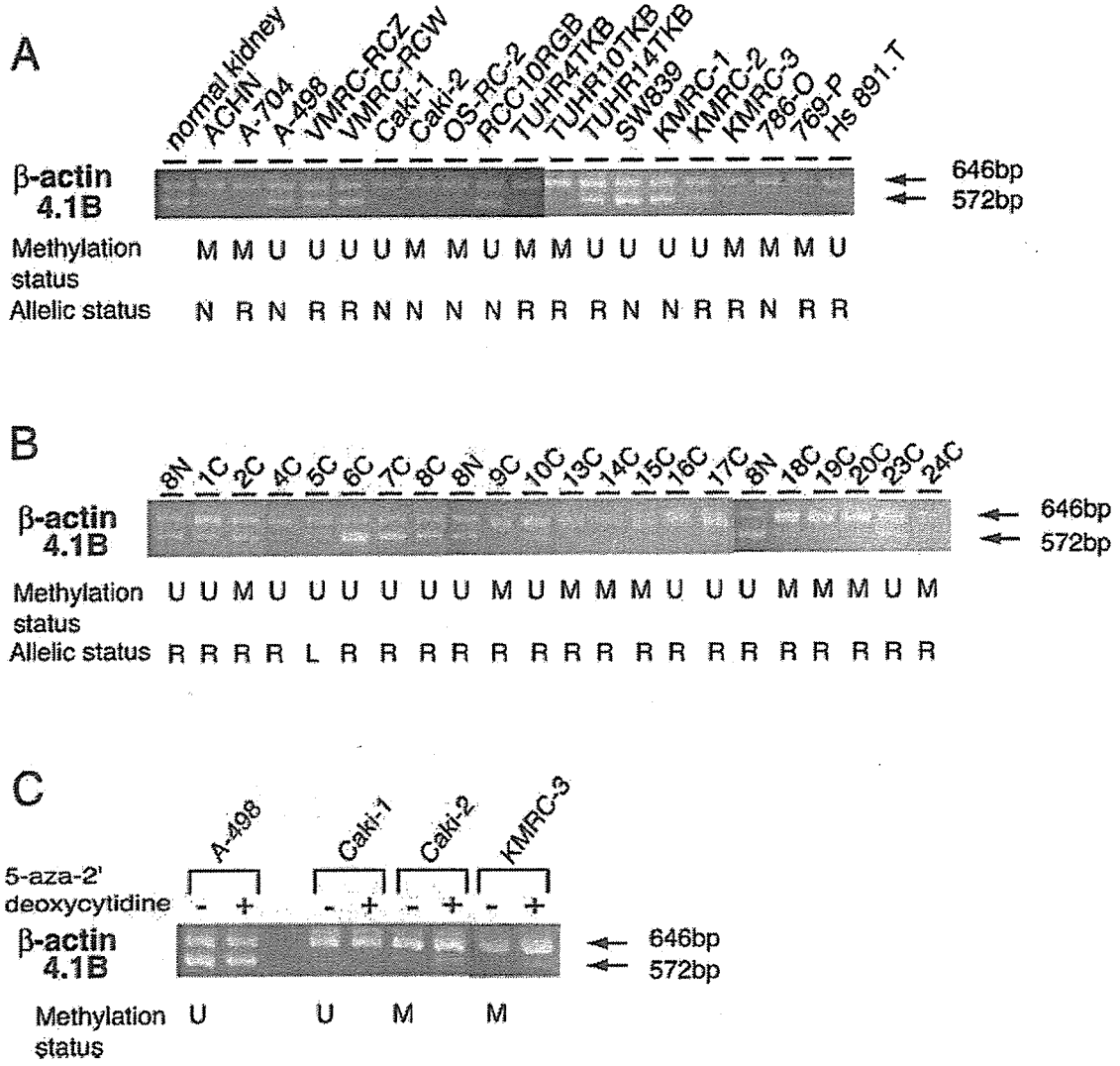

FIGURE 1 – Expression of the *4.1B* gene in RCC. (*a*) and (*b*): RT-PCR analysis of 4.1B and β-actin in RCC cell lines (*a*) and surgically resected RCCC (*b*). C and N in (*b*) indicate cDNA from a cancerous and noncancerous portion of the kidney, respectively. The results of methylation status determined in Figure 2 and allelic status are included as a reference. M and U indicate the hypermethylated and unmethylated promoter of the *4.1B*, respectively. R and L indicate retention and loss of heterozygosity, respectively. N in (*a*) indicates not informative. (*c*): RT-PCR analysis of 4.1B and β-actin in RCC cells treated with 5-aza-2′deoxycytidine (+) or PBS (−).

fite-treated DNA. As shown in Figures 2*a* and 2*c*, 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. 2*a* and 2*d*). Next, we examined the methylation status of the *4.1B* in surgically resected RCCC. As shown in Figure 2*e*, 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. *4.1B* promoter methylation strongly correlated with loss of 4.1B expression in a subset of surgically resected RCCC examined ($p = 0.0063$, Fig. 1*b*, Table I).

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*).

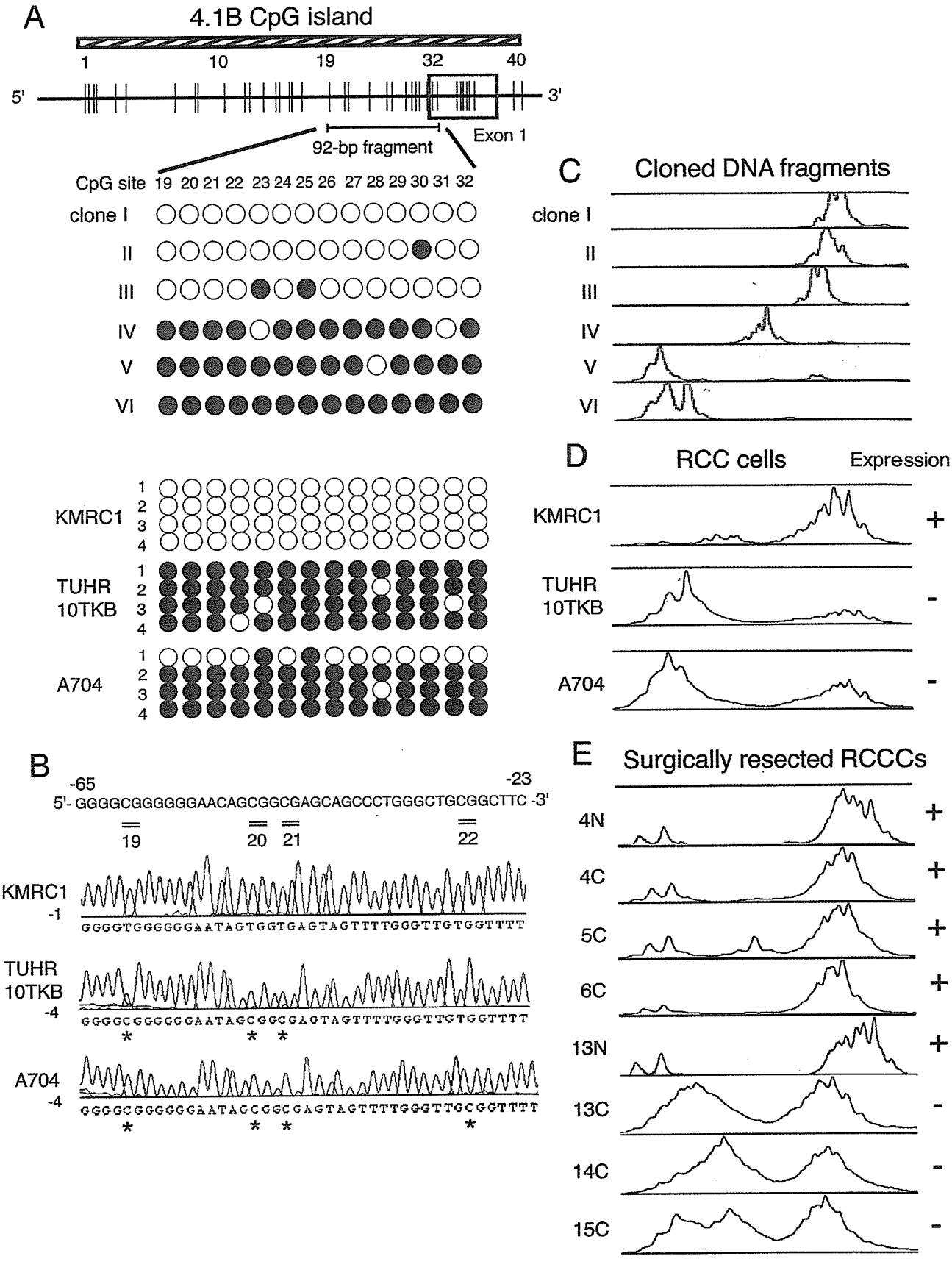
A
4.1B CpG island
1
10
19
32
40
5'
3'
92-bp fragment
Exon 1
CpG site 19 20 21 22 23 24 25 26 27 28 29 30 31 32
clone I
II
III
IV
V
VI
KMRC1
TUHR 10TKB
A704
B
-65
-23
5'- GGGGCGGGGGGAACAGCGGCGAGCAGCCCTGGGCTGCGGCTTC -3'
19
20 21
22
KMRC1
-1
GGGGTGGGGGGAATAGTGGTGAGTAGTTTTGGGTTGTGGTTTT
TUHR 10TKB
-4
GGGGCGGGGGGAATAGCGGCGAGTAGTTTTGGGTTGTGGTTTT
A704
-4
GGGGCGGGGGGAATAGCGGCGAGTAGTTTTGGGTTGCGGTTTT
C
Cloned DNA fragments
clone I
II
III
IV
V
VI
D
RCC cells
Expression
KMRC1
+
TUHR 10TKB
-
A704
-
E
Surgically resected RCCCs
4N
+
4C
+
5C
+
6C
+
13N
+
13C
-
14C
-
15C
-

