による小線源治療への応用を目的に、ファントムを用いた治療計画時間の解析を行い、そのプロセスにおけるQA・QCの要件解析を行った。

具体的には放射線治療計画について計画画像の取得より情報入力など、治療計画stepを見直し、それぞれに関与しているparameterと必要時間の解析により、放射線治療計画時間の短縮について検討した。さらに、放射線治療計画のQA・QCのシステム化について検証プロセスとシステム化の要件を解析した。

（倫理面への配慮）

本研究における技術的な検討においてはファントムを用いて検討した。今後の臨床への応用においては、臨床試験を計画しIRBの審査を経て十分に倫理的な配慮を尽くして行うことを計画している。

C.研究結果

1．放射線治療計画時間の短縮

本年度の研究の成果として治療計画時間短縮を目的に、放射線治療計画に関するparameterの解析を行った。その成果として、①高速CTによるthin－slice撮像による呼吸位相の誤差をおさえた計画画像の取得、②腫瘍形状に合わせたTarget Volume設定の実現により、治療計画装置への情報入力時間が短縮可能となった。さらなる治療計画時間の短縮を目的として、③治療計画Parameter選択、④治療計画作成にあたってのPrePlanとPostPlanの導入について検討を行い計画時間短縮の可能性が示唆された。

1－①高速CTによるthin－slice撮像

高速CTの放射線治療計画への応用は、外照射の三次元治療計画で研究が進んでおり、胸部の治療計画への応用では吸気時画像と呼気時画像の重ね合わせによるTarget Volume設定やmargin設定などに応用されている。しかしながら小線源治療計画装置では使用できるCTの枚数に制限があることが、臨床応用上の問題である。本研究では、あらかじめ治療計画に必要な範囲を特定し、制限範囲内での治療計画画像の取得を行う条件を検討しておくことにより、thin－slice撮像による呼吸位相の誤差をおさえた計画画像の取得が可能であることを明らかにした。

1－②Target Volume設定

治療計画装置に入力するTarget Volumeに関してはこれまでの研究により、腫瘍の位置関係により必要な設定を事前に施行することで、時間短縮に寄与することが明らかとなっている。さらに、本年度は入力方法の改善と設定の最適化を施行し、腫瘍形状に合わせたTarget Volume設定の実現により、治療計画装置への情報入力時間が短縮可能となった。

1－③治療計画Parameter選択

治療計画に使用するparameterについては、これまでの検討により定位放射線治療など外照射で使用されているparameterが本治療においても応用可能であることを示してきた。本年度は、治療計画における所要時間短縮を目的としてparameterの選択を検討した。全てのparameterが治療計画の最適化時に使用されることはなく、主として容積線量ヒストグラム（Dose-Volume Histogram; DVH）におけるRisk臓器の線量が重要であることが明らかとなり、次いでConformity Index（CI）により治療計画を選択する設定とすることで、治療計画時間の短縮に寄与することが明らかとなった。

1－④PrePlanとPostPlanの導入

PrePlanとPostPlanの考え方は、主として前立腺癌の永久刺入による小線源治療で広く使用されている。事前に治療計画を作成し、これに従って小線源を配置し、刺入終了後に再度治療計画を施行している。本研究における小線源治療においては小腫瘍を対象としているため、事前の治療計画を施行しても実際の治療時と対象腫瘍の大きさが異なる可能性が小さいため、治療計画時間の短縮目的でのPrePlanの作成による治療計画時間の短縮が期待される。実際に、ファントムを用いた検証では、事前に作成したPrePlanを実際の治療時にみたてて撮影しなおした計画画像に対する

治療計画として使用することにより、治療時間の短縮が可能であることが示唆されている。

２．治療計画の QA・QC システム化

治療計画の QA・QC システム化を目的とした検証プロセスとシステム化の要件に関する検討を行った。日本放射線腫瘍学会およびアメリカ Image-Guided Brachytherapy Working Group の小線源治療ガイドラインにおける小線源治療計画の QA・QC を参照し、本治療における QA・QC システムの要件についての解析と整理を行い、システム化に必要な項目について検討した。本研究における小線源治療においては、治療計画画像取得のプロセスや、治療計画装置における DVH の利用など治療最適化プロセスにおいて、新たな QA・QC システムの開発が必要となることが、明らかとなった。

D. 考察

磁気を応用した診断・治療装置と高線量率イリジウム遠隔操作装置の組み合わせによる、肺癌をはじめとした小型腫瘍に対する小線源治療では、治療計画に三次元治療計画装置を使用し、Inverse planning 法を治療計画の最適化に応用している。H17 までの本研究は最適化計算の時間短縮を可能としたが、依然として治療計画に要する時間が臨床応用に関しての問題点であった。

本年度の研究の成果として、治療計画時間短縮を目的に、放射線治療計画に関する parameter の解析を行い、①高速 CT による計画画像の取得、②Target Volume 設定の改善により治療計画装置への情報入力時間が短縮可能となっている。また、③治療計画 Parameter 選択、④治療計画作成にあたっての PrePlan と PostPlan の導入について検討を行い計画時間短縮の可能性が示唆されている。本研究による小線源治療方法のもっとも大きな課題が治療計画に必要な時間の短縮であり、本年度の成果は今後の臨床応用を考える場合、大きな影響を与えることが予想される。

