では1998年から2005年まで約360例の乳癌症例に術前化学療法を実施してきた．術前化学療法の原発巣における効果は，約85%以上の症例がPR以上であった．約25%の症例は原発巣がCRとなったが，これらの症例の腋窩リンパ節転移陽性率は25%で，早期乳癌のそれとほぼ同程度まで低下していることが確認された．このような術前化学療法が著効した症例に対して早期乳癌と同様にセンチネルリンパ節生検を実施し，腋窩郭清を省略することが可能かどうかを明らかにすることは非常に重要な課題である．

1．術前化学療法後のセンチネルリンパ節生検における問題点

術前化学療法後のセンチネルリンパ節生検に関してはいまだ十分なエビデンスは得られていない．これまでの報告例はいずれも単一施設で少数例の結果であり大規模な臨床試験は行われていない．早期乳癌症例に対するセンチネルリンパ節生検と比較すると，術前化学療法後の症例の問題点は，①腫瘍径の大きな症例が対象になる，②腋窩リンパ節転移の存在する，または存在した症例がより多く含まれる，③術前化学療法が腫瘍－リンパ管－リンパ節の流れに影響を与える可能性がある，④術前化学療法は転移陽性であったセンチネルリンパ節とノンセンチネルリンパ節に同程度の効果があるのか？⑤術前化学療法後のn0の意義がまだ明らかになっていない，などが挙げられる．これらの要因が術前化学療法のセンチネルリンパ節生検の妥当性を検証するうえで問題点となってきた．

1）海外での成績

術前化学療法後のセンチネルリンパ節生検のこれまで報告されてきた単一施設の成績を**表1**にまとめた[6~13]．症例数は15例から51例といずれも少数例での報告となっている．腫瘍径は平均で3.3 cmから5.5cmで，T1からT4まで対象とし，また，リンパ節転移が認められる症例も含めた試験も報告されている．これらのセンチネルリンパ節の同定率は84%から93%程度で，早期乳癌の成績よりやや低い程度である．偽陰性率は，0%から33%とばらつきを認める．これら7施設の報告をまとめると全体としての同定率は88.7%で，偽陰性率は5.3%である．ただし，偽陰性率に関してはNasonらの15例での33%という報告と少数例を対象にした0%という報告を除けば10%～15%程度という成績が臨床的にも妥当なのではないかと推測する．

術前化学療法後のセンチネルリンパ節生検のこれまで報告されてきた多施設の成績を**表2**にまとめた[14~17]．MamounasらはNational Surgical Adjuvant Breast and Bowel Project randomized trial (NSABP B-27) のAC 4サイクルにdocetaxelを加えた術前化学療法後にセンチネルリンパ節生検が試みられた428例の成績を報告している[14]．試験が多施設にわたるためセンチネルリンパ節生検手技は，まちまちであるが全体としての同定率は85%，偽陰性率は11%という結果である．その他の3つの多施設からの報告も同定率が90%前後，偽陰性率が10%前後と早期乳癌に対するセンチネルリンパ節生検の成績と遜色のない結果が報告されている．

また，これらの結果からわかることは，術前化学療法後にセンチネルリンパ節生検を行う際には，色

表1　術前化学療法後センチネルリンパ節生検－単施設の成績－

	症例数	病期	平均腫瘍径(cm)	同定数(%)	偽陰性数(%)
Breslin et al.,2000[6]	51	II or III	5.0	43(84.3)	3(12)
Miller et al.,2002[7]	35	T1-3N0	3.5	30(86.0)	0(0)
Stearns et al.,2000[8]	34	T3-4, any N	5.0	29(85.0)	3(14)
Haid et al.,2001[9]	33	T1-3, any N	3.3	29(88.0)	0(0)
Julian et al.,2002[10]	31	I or II	NS	29(93.5)	0(0)
Tafra et al.,2001[11]	29	Any T, N0	NS	27(93.0)	0(0)
Nason et al.,2000[12]	15	T2-4, N0	NS	13(87.0)	3(33)
Shimazu et al.,2004[13]	47	II or III	4.5	44(93.6)	4(12)
Kinoshita et al., 2005	88	T2-4, any N	4.9	81(92.0)	3(9)

表2　術前化学療法後センチネルリンパ節生検－多施設の成績－

	症例数	手技(色素/RI)	同定率(%)	偽陰性率(%)
Mamounas et al[14]	428	Blue dye	78	14
(NSABP B-27)		Radiocolloid	89	5
		Combination	88	9
		All techniques	85	11
Krag et al[15]	443	Radiocolloid	93	11
Tafra et al[16]	529	Combination	87	13
McMaster et al[17]	806	Blue dye or Radiocolloid	86	12
		Combination	90	6
		All Techniques	88	7

表3　患者背景

	症例数
平均年齢(歳)	50.2 (27-77)
平均腫瘍径 (cm)*	4.91 (2.7-12)
T分類*	
T2	54 (61%)
T3	28 (32%)
T4	6 (7%)
N分類*	
N0	46 (52%)
N1	34 (39%)
N2	8 (9%)
組織型	
浸潤性乳管癌	86 (98%)
浸潤性小葉癌	2 (2%)
術前化学療法	
FEC plus paclitaxel	85 (97%)
paclitaxel alone	3 (3%)
臨床的腫瘍効果	
CR	45 (51%)
PR	35 (40%)
NC	8 (9%)
病理組織学的腫瘍効果	
pCR	34 (39%)
pINV	54 (61%)
リンパ節転移	
陰性	38 (43%)
陽性	50 (57%)

*化学療法前
pCR＝pathological complete response；pINV＝pathological invasive

素法単独より色素法にRI法を併用した方が成績がよいということである．

2) 国立がんセンターの成績

当院では，早期乳癌に対するセンチネルリンパ節生検のfeasibility studyを終了後，2003年7月より術前化学療法後の乳癌症例に対するセンチネルリンパ節生検のfeasibility studyを開始し，その成績を報告してきた．本試験は単一の外科医，手技により実施された．

腫瘍径3cm以上あるいは腋窩リンパ節転移を認める乳癌症例を対象に術前化学療法として，①FEC/ACを4サイクル，②weekly paclitaxelを12サイクルを組み合わせたものを原則とし，高齢者にのみ②だ

表4　国立がんセンターにおけるセンチネルリンパ節生検の成績

センチネルリンパ節の転移	非センチネルリンパ節の転移	
	陽性	陰性
陽性	16	14
陰性	3	48

False negative rate, 9.1%; overall accuracy, 96.3%; negative predictive value, 94.1%; positive predictive value, 100%

け実施した．術前化学療法後に原発巣がPR以上の効果を示し，かつ，治療後腋窩リンパ節転移が陰性であった88例をセンチネルリンパ節生検の対象とした．これらの平均腫瘍径は4.9cm（2.5cm～12.0cm）で，T4が6例，治療前に明らかにリンパ節転移を認めた42例も対象となっている(表3)．センチネルリンパ節生検は，色素－RI法を用いたものが80例で，色素法単独が8例となっている．結果として，センチネルリンパ節が同定できた症例は80例で，同定率は92%となる．これらの症例のセンチネルリンパ節とノンセンチネルリンパ節の転移の有無をまとめたものを表4に示す．センチネルリンパ節に転移を認めず，ノンセンチネルリンパ節に転移を認めたものは3例で偽陰性率は9%であり，全体として96%の症例においてセンチネルリンパ節が腋窩リンパ節全体の状況を正確に反映していることが証明された．臨床的諸因子とセンチネルリンパ節の同定率との関連を検討したが，治療前のリンパ節転移の有無，臨床的治療効果，病理組織学的治療効果は関連せず，唯一，T4d（炎症性乳癌）症例のみがセンチネルリンパ節の同定を困難にしていることが明らかとなった．一方，センチネルリンパ節が同定できた症例中，偽陰性になった症例は3例のみであったため，術前化学療法も含めてこれらに影響を与える因子は明らかではなかった．