FIGURE 2.

TABLE I – METHYLATION AND EXPRESSION STATUS OF 4.1B AND CLINICOPATHOLOGICAL CHARACTERISTICS IN RCCC

		4.1B Promoter		
	Number of cases	Hypermethylation (%)	No methylation (%)	*p*-value
4.1B expression				
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)	0.006[1]
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)	0.004[2]
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)	NS[1]
Gender				
Male	37	17 (46)	20 (54)	
Female	18	8 (44)	10 (56)	NS[1]
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)	NS[1]
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)	NS[1]
pT4	0	0 (0)	0 (0)	
pN0	54	25 (46)	29 (54)	
pN1,pN2	1	0 (0)	1 (100)	NS[1]
pM0	53	23 (43)	30 (57)	
pM1	2	2 (100)	0 (0)	NS[1]
Nuclear grade				
G1	22	5 (23)	17 (77)	
G2	27	17 (63)	10 (37)	
G3	6	3 (50)	3 (50)	0.017[1]

NS, not significant.
[1]Mann–Whitney *U*-test.–[2]Kruskal–Wallis test.

We then examined the role of promoter methylation in gene silencing of the *4.1B* gene by treating RCC cells with the demethylating 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. 1*c*).

LOH analysis of the 4.1B gene

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 3*a*, 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. 3*b*). 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. 3*c*). 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

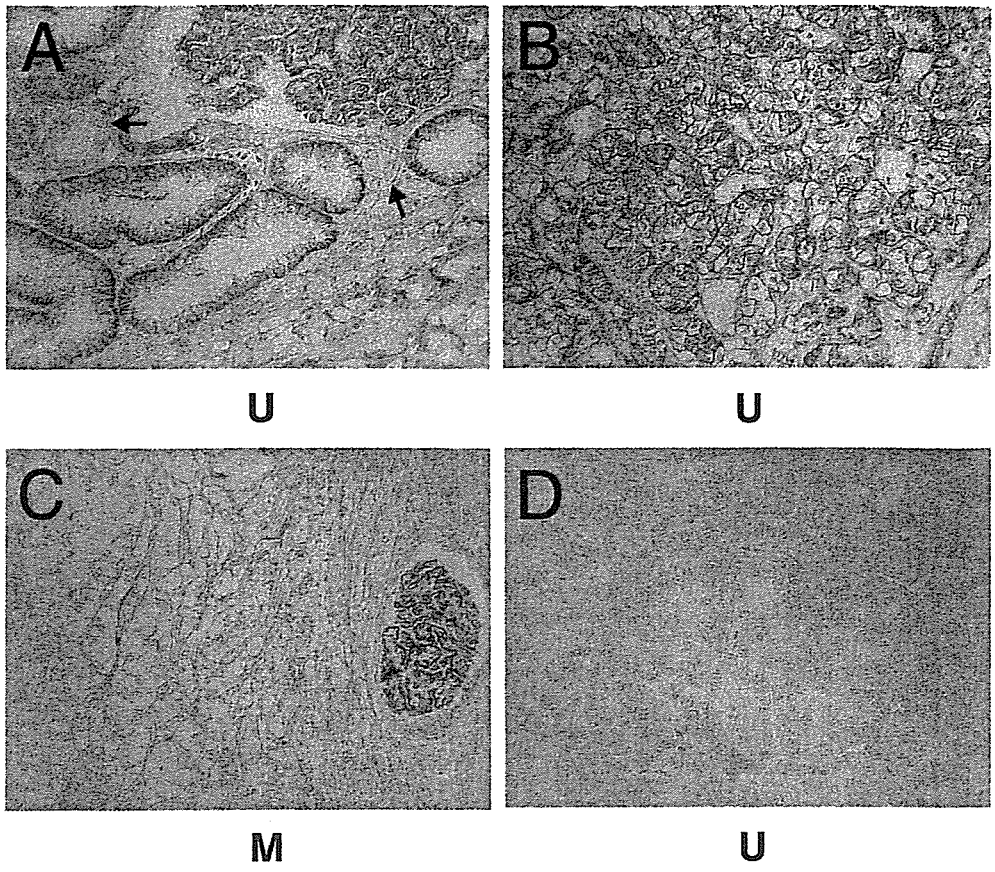

FIGURE 3 – Immunohistochemical analysis of 4.1B protein in human normal kidney (*a*) and surgically resected RCCC (*b*)–(*d*). (*a*) Expression of 4.1B is detected along the basolateral membrane of the proximal convoluted tubules and in the basement membrane of the glomeruli, but not in the distal convoluted tubules (arrows). (*b*): RCCC7C. 4.1B is detected along the cell membrane (membrane expression). (*c*) RCCC19C. 4.1B expression is absent (no expression). The basement membrane of the glomeruli (right) serves as a positive control. (*d*) RCCC5C. 4.1B is present diffusely in the cytoplasm (aberrant expression). M and U indicate tumors with hypermethylated and unmethylated *4.1B* promoter, respectively. Original magnifications, ×400.

diffusely in the cytoplasm, but not at the cell membrane (Fig. 3*d*). Including these tumors with aberrant protein localization, 4.1B expression was abrogated in a total of 11 of 20 surgically resected RCCC (55%).