さらに、治療計画の QA・QC システム化を目的とした検証プロセスとシステム化の要件解析に関しては、本研究における小線源治療計画のみならず、より広範な放射線治療の QA・QC において有用であることが予想される。すなわち、CT を応用した治療計画が今後さまざまな領域の放射線治療で主流となっていくことが想定されており、リアルタイムの治療計画は Image-guided Radiotherapy の実施においてますます重要な課題となっている。本研究の内容は、小線源治療のみならず放射線治療の各分野で広く応用可能と考えられる。治療の安全性を担保し、円滑な治療計画の立案・検証・実施を遂行する際に、放射線治療計画の QA・QC に関するシステム化は重要な役割を果たすと考えられる。本研究により得られた成果の臨床応用により、より高精度な小線源治療の治療計画の実現が可能となったと考えられる。

E. 結論

本研究では磁気を応用した診断・治療装置と高線量率イリジウム遠隔操作装置の組み合わせによる、肺癌をはじめとした小型腫瘍に対する小線源治療に必要な、治療計画の標準化を検討してきた。

本年度の成果により、これまでの研究の結果で明らかとなった臨床応用上の課題である治療計画に要する時間の短縮について、治療計画画像の取得より最適化のプロセスまで時間短縮に関する検討により臨床応用可能な時間短縮を図った。さらに、放射線治療計画の QA・QC のシステム化について検討を行い、新たにシステム開発が必要な項目を明確化した。

本研究により得られた成果の臨床応用により、より高精度な小線源治療の治療計画の実現が可能となったと考えられる。

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H. 知的財産権の出願・登録状況

１．特許取得

情報の記録方法及び情報記録装置。
特許第 3845725 号, 2006 年 9 月 1 日

２．実用新案登録

なし

３．その他

なし

研究成果の刊行に関する一覧表

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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′-GGTGCGGAGGGAGGTCACTGACAAGGAACA G-3′ and 5′-CGCTCCCACATTCATCTGGGTCATAGTCTCCG AG-3′ for DAL-1 (1.0 μM, each) and 5′-GGTGCGGAGGGA GGTCACTGACAAGGAACAG-3′ and 5′-CGCTCCCACATTC ATCTGGGTCATAGTCTCCGAG-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-

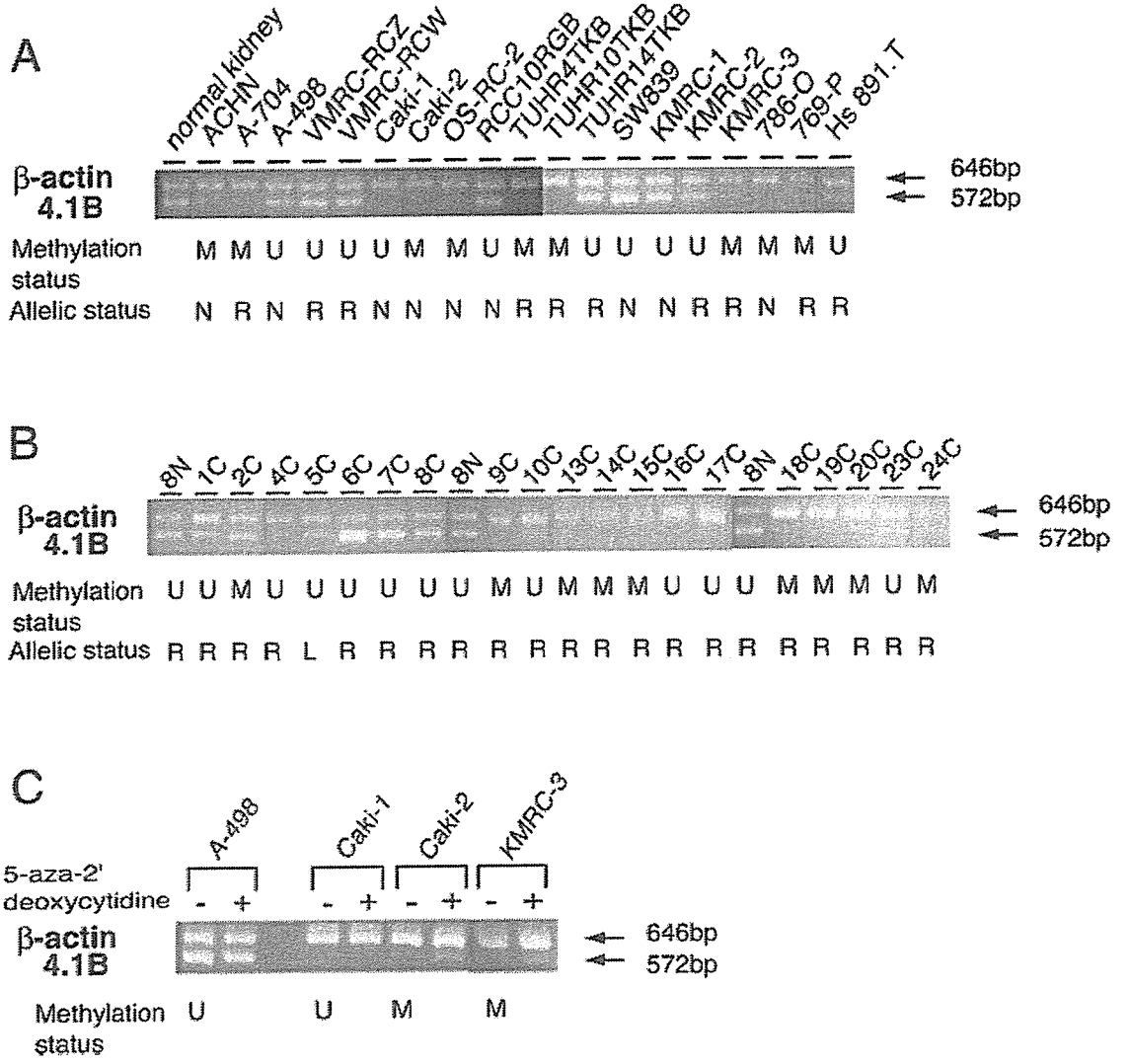

FIGURE 1 – Expression of the *4.1B* gene in RCC. (*a*) and (*b*): RT-PCR analysis of 4.1B and β-actin in RCC cell lines (*a*) and surgically resected RCCC (*b*). C and N in (*b*) indicate cDNA from a cancerous and noncancerous portion of the kidney, respectively. The results of methylation status determined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