まとめ

当院での術前化学療法後センチネルリンパ節生検の結果から，炎症性乳癌以外の術前化学療法が著効した症例において，センチネルリンパ節生検は十分に安全に実施できると結論づけられた．同定率は92%，偽陰性率は9%で，早期乳癌における成績と遜色のないものとなった．海外における最近の報告や多施設からの報告は，当院の結果を支持するものである．一方，2005年度にJournal of Clinical Oncology（JCO）に発表されたAmerican Society of Clinical Oncology（ASCO）のガイドラインでは，Preoperative systemic therapy後のセンチネルリンパ節生検に関して，①技術的には安全に実施することはできる，②Preoperative systemic therapy後のn0の意義が明らかでない，③これらの症例では，正確な腋窩リンパ節の転移状況の把握が治療方針を決める際に重要であること，④エビデンスが十分でない，ことより推奨されていない．正確な腋窩リンパ節の情報を得るという目的からするとセンチネルリンパ節生検をPreoperative systemic therapyの前に施行し，Preoperative systemic therapy後に実施する場合でもN0症例に限られるべきだと強調している[18]．

当院での成績から，強力で安定した化学療法の後，色素－RI法を用い熟練した手技のもとにセンチネルリンパ節生検は，安全に実施できることが確認された．術前化学療法が著効した乳癌症例では，腋窩リンパ節陽性率が25%程度になることから術前化学療法後にセンチネルリンパ節生検を実施することに意義があるものと考える．ただし，本対象が進行癌であるということを十分に認識し，腫瘍内科医，病理医，放射線診断医との連携のもとに，慎重に適応を決めて本手技を修練，実施することが望まれる．

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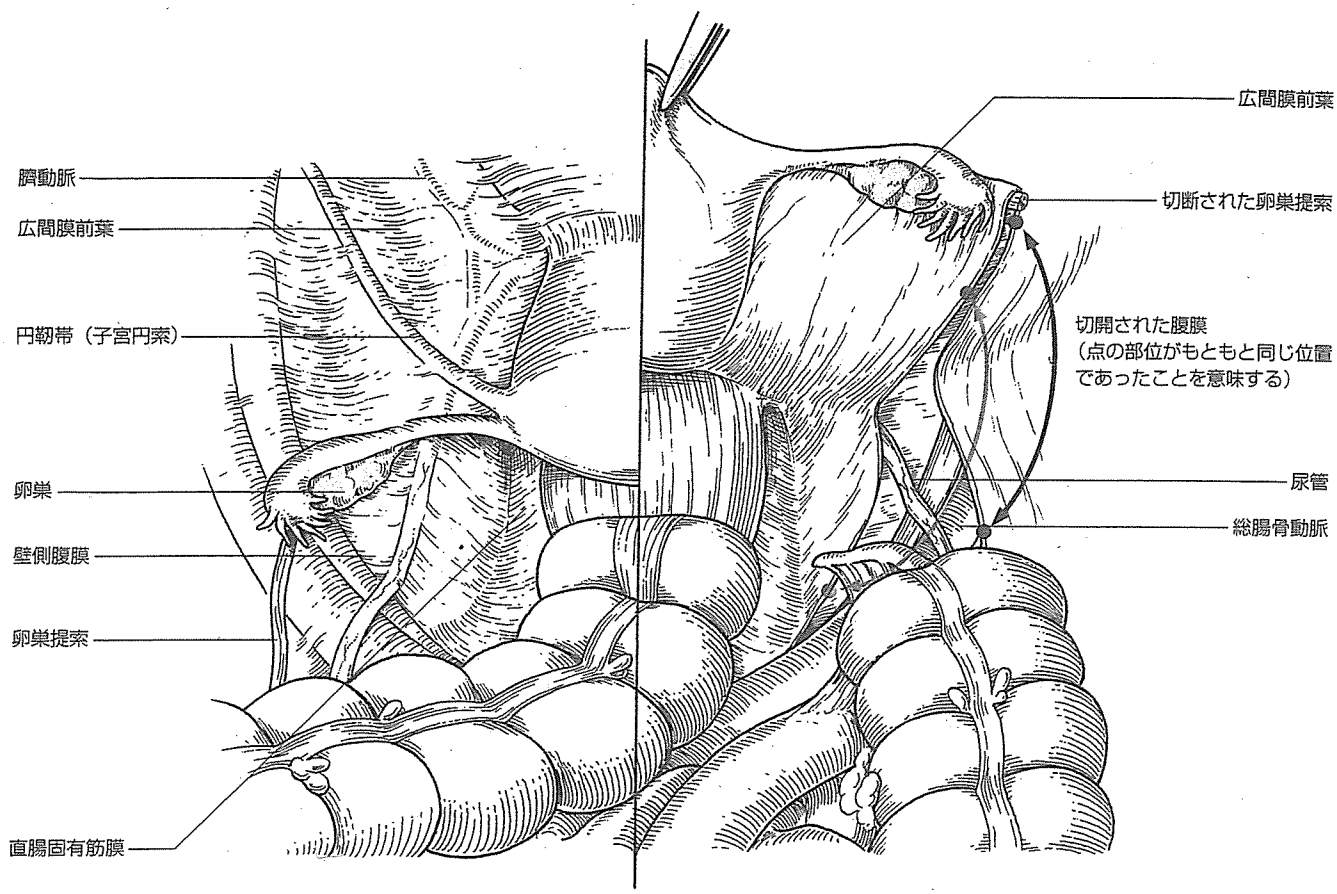
骨盤内手術(前立腺全摘除術，膀胱全摘除術)
膀胱全摘除術〔女性〕



[7-71] 子宮周囲の構造と腹膜，広間膜の関係
右半分は子宮を膀胱側へ持ち上げた状態

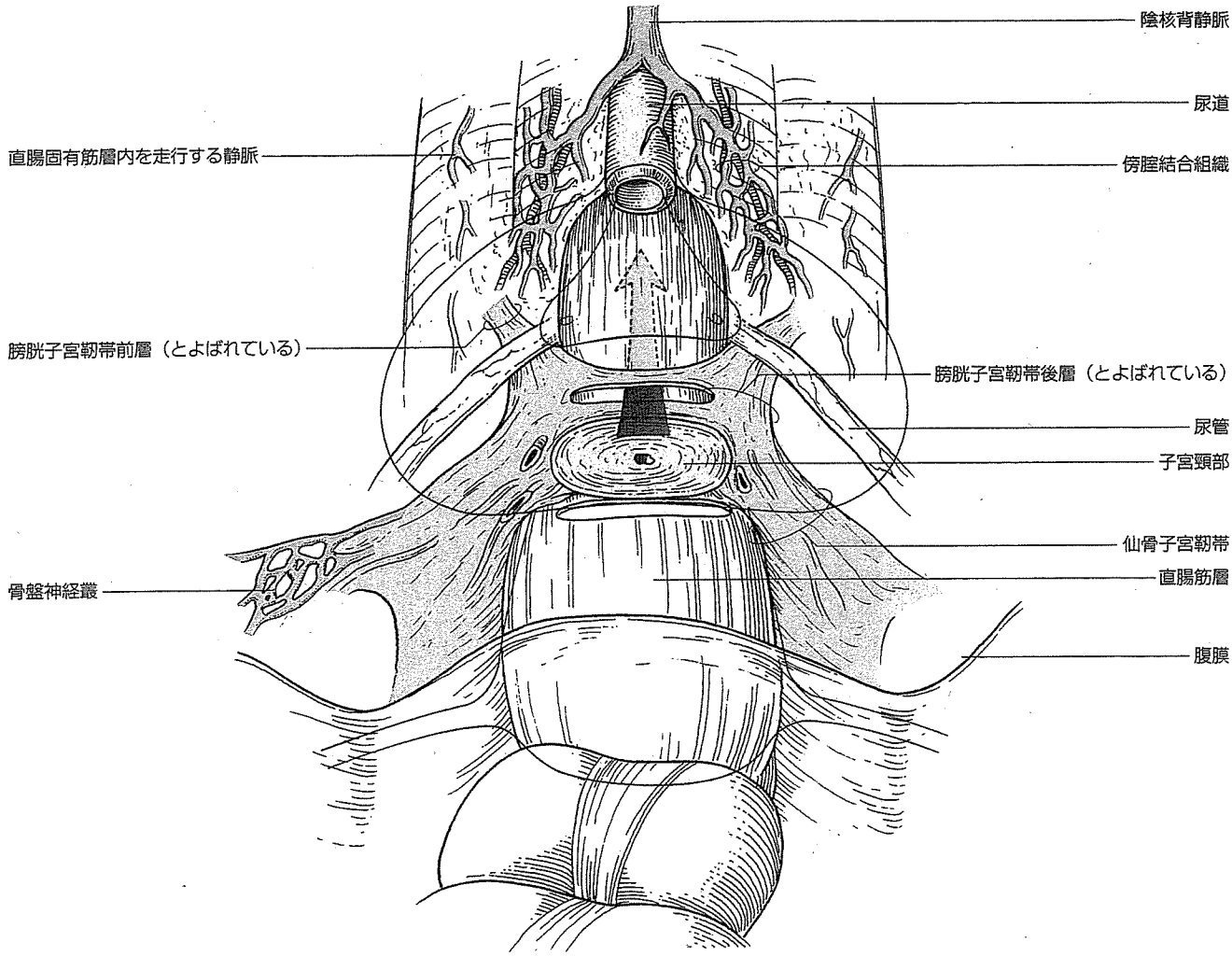
女性の膀胱全摘除術を安全かつ確実に施行するうえで大切な点は子宮，卵巣を取り巻く腹膜とこれを支える索状物の構造とそれを取り巻く血管系の理解，ならびにそれらに対する定型的な処理方法を理解することである。骨盤内臓器の摘出においては静脈叢をいかに処理するかが重要である。前立腺全摘におけるSantorini静脈叢に対するバンチング処理のように，直腸切断術などにおける仙骨静脈叢，広汎子宮全摘における基靱帯を含む傍子宮結合組織などは解剖学的な関係から処理法が決まっており，その方法と考え方を理解する必要がある。