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. 1*a* and 1*b*). In this regard, treatment of Caki-1 cells with 5-aza-2′-deoxycytidine did not restore 4.1B expression (Fig. 1*c*). These results suggest that some mechanisms other than promoter methylation, such as histone deacetyla-

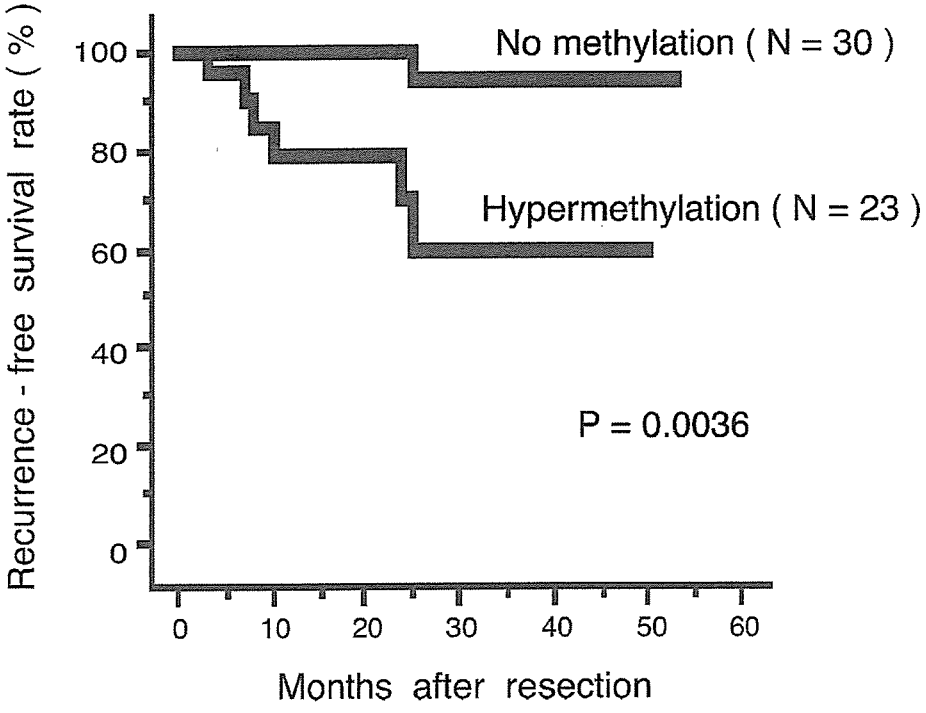

FIGURE 4 – Recurrence-free survival of the patients who received complete resection of RCCC with hypermethylated and unmethylated *4.1B* promoters. Intervals between the primary surgical resection and the metastatic recurrence at the lung, bone, liver, or pancreas are plotted in the Kaplan–Meier analysis.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

TABLE II – PROGNOSTIC VALUE OF *4.1B* METHYLATION STATUS, PATHOLOGICAL STAGE AND NUCLEAR GRADE FOR RECURRENCE-FREE SURVIVAL IN RCCC

Variable	Kaplan–Meier analysis	Multivariate proportional hazard analysis		
	p-value	Relative risk	95% confidence interval	*p*-value
4.1B methylation status[1] (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

[1]U, no methylation; M, hypermethylation.

tion and deficiency of transcription factors, might be involved in the regulation of 4.1B expression in additional populations of RCCC.

Immunohistochemical studies using anti-4.1B antibody provided information about 4.1B expression, but also 4.1B subcellular localization in primary RCCC. In this study, we found a group of tumors with 4.1B mislocalization, in addition to RCCC 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 RCCC 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 RCCC. It has been speculated that the DNA methylation changes are rather rare events in RCCC in comparison with other major malignancies.[27,28] In fact, previous studies have reported that the incidences of hypermethylation in representative tumor suppressor genes, including the *VHL*, *p16/CDKN2A*, *p14/ARF* and *APC* genes, are less than 16% in RCCC.[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 RCCC, respectively, suggesting that several critical genes are inactivated frequently by methylation in RCCC 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 RCCC 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 RCCC 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 RCCC cells to distant organs. Our

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.

Acknowledgements

We are grateful to Dr. E. Surace for his technical assistance. This work is supported in part by Grant-in-Aid for the Third-Term Comprehensive Control Research for Cancer from the Ministry of Health, Labor, and Welfare of Japan; a Grant-in-Aid for Special Projects for Cancer Research from the Ministry of Education, Culture, Science, Sports, and Technology of Japan; a Grant from the Program for the Promotion of Fundamental Studies in Health Sciences of Pharmaceutical and Medical Devices Agency (PMDA) of Japan to Y. M. and a Grant from the National Institutes of Health (NS41520) to D.H.G. D. Y., S. K., and M. S.-Y. are recipients of Research Resident Fellowships from the Foundation for the Promotion of Cancer Research of Japan. M. M. is a recipient of Research Fellowships from PMDA.

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治療学 Biomedicine & Therapeutics 2005年11月10日発行 vol. 39 no. 11 別刷

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suggestion

尿道カテーテルについて

ある種の排尿障害の改善の目的で，また術後の尿流確保の目的などで，一時的に尿道内にチューブを挿入し留置することは，泌尿器科領域の基本的な診療手技の一つだ。

今から30年以上前，私が泌尿器科の臨床に加わるようになった頃は，ネラトンカテーテルというゴム製のカテーテルを経尿道的に膀胱に留置するのが普通だった。そのままではカテーテルが身体から脱落するので，ペニス包帯，略してペニ包という手技でカテーテルをペニスに固定していた。すなわち，ガーゼの端を2本，ヒモ状に切って細くより合せ，これでネラトンカテーテルのペニス先端部から出た部分を，交叉させたヒモで十字状にしばる。別にガーゼ1枚をバイアスに折りたたんで3 cmほどのガーゼのベルトを作り，先ほどのネラトンカテーテルをしばったひもをペニスの冠状溝周囲にこのガーゼで固定する。器用に実施された場合，でき上がりも美しく，固定も何日も長持ちした。

こんな時代に，ある日，米国からフォーリーカテーテルなるものが紹介された。バルーンの部分の材質の薄さ，耐久性，これを膨らませたり，しぼませたりする，水の通路の細さ，いったん注入した水が抜けないための注入口の逆流防止の機構，いずれも美しい！と思うほど完成度の高い医療器具だった。以来，ペニ包はあっという間に姿を消し，男女ともにフォーリーカテーテルが使われるようになった。材質もシリコンとなり，口径も多様となり，内腔がつぶれないようバネの入ったもの，3チャネルのものなど，さまざまな工夫がなされ，今日に至っている。術後の尿道を介した尿流の確保に必然の医療機器として，尿閉の患者さんその他，患者さんはもちろん私どもも大いにその便利さの恩恵に浴してきた。

平成17（2005）年5月，私は偶然に発見された左腎癌に対して小開腹による腎部分切除術を受けた。経過は順調で1週間で退院し，2週間目にはジュネーブに5日間の出張をするなど，いたって元気にしている。しかし，振り返って術後の1週間の生活を考えてみると，硬膜外麻酔チューブから持続注入される稀釈モルフィンのおかげで創の痛みはほとんどなく，第1日目からどんどん歩行できた。最も苦痛だったのは，5日間留置されたフォーリーカテーテルの違和感だった。体動時に思いがけず引っぱられたり，絶えず覚える尿道の違和感，ときどき襲ってくる強い尿意などは想定外だった。だから5日目にフォーリーカテーテルも硬膜外チューブも，すべてのチューブ，カテーテル類が抜去されたときの爽快感は大変なものだった。朝，夕，病棟内を飛ぶように10周した。

私はフォーリーカテーテルを究極の医療器具の一つと考え，それを必要とされる患者さんに使用するのは当然と考えていた。しかし実際に自ら体験してみると，上述したように，その違和感は大変に強かった。これを今まで想像することなく，よく説明することなく，患者さんに使用していたことの想像力の欠如，自らの不明を恥じた。

フォーリーカテーテルを必要とする医療状況は今後も変わらないだろう。だとしたら，術後の一定期間，尿を体外に誘導し，正確に尿量が測定でき，かつ自ら排尿しないでよい，とする目的のためには，さらなる技術的工夫がありうるのではないだろうか？　口径の工夫，材質の工夫，膀胱からの脱落防止機構の工夫……。

今，世の中にはトランスレーショナル・リサーチという言葉が大はやりである。しかし，上に述べたようなリバース・トランスレーショナル・リサーチもありうるのではないか？　いや，あるべきと思う。当り前と思われている技術，たとえば腹部手術後に術後一定期間留置される経鼻胃管は，多くの患者さんが苦痛を訴える。百年間，進歩のない技術はおかしいと思う。