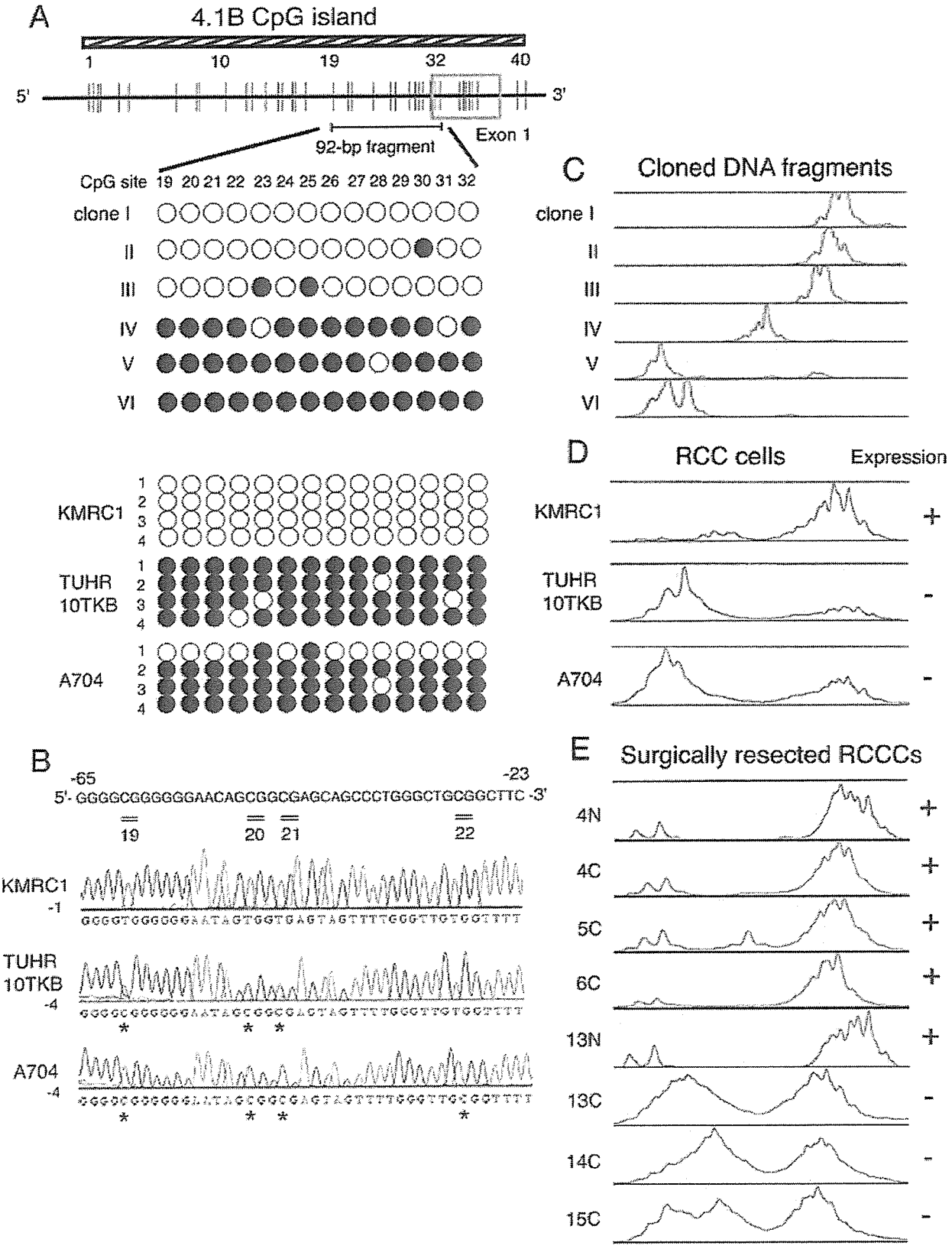
A
4.1B CpG island
1
10
19
32
40
5'
3'
92-bp fragment
Exon 1
CpG site 19 20 21 22 23 24 25 26 27 28 29 30 31 32
clone I
II
III
IV
V
VI
KMRC1
TUHR
10TKB
A704
B
-65
-23
5'- GGGGCGGGGGGAACAGCGGCGAGCAGCCCTGGGCTGCGGCTTC -3'
19
20
21
22
KMRC1
-1
TUHR
10TKB
-4
A704
-4
C
Cloned DNA fragments
clone I
II
III
IV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