本項では女性の膀胱全摘除術における手術法の基礎となる解剖といくつかの場面で実際の処理方法について手術手順に沿って解説する。

女性の膀胱全摘除術に必要な解剖の理解

広間膜の理解

女性の膀胱全摘除術を施行するうえで大切なポイントとして子宮，あるいは卵巣を被覆する腹膜，つまり広間膜とその中を走行する血管，尿管，靱帯を理解することである。子宮は子宮円索(円靱帯)，卵巣は卵巣提索(卵巣静脈)により固定されている。広間膜は腹膜が折り返って子宮，卵管をサンドイッチしていると理解することである。腹膜はそのまま前方では膀胱を，後方では直腸，あるいは骨盤壁を被覆している。[7-71]の左半分は腹膜を被った状態，右半分は卵巣提索も切断，広間膜を切開し後腹膜を走行する尿管を露出した状態を



[7-72] 子宮頸部を支える“靭帯”と周囲の構造（基靭帯は除いてある）

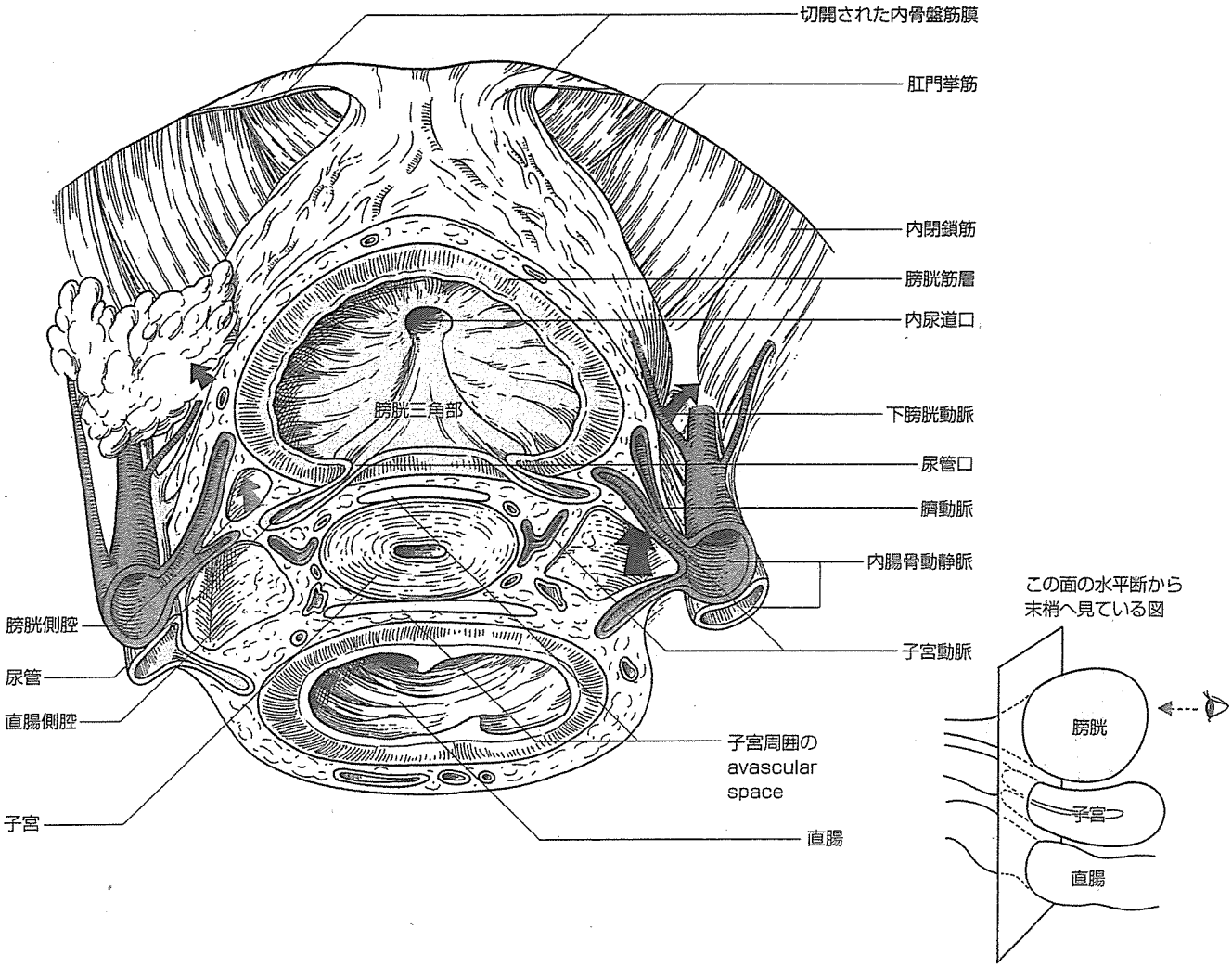
示す。特に注意したい点は右半分に記載したように広間膜前葉，後葉のもともとの腹側の付着部位との関係と尿管の走行の関係である。このことを理解していれば，膀胱全摘除術において尿管を処理するための腹膜の切開線は容易に想定できる。

子宮頸部の周囲構造

子宮の周囲支持組織は婦人科的には“靱帯”とよばれている。これは1921年に岡林らにより発表された子宮の結合組織，支持組織が前・中・後の3部からなるという見知に基づいた概念であり，その後も継続されている概念である。泌尿器科医にはあまりなじみのないものかもしれないが，この概念の理解は女性の膀胱全摘除術には必要である。

子宮を前・中・後より支持する靱帯とはそれぞれ膀胱子宮靱帯，基靱帯，仙骨子宮靱帯である。膀胱子宮靱帯は泌尿器科的には神経血管束を含む索状物とよんでいる膀胱外側の血管茎と同様と理解してよいと思われる。この靱帯とはいったいどのような構造物をさしているかということを理解するのが重要である。

膀胱，尿道，膣，直腸，これらの骨盤内臓器の外側はlateral pelvic fasciaともよぶべき構造膜で覆われており，血管系あるいは神経系は内腸骨動静脈あるいは骨盤神経叢から走行して直腸，子宮，膣，膀胱に流出入している。これら神経血管系の間には脂肪織が存在するわけであるが，これもある程度，規則性をもって走行しており，その間は比較的avasculuarな構造となっている。これを隔壁する組織として膀胱の神経血管束＝膀胱子宮靱帯，基靱帯，仙骨子宮靱帯が存在する。○○靱帯とはつまり，血管，神経が各々の臓器に対して分枝を与える，あるいは合流する部位を盲目的に処理すると出血しやすいため靱帯とよんで注意を喚起していると解釈される。[**7-72**]に子宮頸部を支える靱帯の関係と骨盤神経，傍膣結合組織，尿管，尿道との位置関係を模式的，立体的に図示した。基靱帯は子宮動脈を中心とする子宮と内腸骨動静脈との間の索状物であり次の[**7-73**]で説明する。図中矢印は膀胱子宮窩の腹膜を切開することを意味している。