私たちは，想像力を豊かにして常識を疑い，地味な研究にも取り組むべきだと思う。分子生物学や，最先端の機器を使った研究のみが，臨床研究ではないはずである。心したいものである。

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Reprinted from
Jpn J Clin Oncol 2005;35(11)667–671
doi:10.1093/jjco/hyi177

A Flexible Endoscopic Surgical System: First Report on a Conceptual Design of the System Validated by Experiments

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Received May 17, 2005; accepted August 25, 2005; published online November 8, 2005

Background: Surgery is a standard diagnostic and therapeutic procedure. However, its technical difficulty and invasiveness pose problems that are yet to be solved even by current surgical robots. Flexible endoscopes can access regions deep inside the body with less invasiveness than surgical approaches. Conceptually, this ability can be a solution to some of the surgical problems.
Methods: A flexible (surgical) endoscopic surgical system was developed consisting of an outer and two inner endoscopes introduced through two larger working channels of the outer endoscope. The concept of the system as a surgical instrument was assessed by animal experiments.
Results: Gastric mucosa of the swine could be successfully resected using the flexible endoscopic surgical system, thereby showing us the prospect and directions for further development of the system.
Conclusion: The concept of a flexible endoscopic surgical system is considered to offer some solutions for problems in surgery.

Key words: surgical robot – endoscopic surgery – surgery – robotics – endoscope

INTRODUCTION

We recently reported a new concept for endoscopic mucosal resection of gastric cancer with the use of a magnetic anchor. The anchor consisted of microforceps and a magnetic weight in order to grasp, stabilize and pull up the gastric mucosa (1). During the experiments, we thought that the procedure would be easier if one more endoscope was present to hold and stabilize the mucosa instead of the magnetic anchor.

Concerning flexible endoscopes, there are some ultrathin endoscopes that can be inserted into the working channels of standard endoscopes, such as gastrointestinal endoscopes. If the outer endoscope is able to contain larger and multiple working channels, several thin endoscopes could be inserted through the outer endoscope. This would allow for the resecting procedures. Such a system could also be applied to the fields where current surgical robots are targeting.

One of the problems with current surgical robots is inaccessibility to regions located deep inside the body, particularly regions reached through narrow and winding routes, such as the digestive tracts and blood vessels. However, some early gastric cancers can be resected endoscopically with much less invasiveness than surgery. These surgeries cannot be performed by current surgical robot systems because those regions were not originally considered places for the systems to operate.

An experimental flexible endoscopic surgical system was developed to cope with these problems of accessibility, consisting of a flexible outer endoscope with two working channels through which two inner flexible endoscopes could be inserted. These inner endoscopes were designed to have similar functions as flexible gastrointestinal endoscopes allowing for performance of standard endoscopic procedures even when introduced through the outer endoscope.

The uses of the flexible endoscopic system as a surgical instrument, as well as its functionality, were confirmed during gastric mucosal resection of the swine. This is in contrast to the current limitations for surgical robotics in terms of lesion access.

MATERIALS AND METHODS

FLEXIBLE SURGICAL ENDOSCOPE

As shown in Fig. 1, the flexible surgical endoscope consists of an outer flexible endoscope and two inner flexible endoscopes inserted into the working channel of the outer endoscope. The specifications of these endoscopes are listed in Table 1.

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The outer endoscope also has a 2.8 mm working channel and a charge coupled device (CCD) enabling the endoscope to operate in a similar fashion as standard gastrointestinal endoscopes. The endoscopic images are observed on cathode ray tube (CRT) monitors in the same manner as video-endoscopes.

Each of the inner endoscopes has a 2.0 mm working channel allowing accessories such as forceps and an electrocautery tip to be introduced and used. Unlike the outer endoscope, the inner endoscopes have optic fiber bundles for image visualization, instead of a CCD. These endoscopic images are also observed on CRT monitors. However, a video-adaptor, i.e. a small CCD video camera, must be connected onto each eye piece of the inner endoscopes in order to view the image on the monitors.

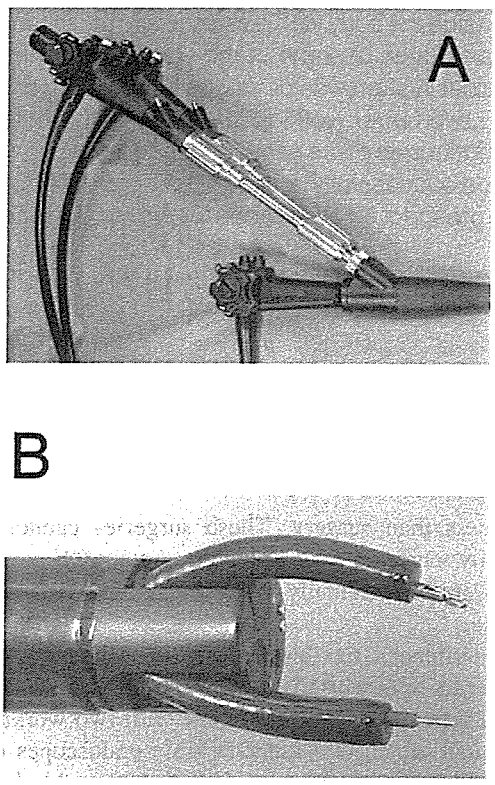

Figure 1. The flexible endoscopic surgical system. (A) The inner endoscope is inserted through a telescopic connecting device, which connects to the opening for the working channel of the outer endoscope near its control section. (B) At the tip of the outer endoscope two inner endoscopes protrude laterally, obtaining a certain distance between the two endoscopes.

Table 1. Specifications of the flexible endoscopic surgical system

	Outer endoscope	Inner endoscope
Total length (mm)	975	1395
Working length (mm)	665	1050
Insertion portion diameter (mm)	20	4.9
Tip bending (degree) (up/down, right/left)	210/120, 120/120	210/120, 120/120
Field of view (degree)	140	120
Depth of field (mm)	4–100	3–50
Channel diameter (mm)	7, 7, 2.8	2

These combined endoscopes are manipulated manually by three physicians together with the help of several assistants. The system, as a whole, operates similar to surgical robotic systems.

Physicians

Two series of experiments were conducted. The first series was performed by a senior endoscopist and three resident physicians in order to assess the system with consideration to its endoscopic nature. The senior endoscopist was trained within the specialty of internal medicine, whereas one of the resident physicians was in training for internal medicine and the other two were for surgery.

The purpose of the subsequent series was to assess the concept of the flexible surgical endoscope from the viewpoint of surgeons. Consequently, the procedure was performed by two senior endoscopists, one having more than 15 year experience as a surgeon and the other having some surgical training, in addition to two residents who were in training for surgery.

These two series were performed on separate occasions, with none of the physicians performing in both series.

Test Subject

Three female swine, under intravenous anesthesia, were laid on an examination table in the left lateral position. Within the first experiment, a 35.6 kg and a 34.1 kg swine were used. In the following experiment, a 41.8 kg swine was used. During these experiments, the law for the humane treatment and management of animals was observed.

Procedure

The procedure was similar to standard endoscopic mucosal resection with the exception of one more endoscope for stabilization of the gastric mucosa.

First, an incision was made in the mucosa surrounding the region of stomach intended for resection (2,3). The outer endoscope was inserted through the esophagus into the gastric cavity. Subsequently, using the telescopic connecting devices (Fig. 1), the inner endoscopes were inserted into the working channels of the outer endoscope and introduced into the gastric cavity.

The outer endoscope was placed near the region in which the first incision was made. Thereafter, the resecting procedure was performed using an electrosurgical knife through one of the working channels of the inner endoscopes, whereas the other contained forceps. Within the procedure, the operator decided which side of the working channels would use the electrosurgical knife.

These procedures were observed on three CRT monitors, each of which was connected to its endoscopic counter part.

The resecting procedures were performed on the anterior wall of the gastric angle, the anterior wall of the middle gastric body and the greater curvature of the middle gastric body in the

first series for the assessment of endoscopic features. Within the following series, the resecting procedures were performed on two regions adjacent to the greater curvature of the lower gastric body.