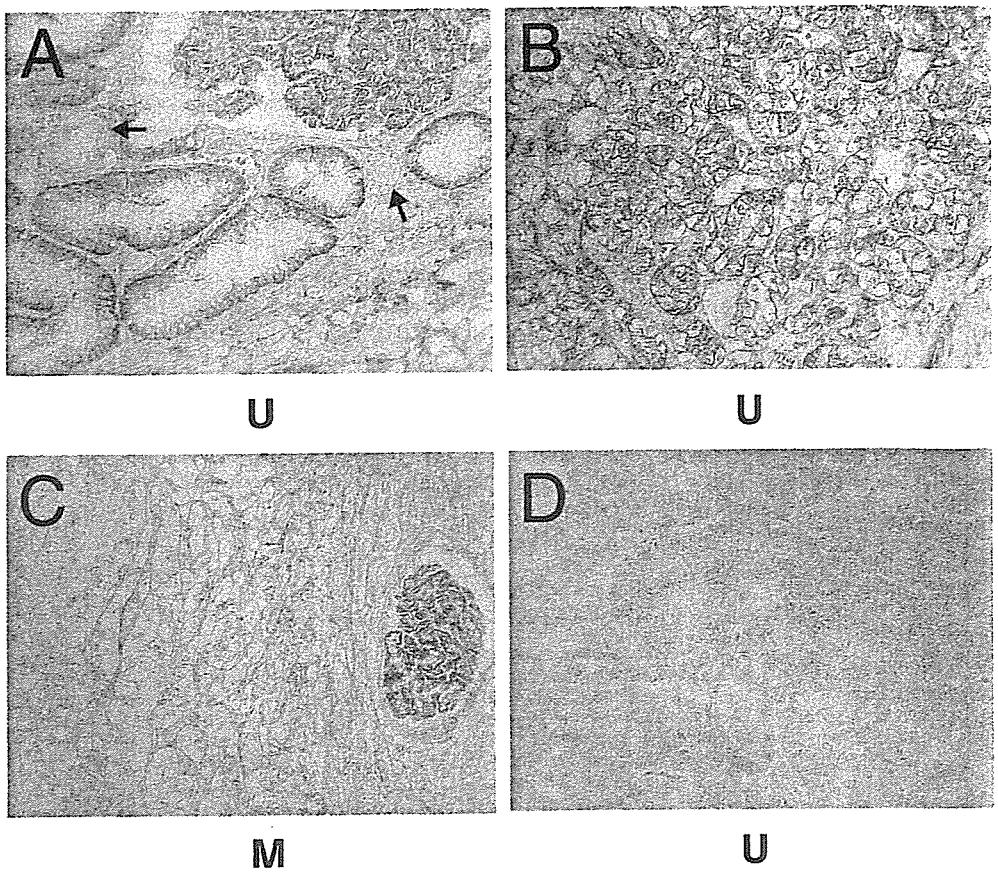

FIGURE 3 – Immunohistochemical analysis of 4.1B protein in human normal kidney (*a*) and surgically resected RCCC (*b*)–(*d*). (*a*) Expression of 4.1B is detected along the basolateral membrane of the proximal convoluted tubules and in the basement membrane of the glomeruli, but not in the distal convoluted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

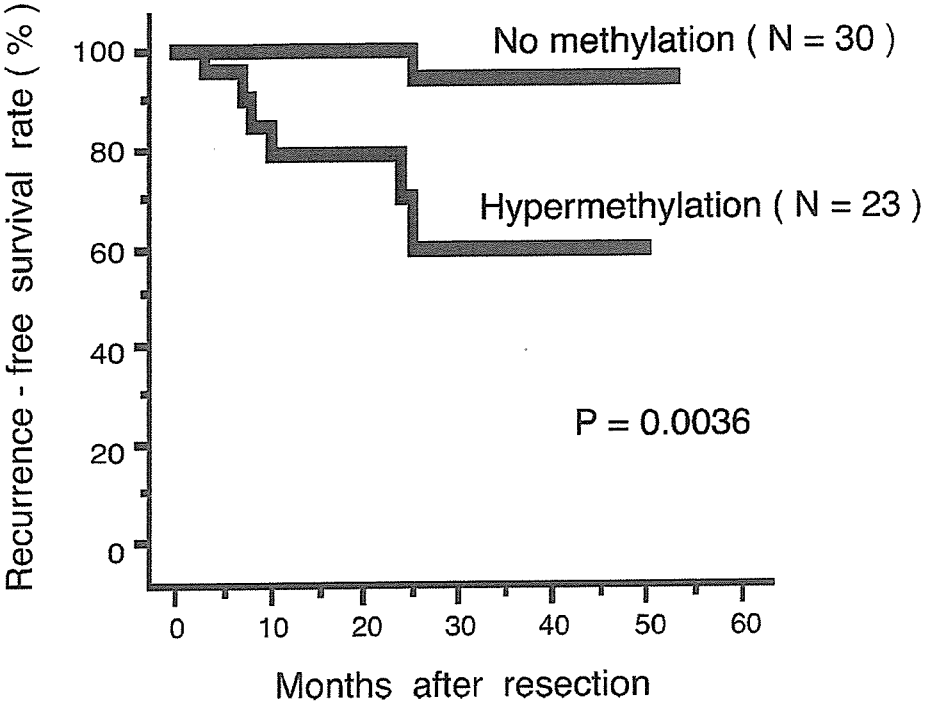

FIGURE 4 – Recurrence-free survival of the patients who received complete resection of RCCC with hypermethylated and unmethylated *4.1B* promoters. Intervals between the primary surgical resection and the metastatic recurrence at the lung, bone, liver, or pancreas are plotted in the Kaplan–Meier analysis. 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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A Multicenter Randomized Controlled Trial to Evaluate the Effect of Adjuvant Cisplatin and 5-Fluorouracil Therapy after Curative Resection in Cases of Pancreatic Cancer

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Background: There have been few randomized controlled clinical trials until now to determine the effectiveness of adjuvant treatments for pancreatic cancer, and the results reported so far are inconsistent.