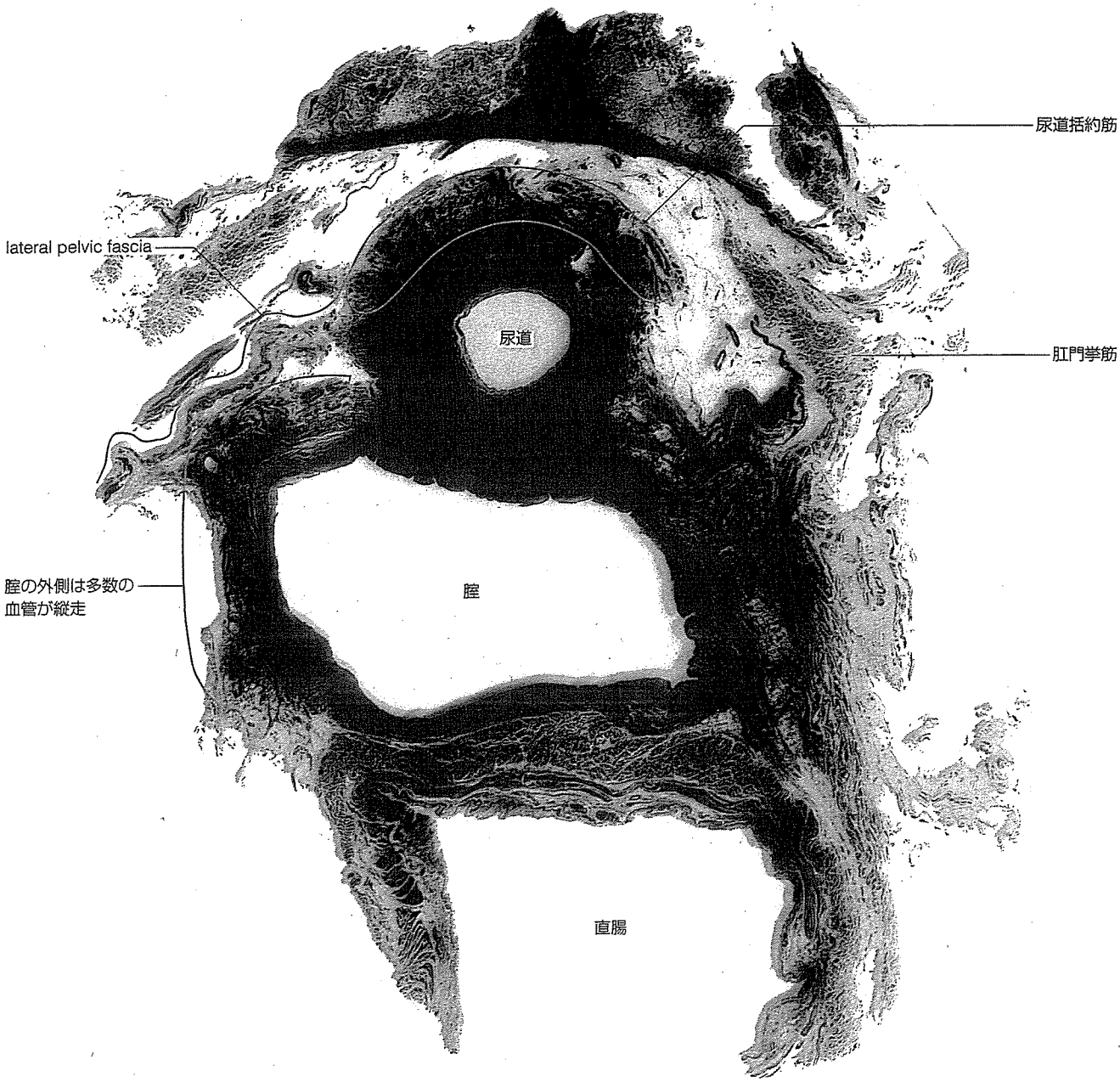
[7-73] 基靭帯

基靭帯と周囲構造

[7-73]に子宮動脈，尿管口が同一横断面で切断されたという仮定で，側腔の構造を模式的かつ立体的に示した。基靱帯は仙骨神経叢から立ち上がってくる神経系と内腸骨動静脈からの子宮への分枝により構成される。子宮動脈と臍動脈との間に構成される空隙を直腸側腔と平行に展開すると膀胱側腔とよばれる腔が形成される(図中の⇨と⇨のライン)。つまり膀胱側腔を展開してはじめて同定できる索条物が基靭帯とよばれている構造物である。基本的には内腸骨血管，骨盤神経叢から子宮に向かい走行する動静脈，神経が含まれる。膀胱全摘では通常子宮とともに切除されるために実際には基靱帯を単独で認識することはなく，泌尿器科医にとっては側方の血管茎として理解され，これを処理する段階で自動的に切断される。

骨盤内臓器はlateral pelvic fasicaに被覆されており，その間を血管神経系が走行し，その間にはavascularな構造より構成されていることは既に述べた。直腸側腔を理解するためには直腸側腔の"入り口"(図中の⇨)とその"出口"ともいうべき肛門挙筋，梨状筋，直腸間膜により構成されるポケット状の陥凹部(図中の⇨)の立体関係の理解が重要である。直腸は仙骨に沿って一度背側に向かい，その後，肛門に向かい腹側に走行する。このポケット状の陥凹部は直腸の走行が角度の変わる部位である。このことを理解することは直腸側腔を展開する場合，正しい方向を理解するうえで重要である。

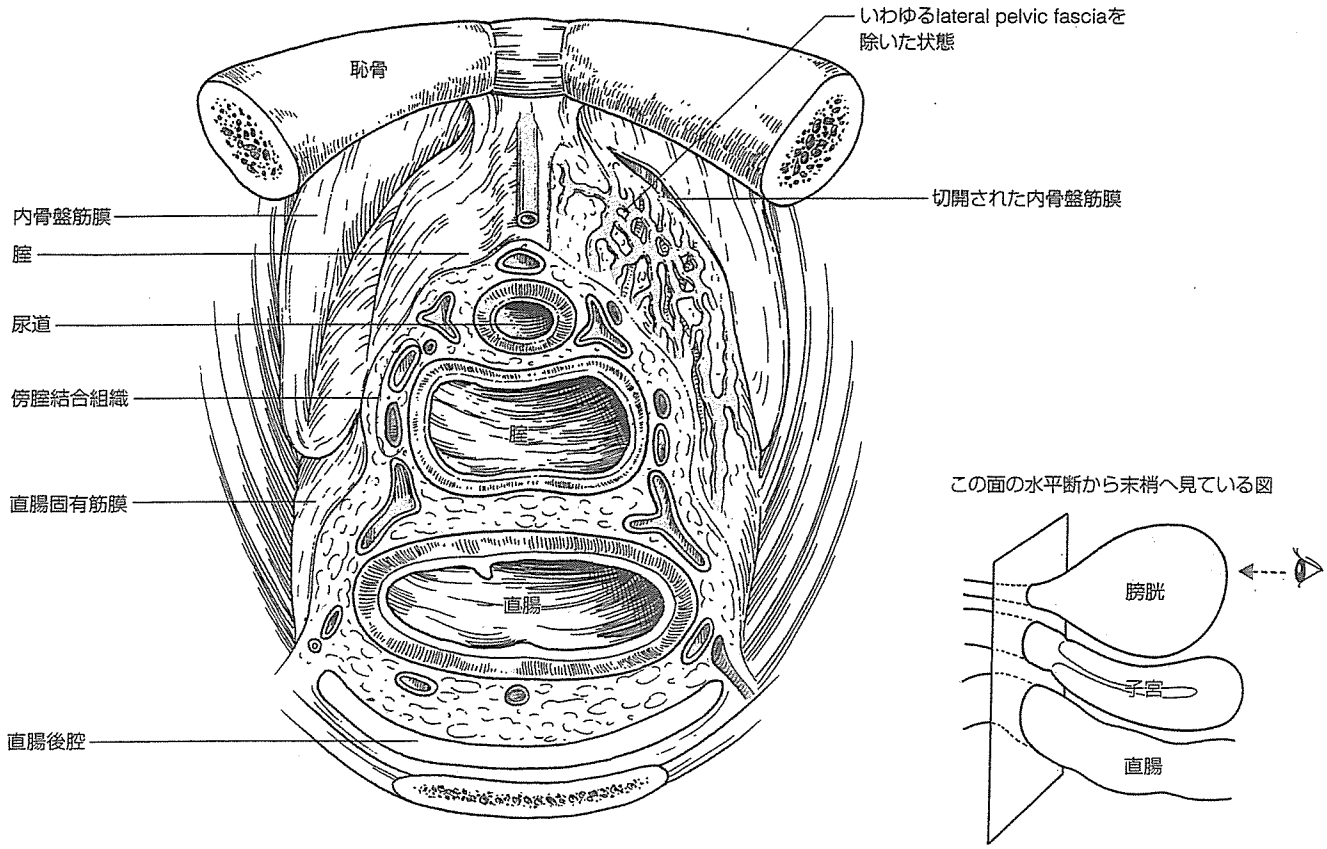
直腸側腔とは要は直腸固有筋膜の上のスペースである。この空間を展開することにより，基本的に膀胱，子宮への血管神経系は腹側に剥離されることになる。膀胱前腔，つまり内骨盤筋膜と膀胱との間で構成されるスペースを中枢に向かいていねいに展開し，内腸骨血管を露出することで，膀胱子宮への血管茎が板状に把持することが可能となる。また女性の膀胱はときに外側で後方に落ち込んでいることがあり，血管茎の切除を盲目的に施行していると膀胱を切り込んでしまう危険を回避するためにも重要である。