RESULTS

Concerning insertion of the outer endoscope through the esophagus into the gastric cavity, some difficulties were encountered owing to the large diameter of the outer endoscope and the relatively small size of the swine in both experimental series. However, the outer endoscope was introduced into the gastric cavity.

As for insertion of the inner endoscopes through the working channels of the outer endoscope, there were no difficulties experienced, even when the outer endoscope was bent due to insertion through the esophagus. Access to regions of the gastric wall was limited to the greater curvature due to the rigidity of the outer endoscope.

Maneuverability of the flexible endoscopic surgery system was satisfactory regarding the experiments were the first experiences for the physicians involved, despite some problems to solve.

The images from the outer endoscope were similar to those of standard gastrointestinal video-endoscopes due to the CCD system used in the outer endoscope. However, the images from the inner endoscopes were inferior to those of the outer endoscope. This inferiority was attributed to the limited number of optical fibers within the inner endoscope and deterioration of the image caused by conversion from optical images to electrical images through the use of a video-adapter. Consequently, during most of the procedure, endoscopic images were mainly observed using the monitor for the outer endoscope.

Some differences in use of the inner endoscopes for the resecting procedures between the first series and the second series were noticed. In the first series, the physicians appeared to have difficulties in some of the procedures such as accessing the mucosa, stabilization of the mucosal flap and resection procedures. These procedures were considered standard techniques for actual surgery, which means surgical experiences are required even to maneuver the flexible endoscopic surgical system.

Within the second series conducted by endoscopists with surgical experience, the resecting procedures were satisfactory, despite the fact this was their first experience using the system (Fig. 2). Through cooperation between the operator and assistants using verbal commands, manipulation of the inner endoscopes and the outer endoscope could be achieved. The functions of the inner endoscopes could be modified by changing the instruments inserted into the working channels. The flexible nature of the inner endoscopes allowed additional functions such as stabilization of the gastric wall by the longitudinal flank of the endoscope, as shown in Fig. 2C.

Within all the experiments, resecting procedures were completed without any complications such as perforation of the gastric wall. Consequently, five mucosal pieces, with sizes of

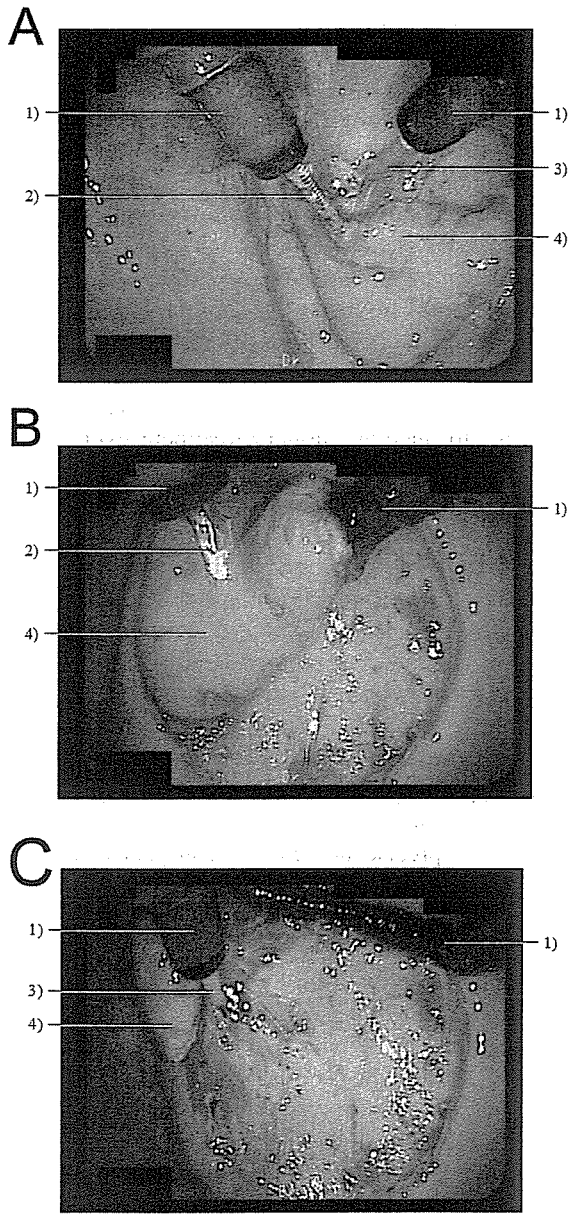

Figure 2. Images of the resecting procedures. (1) Inner endoscope, (2) forceps, (3) electrosurgical knife and (4) mucosal flap. (A) The right inner endoscope, with an electrosurgical knife introduced through its working channel, was maneuvered by the operator. The left inner endoscope, with forceps, was maneuvered by an assistant. (B) The tip of the right inner endoscope is holding up the mucosal flap in order to assist the forceps of the left inner endoscope to grasp the mucosal flap. (C) The right inner endoscope is pulling up the mucosal flap using forceps concealed in this image. In addition, using the flexibility of the endoscope, the gastric wall is stabilized by the longitudinal flank of the inner endoscope.

2.8×1.6 cm^2, 2.8×2.7 cm^2 and 2.6×2.0 cm^2 in the first series, and 3.2×2.7 cm^2 and 4.0×3.4 cm^2 in the second series were each resected in a single piece.

DISCUSSION

Surgical procedures are good options for diagnosis and treatment providing several advantages over non-surgical

approaches, especially in cases of malignant diseases. Although surgery is well accepted as a standard procedure in medicine there are still some problems left unsettled.

The technical difficulty of surgery is a common problem particularly for trainees, but even for experienced surgeons who have some technical limitations. Surgical procedures are difficult for regions deep in the body because the visual field for surgeons is limited, the number of surgical instruments which can be introduced is limited and the movements of these instruments are limited. One of the exemplary regions of this problem is the pelvic cavity, which includes surgery of rectal and prostate cancers.

Invasiveness is an inherent drawback to surgery, discouraging patients to undergo surgical treatment even when it is appropriate. It is true that surgery should be avoided when there are other less invasive alternatives.

Surgical robots such as the da Vinci system and the Zeus system are highly advanced medical instruments allowing for fine movements when appropriately manipulated by surgical experts. These systems are expected to solve some surgical problems such as invasiveness and the difficulty (4–8). Thus far, the systems have been able to solve some of the problems associated with surgery.

As for the invasiveness of surgery, endoscopic surgeries such as laparoscopy can be performed with robotic systems, utilizing smaller incisions than those of other standard open surgical approaches. The precise movements of surgeons are facilitated by robotic systems. However, laparoscopic procedures can be performed even without the robotic systems with the same amount of invasiveness.

Current robotic systems may also pose problems (4–8), such as the limited number of surgeons who can manipulate the system, which is usually one. Additional training for the specific manipulating methods of the systems is another problem, as well as introduction costs. Consequently, it is currently not clear what the benefits of these robotic systems are, especially when assessed from the patient side. Moreover, problems which even surgical experts suffer from have not been solved.

Flexible endoscopes have been developed to cope with the problems of accessing regions through narrow tracts such as the esophagus and the tracheobronchial tree. Even in these regions flexible endoscopes can perform surgical procedures similar to standard surgery. Therefore, endoscopes are naturally considered functional even in other cavities such as the abdomen and pelvic cavities.

It would be easier and more functional to perform an operation using several endoscopic instruments introduced through the end of one endoscope, rather than conducting resection using only one endoscopic instrument introduced into one endoscope, as done in standard endoscopic procedures. The simplest model for this concept is the flexible endoscopic surgical system we developed and examined within these trials.

We assumed that there would be several problems with the flexible endoscopic surgical system when used clinically as it is merely a conceptual model to confirm its feasibility of use. However, despite those problems, the system was able to perform surgical resection. In addition, the problems encountered within the first experiment were inherent in all technical procedures.