Methods: Patients with invasive ductal pancreatic cancer who underwent radical surgery with clear histological margins at 11 Japanese institutions were enrolled and randomly assigned to one of two groups: surgery-alone group (no further treatment after surgery) and the surgery + chemotherapy group [two courses of postoperative adjuvant systemic chemotherapy with cisplatin (80 mg/m^2, Day 1) and 5-fluorouracil (500 mg/m^2/day, Days 1–5)]. Patients with a positive resectional margin or with resected distant metastases were excluded from the trial in order to minimize the influence of residual cancer.

Results: Between 1992 and 2000, 89 patients were randomized into the two arms of the trial (45 patients to the surgery + chemotherapy arm and 44 patients to the surgery-alone arm). Four patients in total were found to be ineligible (three in the surgery + chemotherapy group and one in the surgery-alone group). The baseline characteristics were comparable between the two groups. In the surgery + chemotherapy group, four patients did not receive the adjuvant treatment because of patient refusal. Toxicity was minor and acceptable among the eligible patients in the surgery + chemotherapy group. The estimated 5-year survival rates were 26.4% in the surgery + chemotherapy group and 14.9% in the surgery-alone group, and the median duration of survival was 12.5 months and 15.8 months, respectively. The recurrence rates at 5 years were 73.6 and 80.8%, respectively, in the surgery + chemotherapy and the surgery-alone groups. The differences in the survival and recurrence rates between the two groups were not statistically significant.

Conclusions: Postoperative adjuvant chemotherapy using cisplatin and 5-fluorouracil was safe and well tolerated; however, no clear survival benefit could be demonstrated.

Key words: adjuvant – chemotherapy – clinical trials – pancreatic neoplasms

INTRODUCTION

Pancreatic cancer is the fifth most common cause of death from cancer in Japan (1) and the United States (2), and its incidence is rising. Although radical resection appears to be the only means to obtain a cure, the 5-year survival rate after potentially curative resection remains extremely low, in the range of 5–30% (3–5). Therefore, effective adjuvant therapy is currently being sought. The Gastrointestinal Tumor Study Group (GITSG) performed the first multicenter randomized controlled trial to evaluate the efficacy of adjuvant treatment (6), and they concluded that adjuvant chemoradiotherapy prolonged the postoperative survival of patients with pancreatic cancer. However, the results of a few subsequent randomized controlled trials (7–10) have been inconsistent, and further evidence concerning the effectiveness of adjuvant treatments for pancreatic cancer is awaited. Combination chemotherapy with 5-fluorouracil (5-FU) and cisplatin was considered to be a promising regimen for pancreatic cancer in the early 1990s (11,12). Based on our experience of using

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this regimen in patients with unresectable pancreatic cancer, we expected that it might prove to be suitable for postoperative adjuvant treatment (13). In 1992, we initiated a multicenter randomized controlled trial to evaluate the efficacy of adjuvant chemotherapy with cisplatin and 5-FU after margin-negative resection in patients with pancreatic cancer.

METHODS

Patients and Design

Patients with ductal pancreatic cancer who underwent resectional surgery with histologically clear margins between April 1992 and March 2000 in 11 Japanese institutions were enrolled for the present study. Patients with other pancreatic and periampullary neoplasms, such as intraductal papillary mucinous neoplasm, cystadenocarcinoma and endocrine tumor, were excluded. Presence of distant metastases, even if they were resected, and presence of peritoneal seeding were regarded as criteria for exclusion from the study. After we obtained their written informed consent, the patients were registered with the randomization center by fax within 10 weeks of surgery and were then randomly assigned to one of two groups: the surgery-alone group and the surgery + chemotherapy group. They were stratified according to the institution and tumor stage using the minimization technique. The tumor stage was determined according to either the fourth or fifth edition of the UICC TNM classification, depending on the time of patient registration, and the patients were divided into two categories for stratification as follows: those with tumor in stage I or stage II according to the fifth edition (14) [equivalent to stage I of the fourth edition (15)] were assigned to one group and the remaining patients were included in the other group. Resection procedures and the range of dissection were determined according to institutional policy. Handling and histological examination of the resected specimens were carried out according to the recommendations of the Japan Pancreatic Society (16). Patients in the surgery-alone arm and those in the surgery + chemotherapy arm were followed up at 3 month intervals. Blood tests and imaging by computed tomography or ultrasound were carried out. Diagnosis of recurrence was made based on the imaging findings. Treatment after recurrence was not defined. All data were collected at a central registration office. Three pathologists performed the pathology reviews for the first 18 cases; thereafter, institutional histological diagnoses were relied upon. No external beam radiation was given to any of the patients. Intraoperative irradiation was administered on an institution-by-institution basis, and this was given to all candidates of any institution who opted to use it. The trial was conducted with the approval of the local ethics committee at each institution.

Adjuvant Chemotherapy

Chemotherapy was started within 1 week of randomization. Two courses of treatment with a combination regimen of 5-FU and cisplatin were administered. Cisplatin was administered at a dose of 80 mg/m^2 on the first day of the treatment course; 5-FU was given at a daily dose of 500 mg/m^2 as a continuous infusion for the first 5 days of the treatment course. The second course was repeated 4–8 weeks after the start of the first course. Toxicity was assessed according to the World Health Organization (WHO) guidelines (17). The second course was withheld if toxicity of grade 3 or above severity was observed or if the patient's condition did not improve sufficiently to fit the eligibility criteria for registration within 8 weeks of the start of the initial course.