[7-74] 尿道と腟周囲の構造
剖検例における水平断，Azan染色，マクロ像

尿道と腟周囲の構造

[**7-74**]に尿道周囲における剖検症例の水平断のAzan染色のマクロ像を示す。マクロ像で明らかなように腟壁の外側には多数の血管が縦走し，前方では尿道との境界まで存在している。これらは傍腟結合組織とよばれている。血管系は膜の下に多数存在し，尿道後面外側まで存在していることを意識することが大切である。周囲の構造を模式的かつ立体感をもたして表したのが[**7-75**]である。浅陰核背静脈はlateral pelvic fasicaの上に存在するが，それ以外は尿道，腟，直腸ともにlateral pelvic fasicaに覆われている。そして尿道と腟の境界部外側には男性の神経血管束と同様に多数の血管，神経が走行し，血管は静脈叢を形成している。[**7-75**]の左半分はfasciaに覆われた状態，右半分は膜を除き，血管の状態を表したものである。さらに恥骨尿道靱帯の直下では静脈洞のような状況となっており，この部位では運針を行っても止血が困難なことが多く，ときに信じられないくらいの出血をみることがある。処理法については後述する。



[7-75] 尿道と膣周囲の構造の模式図

実際の手術手技

膀胱前腔の構造と処理方法

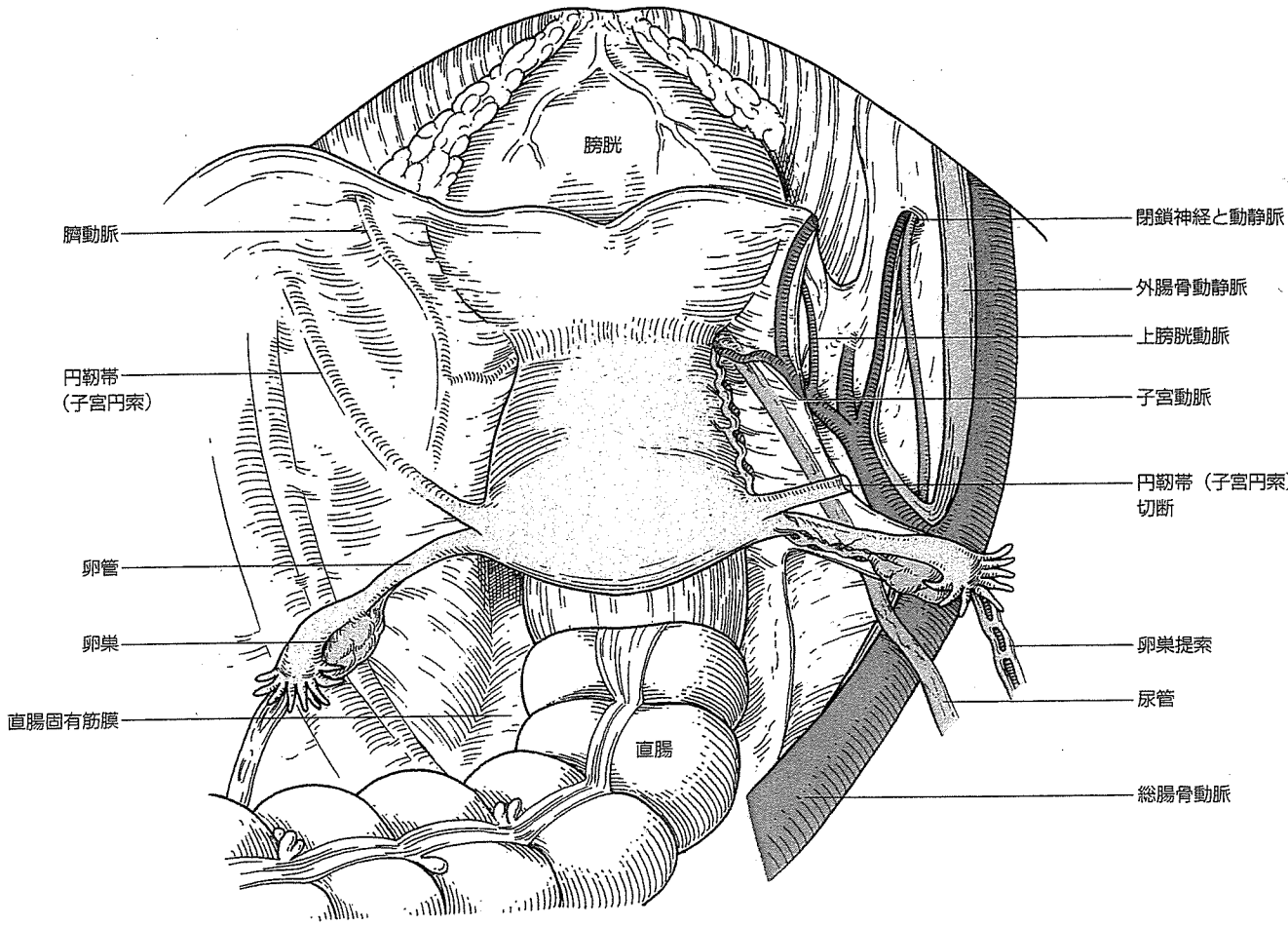
膀胱前腔はRetzius space(Retzius腔)とよばれる空間で構成されており，膀胱壁膜ともいうべき膜構造と内骨盤筋膜の間に脂肪織が存在する。これは男性でも同様である。膀胱前腔を開放することは用手的な剥離でも対応可能であるが，本来の剥離層は膀胱の静脈が膜を1枚かぶった状態になる層である。それより深く剥離すると膀胱筋層を露出することになり，静脈から無用な出血をきたすことになる。また逆に剥離層から離れすぎると脂肪織内の血管に遭遇しこれも無用な出血をきたすことになる。

次に大切なメルクマールは臍動脈である。臍動脈は内腸骨動脈から分岐しており動脈外側に沿って剥離を進めると，郭清すべき外側の組織と膀胱の血管茎との間を展開できる。実際には腹膜の裏で臍動脈を同定し，動脈の外側をクーパーなどで鈍的に中枢に向かい剥離を進めると容易に内腸骨動脈本幹に至ることができる。広間膜を処理してリンパ節郭清を行うことで，[**7-76**]の右の図で示したような状況にする。必要に応じて血管を処理する。