Of interest, these problems showed us that, when indicated for resecting procedures, the flexible endoscopic surgical system is easier to manipulate by surgeons and not by endoscopists despite its endoscopic appearances.

The images of the inner endoscopes were not satisfactory because a CCD was not used in these endoscopes. Consequently, resecting procedures were monitored by images from the outer endoscope which contained the CCD. In this situation, the operator had to control the inner endoscope via observations on the monitor of the outer endoscope. This is different from standard endoscopic procedures in which images are observed on the monitor of the endoscope which the operator is controlling.

In general, it is not easy for trainees to understand appropriate surgical procedures, i.e. where to cut and where to stabilize. Verbal communication during operation is important to facilitate appropriate assistance, which was not adequately utilized in the first series. These issues are to be learned through years of experience and cannot be achieved instantly.

As mentioned above, the difference between the two experiments may reveal that for these flexible endoscopes, surgical experience is an important factor, when the system is indicated for surgical procedures. The limitation of the inner endoscopes, not having CCD may have emphasized this issue. Consequently, the next system is to consist of two inner endoscopes with a CCD for each. This would allow the operators to control the inner endoscopes in such a manner as used for standard gastrointestinal endoscopic procedures.

Furthermore, we think that there should be two styles of design for future flexible endoscopic surgical systems; one with increased surgical maneuverability designed particularly for the techniques of surgeons, the other preserving flexible endoscopic maneuverability for endoscopists. Although it has not been decided yet which design is more appropriate for a future surgical system, endoscopists may be able to become accustomed to the flexible endoscopic surgical system with surgical maneuverability when the system is popularized.

In addition to the merits mentioned above, flexible endoscopic materials can theoretically be made compatible with X-ray systems such as fluoroscopes and computed tomography (CT) systems, exemplified by such procedures as X-ray guided bronchoscopy. In the future, the materials used for flexible endoscopic constructions are expected to acquire compatibility with the magnetic fields of magnetic resonance imaging (MRI) systems.

As mentioned before, limitations in visualization pose surgical problems even for experienced surgeons. This may only partially be solved by the subjective ability of surgeons to presume the identity of invisible objects using their tactile sense and their intuition. Actually, the compatibility with imaging systems was one of the important requirements for the design of the flexible endoscopic surgical system,

allowing visibility of anatomical information invisible to the surgeon's eyes.

In order to make one more step towards the future for less invasive and more effective medical treatments, we believe that future surgical systems should acquire the accessibility to narrow regions located deep inside the body together with the compatibility of imaging systems such as CT and MRI. Thus, from the flexible nature and structural characteristics of a non-jointed, smooth outer sheath, we selected the flexible endoscope as the conceptual basis of development for our system. It is the combination of these and the aforementioned aspects that allows for minimization in invasiveness, through the use of pre-existing natural structures and tracts for lesion access to deep regions, and with the presence of multiple interchangeable inner-scopes, an increase in distal tip functionality at the surgical site. Although there are several factors still to discuss and develop, the concept of the flexible endoscopic surgical system is considered an appropriate development for a future surgical robotic system with this current system being a successful step towards that furture.

Acknowledgments

This study was supported by a Grant-in-Aid for Research on Medical Devices for Analyzing, Supporting and Substituting the Function of Human Body from the Ministry of Health, Labour and Welfare.

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Int. J. Cancer: **118,** 916–923 (2006)

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 RCCC. 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 RCCC by reverse transcription-PCR. Bisulfite sequencing or bisulfite SSCP analyses revealed that the *4.1B* promoter was methylated in 9 of 19 (47%) RCC cells and 25 of 55 (45%) surgically resected RCCC, 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 RCCC.

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 chemo-, 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*.[18,19] 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; SSCP, single-strand conformation polymorphism; TNM, tumor-node-metastasis.

Grant sponsors: the Ministry of Health, Labor, and Welfare, Japan; the Ministry of Education, Culture, Science, Sports, and Technology, Japan; the Program for the Promotion of Fundamental Studies in Health Sciences of Pharmaceutical and Medical Devices Agency (PMDA), Japan; the Foundation for the Promotion of Cancer Research of Japan; the National Institutes of Health; Grant number: NS41520.

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Received 22 March 2005; Accepted after revision 10 June 2005
DOI 10.1002/ijc.21450
Published online 8 September 2005 in Wiley InterScience (www.interscience.wiley.com).

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′-GGTGCGGAGGGAGGTCACTGACAAGGAACAG-3′ and 5′-CGCTCCCACATTCATCTGGGTCATAGTCTCCGAG-3′ for DAL-1 (1.0 μM, each) and 5′-GGTGCGGAGGGAGGTCACTGACAAGGAACAG-3′ and 5′-CGCTCCCACATTCATCTGGGTCATAGTCTCCGAG-3′ 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 examined 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′-CGGAGTTTCGGTGTTTTTTGTAATAGG-3′ and DAL-1 PR2R: 5′-GCGCCGCGACGTAAAAACTAAAC-3′). The PCR products were subcloned to confirm the sequence of at least 4 clones for each sample.

Bisulfite single-strand conformation polymorphism (SSCP) analysis

For SSCP analysis, the 92-bp fragments were amplified by PCR using two primers, PR2F and PR2R, the latter of which was end-labeled with Texas Red. The PCR products were diluted 7 times with a loading buffer (90% deionized formamide, 0.01% New Fuchsin and 10 mM EDTA), heat-denatured for 3 min at 95°C, immediately cooled on ice for 3 min and then loaded onto the gel (0.5× MDE™ Gel Solution; BMA, Rockland, ME). Electrophoresis was carried out for 120 min at 20°C, using SF5200 (Hitachi Electronics Engineering, Tokyo, Japan) with cooling systems. The analysis was repeated 3 times using independent PCR products. The criterion for hypermethylation was met when the ratio of the methylated fragments to the unmethylated fragments was more than 0.4.

Immunohistochemistry

Sections (5-μm thick) of formalin-fixed, paraffin-embedded specimens were obtained from the National Cancer Center Hospital. For antigen retrieval, the section was heated for 5 min at 120°C with 1 mM EDTA in an autoclave after de-paraffinization and dehydration. Nonspecific reactions were blocked with 5% normal donkey serum (NDS) in TBS. All sections were incubated with anti-DAL-1 antibody (diluted with 1% NDS in TBS 1:2,000) at 4°C overnight. This rabbit polyclonal antibody against 18 amino acids in the U2 domain of DAL-1 was generated by D. H. Gutmann (unpublished results). The sections were then incubated with a labeled polymer, horseradish peroxidase (DakoCytomation, Glostrup, Denmark), at room temperature for 1 hr, rinsed gently with TBS, covered with 3,3′-diaminobenzidine (DakoCytomation) and incubated for 3 min. All sections were counterstained with hematoxylin. 4.1B expression was determined as "membrane expression" when 4.1B signals were detected along the cell membrane in more than 80% of the cells and as an "aberrant expression" or "no expression" when the majority of the 4.1B signals were observed diffusely in the cytoplasm or were undetected.

Statistical analysis

The Kruskal–Wallis test and Mann–Whitney *U*-test were used to examine the correlation with clinicopathological characteristics. Recurrence-free survival was analyzed by the Kaplan-Meier method and the Log-rank test. Multivariate analysis was carried out using the Cox proportional hazard model. The software Stat View 5.0 (SAS institute, Cary, NC) was used for the analysis. Differences with p values of less than 0.05 were considered significant.

Results

Loss of 4.1B expression in RCC

We initially examined the expression of the *4.1B* gene in normal kidney and 19 RCC cell lines by RT-PCR. As shown in Figure1*a*, a significant amount of 4.1B mRNA was detected in normal kidney. On the other hand, 10 of 19 (53%) RCC cell lines lacked 4.1B mRNA expression. Next, we analyzed the expression of *4.1B* mRNA in 19 surgically resected RCCC as well as several noncancerous renal tissues from the same patients. Semi-quantitative analysis by RT-PCR revealed that *4.1B* mRNA was absent or markedly reduced in 12 of 19 (63%) of these primary RCCC (Fig. 1*b*). These results suggest that the *4.1B* gene may be a target for inactivation in renal carcinogenesis.