Statistics

The primary endpoint of the study was the duration of survival. Duration of survival was calculated from the date of registration to the date of death due to any cause or was censored at the latest follow-up. The two treatment arms were also compared for recurrence rate. Safety analyses were performed based on data obtained from all the eligible patients who had started chemotherapy. Efficacy analyses were performed according to the intention-to-treat principle. Survival curves were drawn using the Kaplan–Meier technique. Differences in the duration of survival were compared using a two-sided log-rank test, with the significance level set at 5%. The prognostic value of the variables was tested by multivariate analysis using the Cox proportional hazards model. Assuming an overall 2-year survival rate of 15% in the surgery-alone arm, the present study was designed to enroll more than 86 patients in order to detect an absolute increase by 25% (i.e. 40% survival rate for 2 years) in the surgery + chemotherapy arm, at a significance level of 5% with 80% power.

RESULTS

Patient Characteristics

Between April 1992 and March 2000, 89 patients were randomized: 45 patients to the surgery + chemotherapy arm and 44 patients to the surgery-alone arm. Three patients in the surgery + chemotherapy group and one in the surgery-alone group were rated ineligible, resulting in 95.5% compliance. The reasons for ineligibility included resected distant metastases (two cases), histologically positive resection margin (one case) and severe postoperative complication (one case). The baseline characteristics of the patients in the two groups were comparable (Table 1).

Treatment Data

Four patients assigned to the surgery + chemotherapy arm refused treatment after randomization, and the detailed data for three ineligible patients were not available. As a result, a total of 38 patients were evaluated for treatment toxicity. Of these, 31 patients (81.6% of the patients who received chemotherapy) received two courses of chemotherapy, and 7 patients received only one course of chemotherapy. The reasons for treatment discontinuation were patients' withdrawal from the

Table 1. Demographic and clinical data for the patients

	Surgery + chemotherapy	Surgery alone
Gender (M:F)	29:16	21:23
Age (mean ± SD)	60.8 ± 8.1	60.1 ± 8.9
Operative procedure (pancreaticoduodenectomy:others)	37:8	34:10
Intraoperative irradiation (30:0 Gy)	30:15	27:17
Location of the tumor (head:body/tail)	35:9	34:8
Size of the tumor (<4:≥4 cm)	35:10	36:8
Histological type (papillary and well-differentiated:others)	24:21	21:23
Nodal involvement (present:absent)	12:23	9:35
pT (pT1–3:pT4)	36:9	33:11

trial (four cases), development of recurrent disease (two cases) and unresolving leucopenia (one case).

Toxicity

One ineligible patient who was suffering from a severe post-operative complication that was not documented at the time of registration died of sepsis after one course of chemotherapy. Minor toxicity was commonly observed, especially nausea and vomiting, among the 38 eligible patients who actually received the adjuvant chemotherapy. In a few patients, toxicities of grade 3 or higher severity were encountered (Table 2). However, the toxicities were reversible and resolved with conservative treatment alone in all patients.

Recurrence

Seventy-one patients died and 18 patients were alive at the end of the follow-up period. The median follow-up duration for the survivors was 44.8 months. The recurrence status remained unknown in one ineligible patient. Among the remaining 88 randomized patients, 34 (77.3%) in the surgery-alone group and 32 (71.1%) in the surgery + chemotherapy group developed recurrence. The recorded sites of recurrence are shown in Table 3. The liver was the most frequent site of recurrence for metastasis, followed by peritoneal seeding and local recurrence, in both groups. There was no significant advantage of adjuvant chemotherapy in terms of the recurrence rate (Figure 1). The median time to recurrence was 10.2 months in the 44 patients in the surgery-alone group and 8.6 months in the 44 patients in the surgery + chemotherapy group. The 5-year recurrence rates were 80.8 and 73.6%, respectively, in the two groups ($P = 0.80$).

Duration of Survival

In the randomized patients, the cause of death was recurrent disease in 63 patients (32 from the surgery-alone group and 31 from the surgery + chemotherapy group), in-hospital death in 1 patient (from the surgery + chemotherapy group), non-malignant/non-toxicity death in 5 patients (3 from the surgery-alone group and 2 from the surgery + chemotherapy group) and unknown in 2 patients (1 from each group). The duration of survival was not influenced by adjuvant chemotherapy in either the randomized or the eligible patients. The survival curves of all the randomized patients are shown in Figure 2. The median survival was 15.8 months in the surgery-alone group and 12.5 months in the surgery + chemotherapy group, and the 5-year survival rate was 14.9% in the surgery-alone group and 26.4% in the surgery + chemotherapy group ($P = 0.94$).

Table 2. Summary of toxicities according to WHO criteria ($n = 38$)

	Grade 1	Grade 2	Grade 3	Grade 4
Nausea/vomiting	15	12	5	0
Leukopenia	14	6	2	0
Granulocytopenia	6	5	3	1
Thrombocytopenia	7	2	0	0
Mucositis	1	1	2	0
Cardiac	0	0	0	0
Hepatic	17	9	3	0
Renal	3	1	0	0