側腔の展開方法

骨盤内リンパ節郭清を施行する際，[**7-73**]で示したように肛門挙筋，梨状筋，直腸間膜により構成されるポケット状の陥凹部があり，通常この部分には特に血管と交通のない脂肪が貯留しており，その部分の脂肪をクーパーなどで鈍的に摘出することで肛門挙筋，直腸間膜などを確認する。このことにより血管系の処理の終点を確認することが可能である。男性では最終的には内骨盤筋膜を切開するが，女性では必ず切開が必要というわけではない。重要な点は内骨盤血管系あるいは骨盤神経系がこの陥凹部では既に分枝を終了しており，トンネルの出口のような状況になっていることを理解することである。

直腸側腔の展開は直腸に緊張をかけ，臓側腹膜と壁側腹膜との移行部を直腸の走行に沿って切開することにより，可能



[7-76] 広間膜，腹膜とその裏の構造

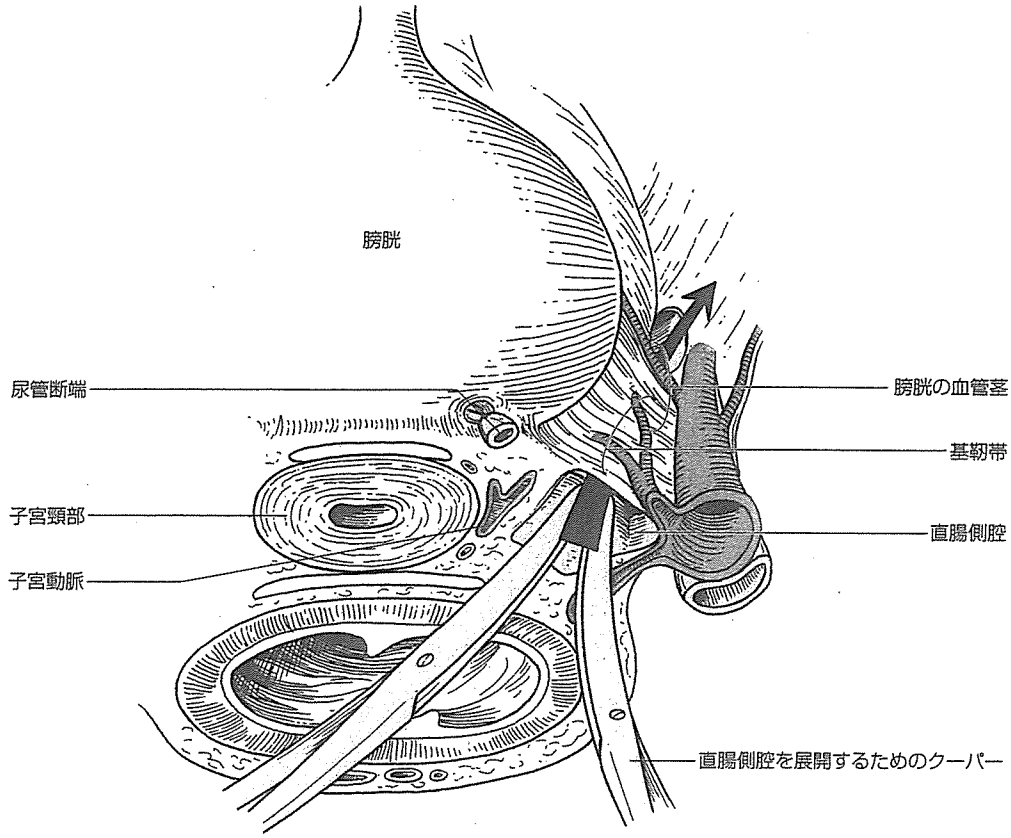
左半分は腹膜を被った状態，右半分は処理された状態

となる。まず尿管断端を確認する。その後方，外側を注意する。脂肪に包まれて展開できるスペースがあるようには見えないが，直腸の腹膜剥離ラインを参考に尿管断端の1～2cm後方で脂肪織にクーパーを鈍的にあてがいながら先ほどの陥凹部に向かい剥離を進めると，剥離層が適切な場合には容易に貫通することができる。[**7-77**]に方法を模式的に示した。このことにより内腸骨血管系あるいは骨盤神経系をすべて外側に展開できる。既に述べたように直腸側腔の展開は単に血管系の処理を確実にする目的のみならず，膀胱を完全に摘出するためにも重要である。膀胱を完全に摘出するとは当たり前ではと思われるが，女性の膀胱はときに想像以上に直腸側面に落ち込んでいることがある。特に子宮全摘を施行された症例ではまさに尿管合流部を含む膀胱が直腸の外側で後方に偏位していることがある。このような症例では直腸側腔を適切に展開することにより，“膀胱を2度切り”“尿管断端の取り残し”などという“情けない”事態を回避することができる。

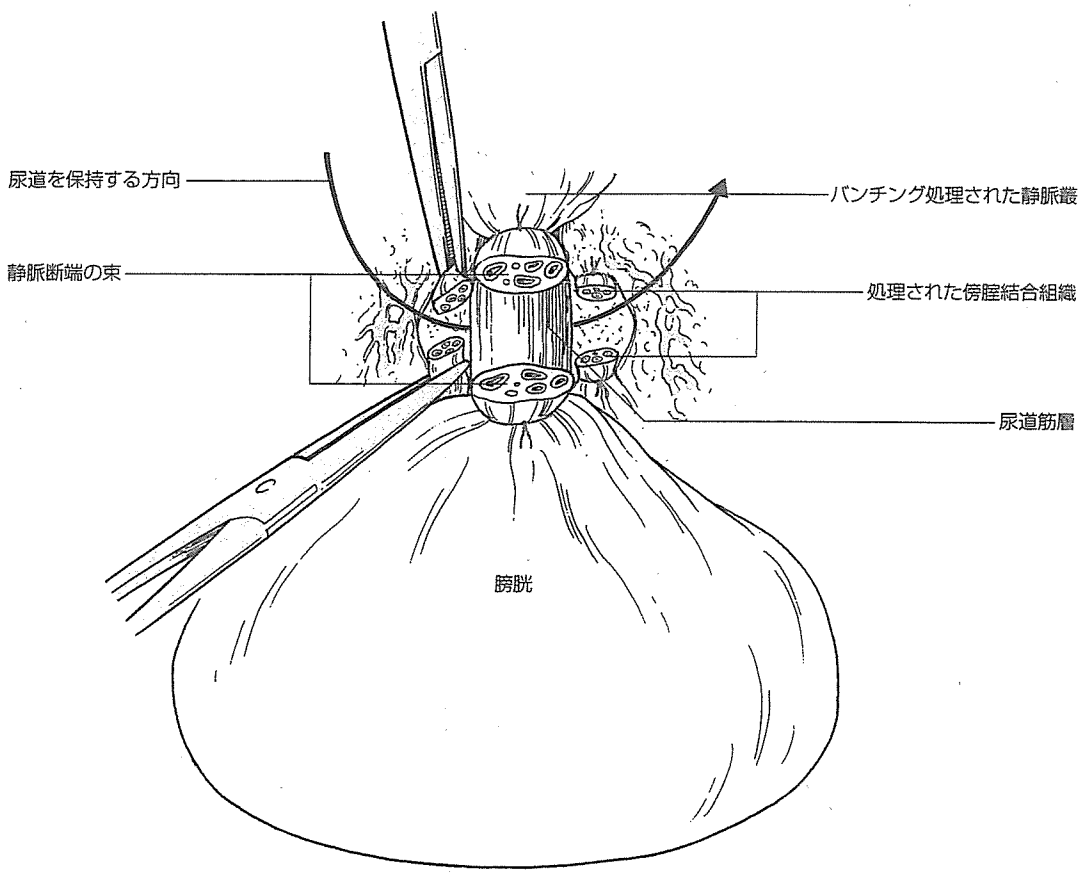
尿道の処理

子宮動脈を処理し，直腸子宮窩の腹膜を切開し，子宮円蓋を越える。次に仙骨子宮靱帯を切断して子宮頸部をこえ腟に至る([**7-72**]参照)。ここまでの処理はそれほど困難なものではない。