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 RCC 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.[24] 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. 2*a* and 2*b*). A similar analysis showed that hypermethylation was observed in 9 of 19 (47%) RCC cell lines, where hypermethylation strongly correlated with loss of 4.1B expression ($p = 0.0004$, Fig. 1*a*). To examine the methylation status of the promoter quantitatively, we analyzed the promoter fragments by SSCP after PCR amplification of the bisul-

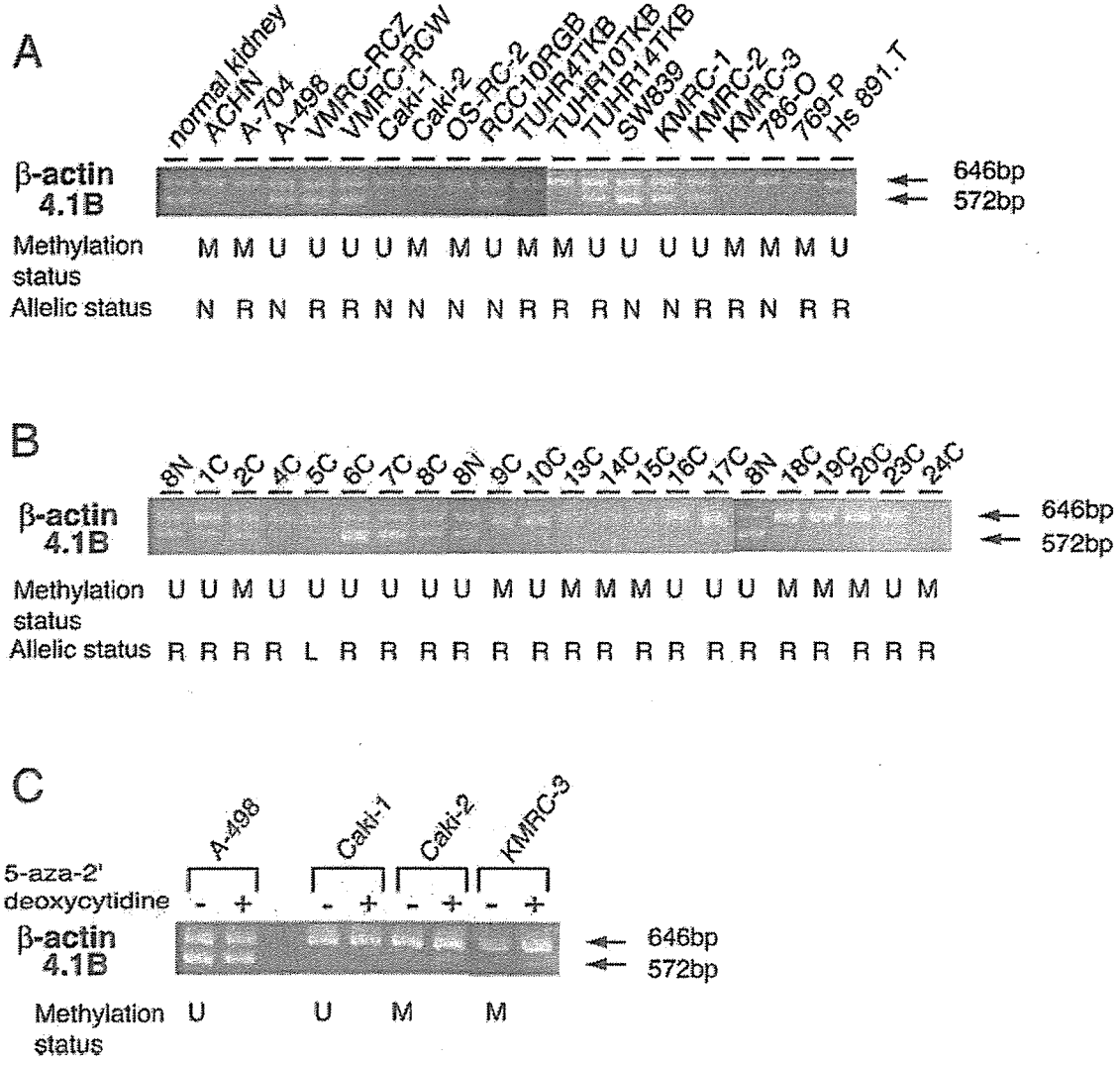

FIGURE 1 – Expression of the *4.1B* gene in RCC. (*a*) and (*b*): RT-PCR analysis of 4.1B and β-actin in RCC cell lines (*a*) and surgically resected RCCC (*b*). C and N in (*b*) indicate cDNA from a cancerous and noncancerous portion of the kidney, respectively. The results of methylation status determined in Figure 2 and allelic status are included as a reference. M and U indicate the hypermethylated and unmethylated promoter of the *4.1B*, respectively. R and L indicate retention and loss of heterozygosity, respectively. N in (*a*) indicates not informative. (*c*): RT-PCR analysis of 4.1B and β-actin in RCC cells treated with 5-aza-2'deoxycytidine (+) or PBS (−).

fite-treated DNA. As shown in Figures 2*a* and 2*c*, 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. 2*a* and 2*d*). Next, we examined the methylation status of the *4.1B* in surgically resected RCCC. As shown in Figure 2*e*, 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. *4.1B* promoter methylation strongly correlated with loss of 4.1B expression in a subset of surgically resected RCCC examined ($p = 0.0063$, Fig. 1*b*, Table I).

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*).