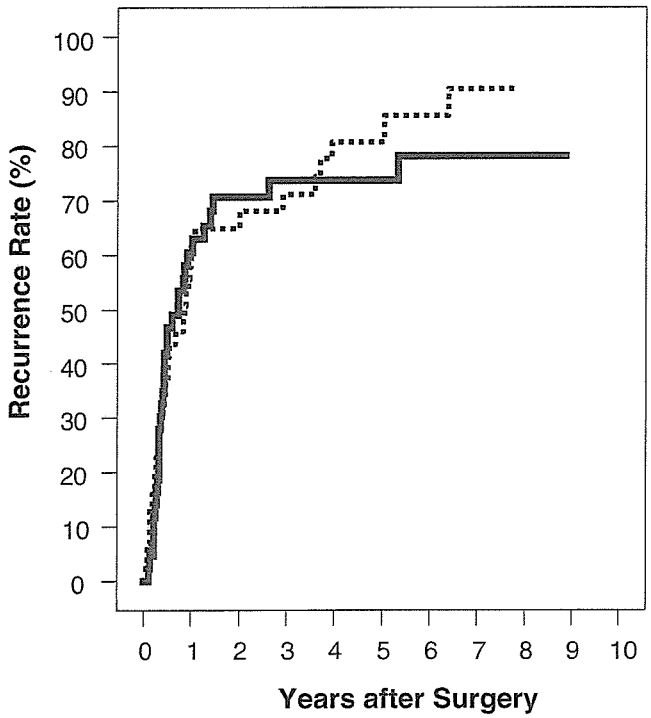
Table 3. Sites of recurrence

	Surgery + chemotherapy	Surgery alone
Liver	20	22
Peritoneum	10	9
Pleura	1	1
Local recurrence	6	7
Lymph node	3	1
Lung	1	1
Bone	1	1
Skin	0	2
Brain	1	0
Number of patients with recurrence	32	34

Prognostic Factors

In order to assess the influence of prognostic factors, the relationship of the outcomes to the following variables were investigated: gender, age, histological type, size of tumor, tumor location, pT factor, nodal involvement, type of operative procedure and administration of intraoperative radiotherapy. Calculation of the correlation coefficients (r) of pairs of variables revealed a close correlation for the tumor location and the type of

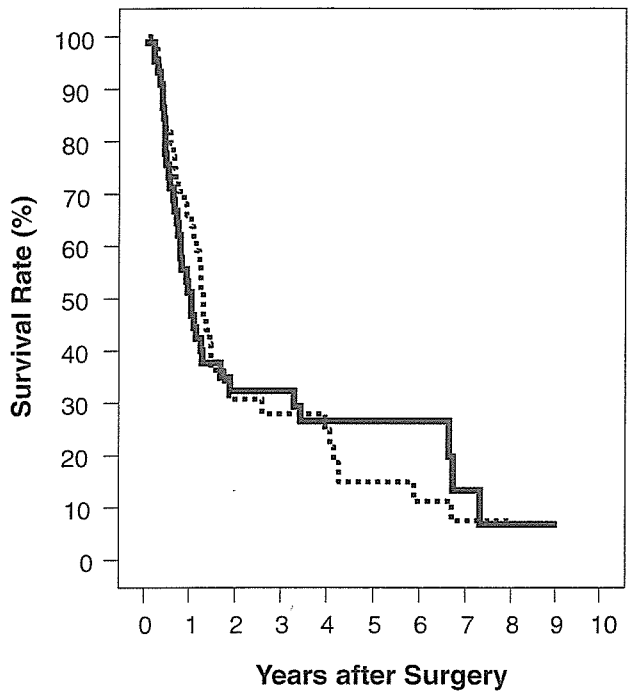
operative procedure ($r = 0.96$), whereas the coefficients for all the other pairs were less than 0.5. Consequently, 'operative procedure' was excluded from the subsequent multivariate analysis. The prognostic value of the remaining variables together with the assigned treatment arm as an additional variable was tested using multivariate analysis. The significant factors determined from this analysis were nodal involvement and the histological type of the tumor (Table 4); the effect of the pT factor was marginal. Consequently, the stage of the disease, the major determinants of which were the nodal status and the pT factor, was determined to be a good prognostic indicator. Patients with tumor in stage I or in stage II according to fifth edition of the UICC TNM classification survived significantly longer than those with more advanced disease (Figure 3). The median survival time in the two groups was 79.7 and 12.6 months, respectively ($P = 0.004$).



No. at risk

Observation	44	18	11	9	6	4	3	2
Chemotherapy	44	16	10	9	6	6	3	2

Figure 1. Cumulative recurrence rate. Solid line: surgery + chemotherapy group; dotted line: surgery-alone group.



No. at risk

Observation	44	29	11	10	8	4	3	2
Chemotherapy	45	23	12	11	7	7	5	2

Figure 2. Cumulative survival rate. Solid line: surgery + chemotherapy group; dotted line: surgery-alone group.

Table 4. Multivariate analysis

Variable	β	SE	*P*	HR	95% CI
Nodal involvement (absent versus present)	1.167	0.348	0.001	3.213	(1.626–6.350)
Histological type (papillary or well-differentiated tubular versus moderately or poorly differentiated tubular)	0.791	0.273	0.004	2.206	(1.291–3.769)
pT factor (pT1–3 versus pT4)	0.528	0.300	0.078	1.695	(0.942–3.050)
Gender (female versus male)	0.393	0.272	0.148	1.482	(0.869–2.526)
Size of tumor (<4 cm versus ⩾4 cm)	0.166	0.172	0.334	1.181	(0.843–1.654)
Age	0.017	0.016	0.282	1.017	(0.986–1.049)
Chemotherapy	−0.053	0.254	0.835	0.948	(0.576–1.561)
Intraoperative radiotherapy	−0.280	0.299	0.349	0.756	(0.421–1.357)
Location of the lesion (head versus body or tail of the pancreas)	−0.354	0.291	0.224	0.702	(0.397–1.241)

SE, standard error; *P*, significance; HR, hazard ratio; CI, confidence interval.