女性の膀胱全摘で最も注意を要するのは尿道周囲の処理ではないかと思っている。位置関係の把握は前立腺も無いこと，骨盤も広いことから容易である。尿道前面には通常脂肪織内に陰核背静脈からの静脈が走っており，尿道前面で容易に剥離処理できる([**7-75**]参照)。男性と異なり，この処理を行うことで女性の尿道前面はさらに容易に位置関係を把握できる。膀胱頸部の位置を挿入したFoleyカテーテルを動かすなどして位置関係を把握する。腟を被覆する内骨盤筋膜を切開しなくても尿道の処理は可能である。われわれが内骨盤筋膜を切開するのは，これを切開することにより，肛門挙筋を腟壁から剥離し，一気に直腸固有筋膜に到達，さらには直腸固有筋膜を切開して直腸筋層を同定することで腟後面の処理を



[7-77] 直腸側腔の展開法



[7-78] 尿道の処理法

より確実にするためである。この操作により傍膣組織の処理を確実に行うことが可能となる。

尿道周囲にはまさに前立腺の周囲のように静脈叢が取り巻いている。特に男性のSantorini静脈叢のように尿道前面で尿生殖隔膜に近いところでは，静脈は静脈洞になっているのではと思われるくらい，一度出血をきたすと運針では止血がなかなか困難な状況に追い込まれることがある。われわれはこの部位を処理するためには，まず膀胱頸部と尿生殖隔膜との中間あたりで尿道の深さを理解して尿道前面をバンチング鉗子で把持。これを収束結紮させ，前面の組織をまさにSantorini静脈叢を切断するように切開し，尿道筋層を確認。そのまま尿道外側とおぼしきラインを設定し，外側で膣壁に向かいメッツェンバウムなどで尿道外側を剥離，この段階で傍膣組織などから出血があるが，尿道外側の組織を鉗子で把持，これを収束結紮することで尿道のみとし，後の膣壁の切開のゴールとして理解できる状態にしている。[**7-78**]に概要を示した。

出血に対する対応として2-0程度の糸で運針してみて，さらに針穴からの止血が不十分の場合は4-0や3-0程度のむしろ細い糸で針穴周囲を運針する。この段階である程度の止血がなされていれば，それ以上止血を追求しないほうがよい。さらに大きく運針して止血を得ようとすると周囲の静脈が裂け，どんどん出血が多くなるという悪循環となる。これがときに起こる大量の出血の原因であると考えている。

(文献はp217を参照)

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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.

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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′-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 Figure 1*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-

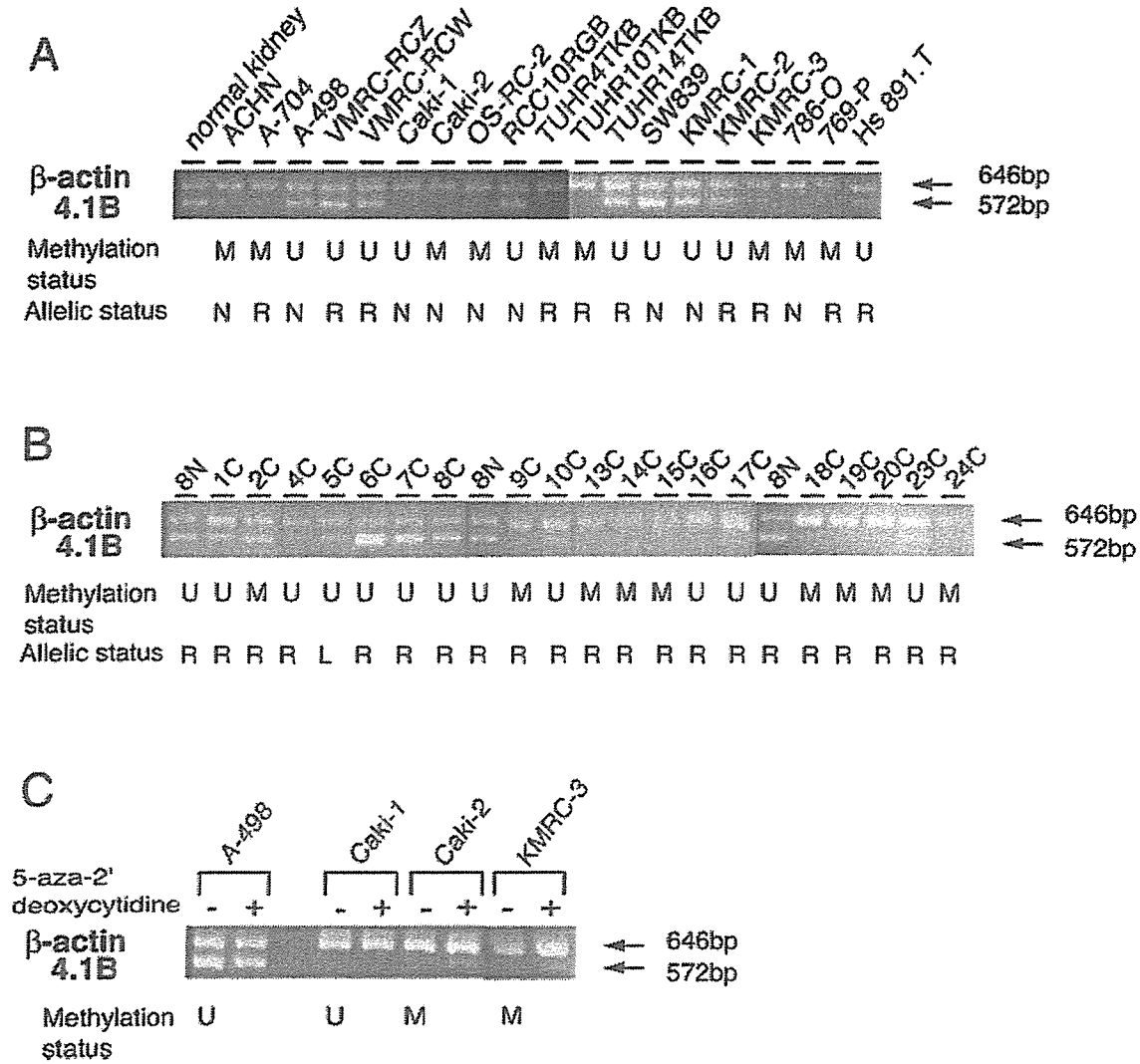

FIGURE 1 – Expression of the *4.1B* gene in RCC. (*a*) and (*b*): RT-PCR analysis of 4.1B and β-actin in RCC cell lines (*a*) and surgically resected RCCC (*b*). C and N in (*b*) indicate cDNA from a cancerous and noncancerous portion of the kidney, respectively. The results of methylation status determined 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*).