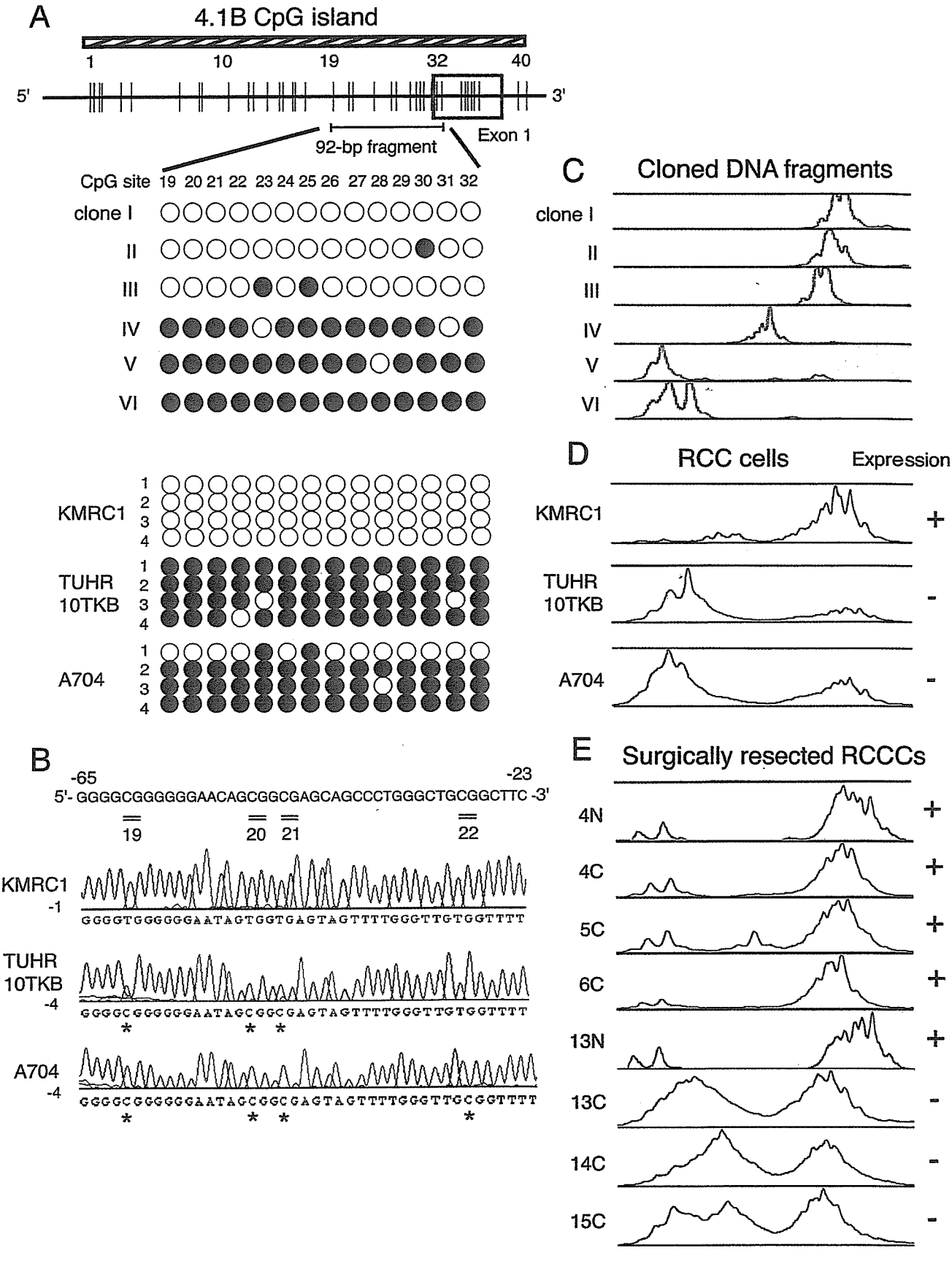
A
4.1B CpG island
1
10
19
32
40
5'
3'
92-bp fragment
Exon 1
CpG site 19 20 21 22 23 24 25 26 27 28 29 30 31 32
clone I
II
III
IV
V
VI
KMRC1
TUHR 10TKB
A704
B
-65
-23
5'- GGGGCGGGGGGAACAGCGGCGAGCAGCCCTGGGCTGCGGCTTC -3'
19
20 21
22
KMRC1 -1
GGGGTGGGGGGAATAGTGGTGAGTAGTTTTGGGTTGTGGTTTT
TUHR 10TKB -4
GGGGCGGGGGGAATAGCGGCGAGTAGTTTTGGGTTGTGGTTTT
A704 -4
GGGGCGGGGGGAATAGCGGCGAGTAGTTTTGGGTTGCGGTTTT
C
Cloned DNA fragments
clone I
II
III
IV
V
VI
D
RCC cells
Expression
KMRC1
+
TUHR 10TKB
-
A704
-
E
Surgically resected RCCCs
4N
+
4C
+
5C
+
6C
+
13N
+
13C
-
14C
-
15C
-

FIGURE 2.

TABLE I – METHYLATION AND EXPRESSION STATUS OF 4.1B AND CLINICOPATHOLOGICAL CHARACTERISTICS IN RCCC

		4.1B Promoter		
	Number of cases	Hypermethylation (%)	No methylation (%)	*p*-value
4.1B expression				
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)	0.006[1]
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)	0.004[2]
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)	NS[1]
Gender				
Male	37	17 (46)	20 (54)	
Female	18	8 (44)	10 (56)	NS[1]
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)	NS[1]
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)	NS[1]
pT4	0	0 (0)	0 (0)	
pN0	54	25 (46)	29 (54)	
pN1,pN2	1	0 (0)	1 (100)	NS[1]
pM0	53	23 (43)	30 (57)	
pM1	2	2 (100)	0 (0)	NS[1]
Nuclear grade				
G1	22	5 (23)	17 (77)	
G2	27	17 (63)	10 (37)	
G3	6	3 (50)	3 (50)	0.017[1]

NS, not significant.
[1]Mann–Whitney *U*-test.–[2]Kruskal–Wallis test.

We then examined the role of promoter methylation in gene silencing of the *4.1B* gene by treating RCC cells with the demethylating 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. 1*c*).

LOH analysis of the 4.1B gene

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 3*a*, 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. 3*b*). 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. 3*c*). 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

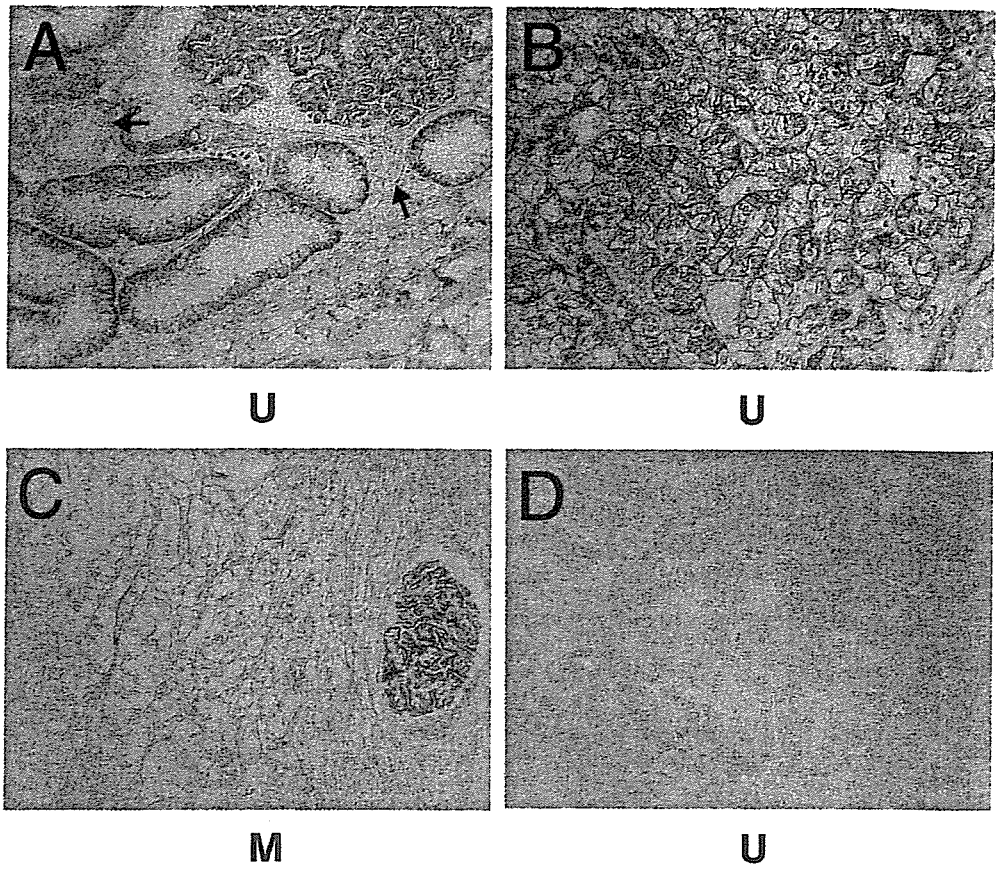

FIGURE 3 – Immunohistochemical analysis of 4.1B protein in human normal kidney (*a*) and surgically resected RCCC (*b*)–(*d*). (*a*) Expression of 4.1B is detected along the basolateral membrane of the proximal convoluted tubules and in the basement membrane of the glomeruli, but not in the distal convoluted tubules (arrows). (*b*): RCCC7C. 4.1B is detected along the cell membrane (membrane expression). (*c*) RCCC19C. 4.1B expression is absent (no expression). The basement membrane of the glomeruli (right) serves as a positive control. (*d*) RCCC5C. 4.1B is present diffusely in the cytoplasm (aberrant expression). M and U indicate tumors with hypermethylated and unmethylated *4.1B* promoter, respectively. Original magnifications, ×400.

diffusely in the cytoplasm, but not at the cell membrane (Fig. 3*d*). Including these tumors with aberrant protein localization, 4.1B expression was abrogated in a total of 11 of 20 surgically resected RCCC (55%).

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. 1*a* and 1*b*). In this regard, treatment of Caki-1 cells with 5-aza-2′-deoxycytidine did not restore 4.1B expression (Fig. 1*c*). These results suggest that some mechanisms other than promoter methylation, such as histone deacetyla-