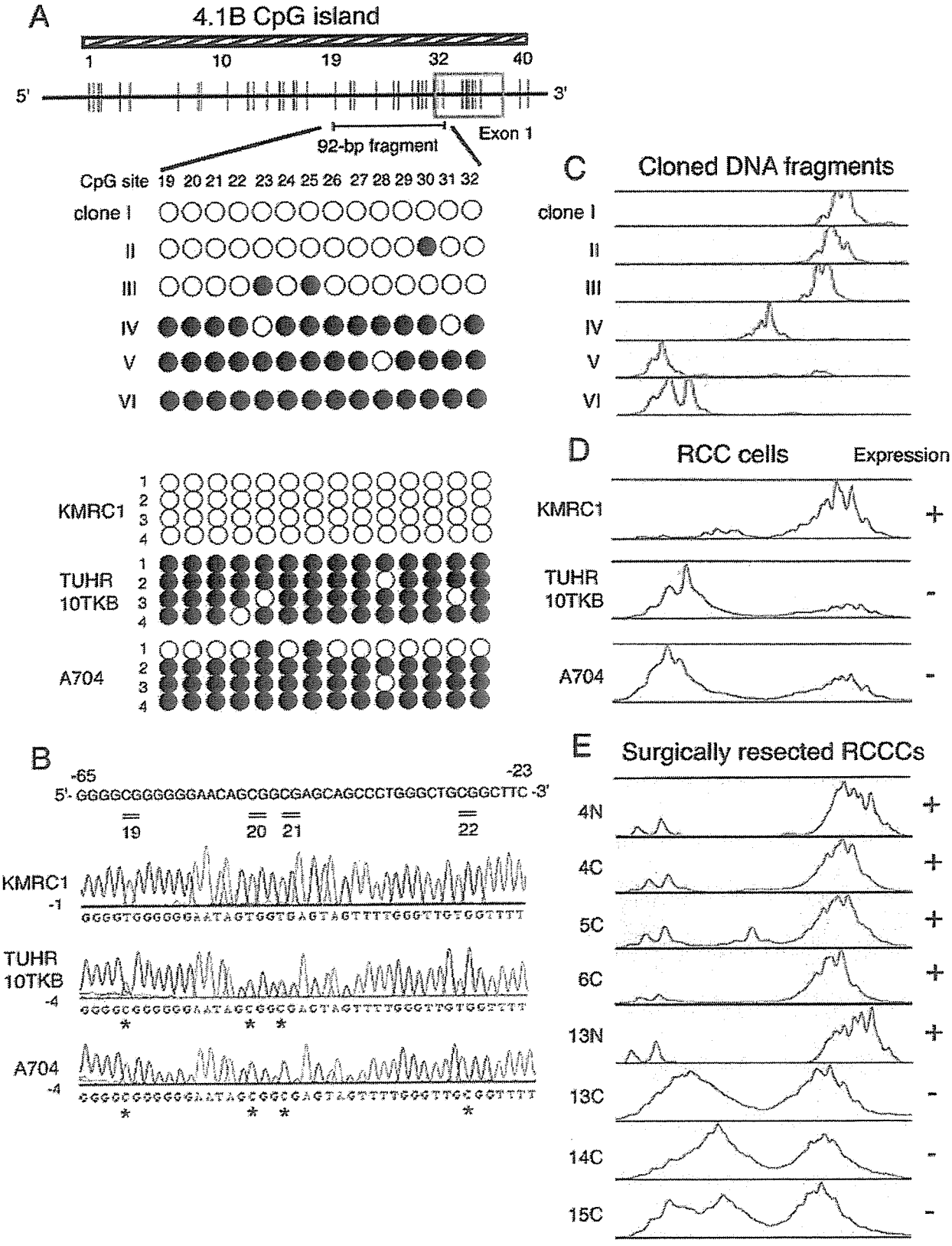
A
4.1B CpG island
1
10
19
32
40
5'
3'
Exon 1
92-bp fragment
CpG site 19 20 21 22 23 24 25 26 27 28 29 30 31 32
clone I
II
III
IV
V
VI
KMRC1
TUHR 10TKB
A704
B
-65
-23
5'- GGGGCGGGGGGAACAGCGGCGAGCAGCCCTGGGCTGCGGCTTC -3'
19
20 21
22
KMRC1 -1
TUHR 10TKB -4
A704 -4
C
Cloned DNA fragments
clone I
II
III
IV
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

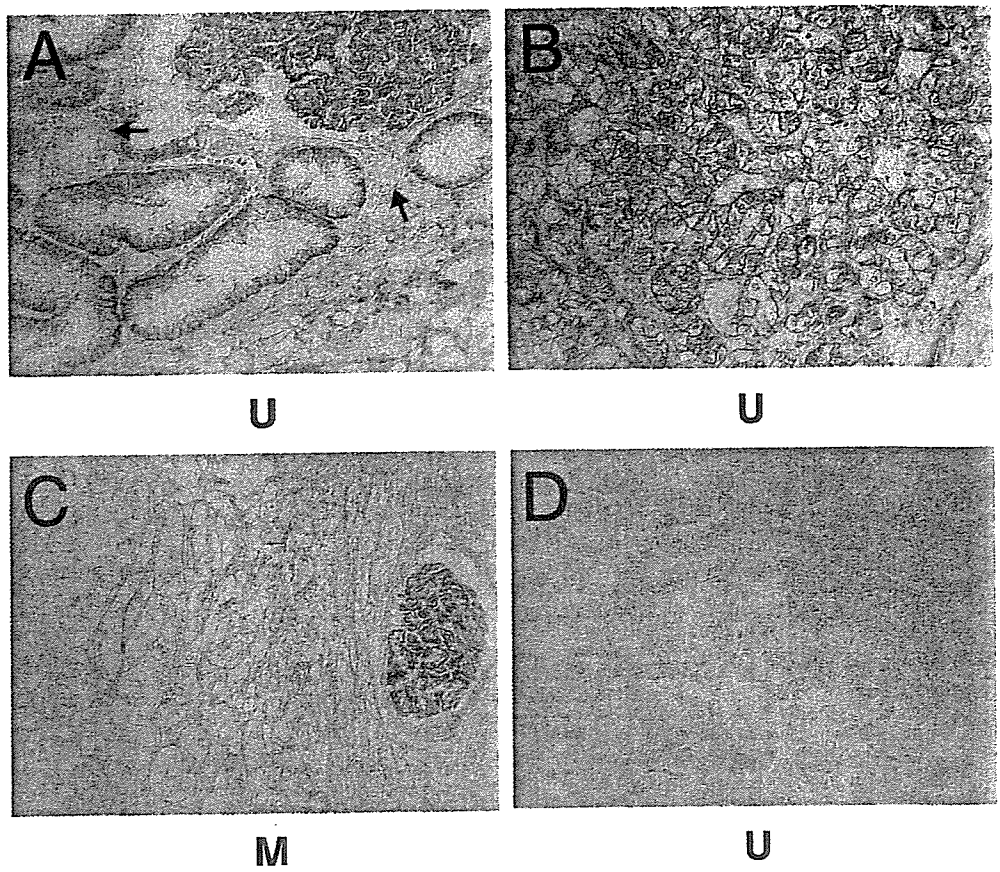

FIGURE 3 – Immunohistochemical analysis of 4.1B protein in human normal kidney (*a*) and surgically resected RCCC (*b*)–(*d*). (*a*) Expression of 4.1B is detected along the basolateral membrane of the proximal convoluted tubules and in the basement membrane of the glomeruli, but not in the distal convoluted 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-

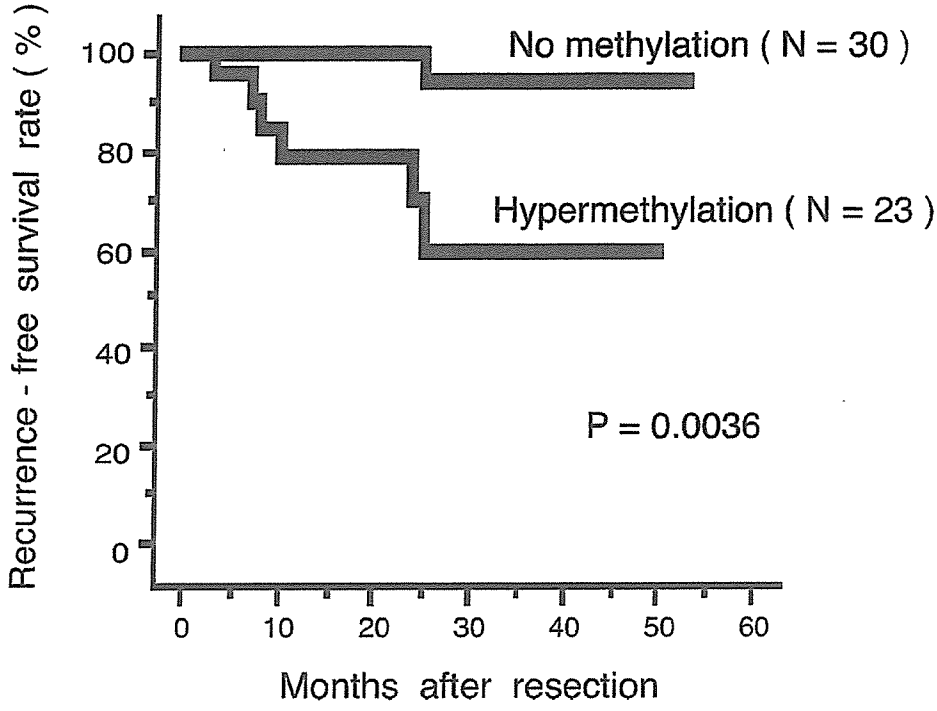

FIGURE 4 – Recurrence-free survival of the patients who received complete resection of RCCC with hypermethylated and unmethylated *4.1B* promoters. Intervals between the primary surgical resection and the metastatic recurrence at the lung, bone, liver, or pancreas are plotted in the Kaplan–Meier analysis.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

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