DISCUSSION

Resectional surgery provides the only chance of cure for patients with pancreatic cancer. However, in most cases the operation is non-curative, and an extremely high recurrence rate is observed. Consequently, a number of adjuvant treatments have been tried in the hope of prolonging survival. GITSG performed the first randomized controlled trial to evaluate the effect of adjuvant treatment in a group of 43 patients with pancreatic cancer. They concluded that adjuvant chemoradiotherapy after curative resection prolonged patient survival (6). However, subsequent larger studies carried out in Europe failed to show any evident benefit of adjuvant chemoradiotherapy (8–10). Similarly, the value of systemic chemotherapy in an adjuvant setting also remains controversial owing to the scarcity of convincing evidence. Only three randomized controlled trials have been reported to assess adjuvant chemotherapy for pancreatic cancer in the literature (Table 5). Bakkevold et al. (7) claimed that adjuvant combination chemotherapy using 5-FU, doxorubicin and mitomycin C (AMF) prolonged the median survival time in a cohort of postoperative patients with pancreatic or ampullary cancer. However, their results were not definitive, because there was no detailed documentation of the data. Takada et al. found no advantage of adjuvant treatment using mitomycin C and 5-FU in a larger number of patients (18). Neoptolemos et al. (9,10) conducted a prospective randomized trial (ESPAC-1) to assess the effects of two types of adjuvant treatments, namely, chemotherapy and chemoradiotherapy. They concluded that chemotherapy alone improved the survival rate and that chemoradiotherapy may even have had an adverse effect on survival. However, their conclusion remains a subject of debate because of the unorthodox and complex design of the study.

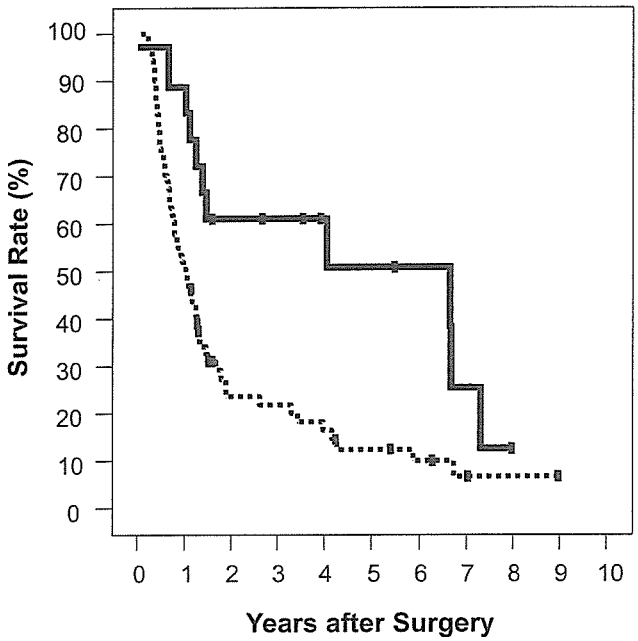


Figure 3. Cumulative survival rate categorized by the disease stage according to the fifth edition of the UICC TNM classification. Solid line: stages I and II; dotted line: stages III, IVa and IVb.

In contrast to the ESPAC-1 trial, our study was simple in design, allowing the comparison of survival between two patient groups: one group with adjuvant chemotherapy and the other group without adjuvant chemotherapy. Patients were stratified according to the institution and stage of the disease in order to minimize the influence of possible prognostic factors. Furthermore, patients with a positive histological margin were excluded from the study with the objective of excluding possible bias introduced by one of the strongest prognostic factors, the status of the resectional margin (19). However, this last criterion did interfere with the rapid recruitment of patients. It took almost 8 years to carry out the registration and randomization of 89 patients. Fortunately, there have been no remarkable changes in the diagnosis or treatment of pancreatic cancer during this period, and the trial could be continued without any major revisions of the protocol. Only two previous trials have evaluated adjuvant treatment for patients with R0 resection (6,7). Both encountered similar difficulties and a smaller number of patients were enrolled

Table 5. Randomized controlled trials of adjuvant chemotherapy for pancreatic cancer

Author	Year of publication	Disorder	Chemotherapy	Number of cases	MST (months)	5-year SR (%)	Significance
Bakkevold et al.	1993	PC and AMP (R0)	AMF	31	23	4	NS with generalized Wilcoxon's test
			Observation	30	11	8	
Takada et al.	2002	PC (R1)	MF	81	NA	11.5	NS with the log-rank test
			Observation	77	NA	18.0	
ESPAC	2004	PC (R1)	5-FU + LV	147	20.1	21	$P = 0.009$ the with log-rank test
			No chemotherapy	142	15.5	8	
Present study		PC (R0)	FP	45	12.5	26.4	NS with the log-rank test
			Observation	44	15.8	14.9	

AMF, doxorubicin + mitomycin C + 5-FU; AMP, ampullary carcinoma; LV, folinic acid; MF, mitomycin C + 5-FU; MST, median survival time; NA, not available; NS, not significant; PC, pancreatic cancer; SR, survival rate.

than in the present study. The patient characteristics, especially the stage distribution and proportion of patients with nodal involvement, were comparable among these trials.

The present study showed that adjuvant combination chemotherapy using 5-FU and cisplatin could be carried out with acceptable safety, as long as the patients met the eligibility criteria, although one patient died of sepsis after a single course of chemotherapy. The monitoring committee, after analysis of the case data, judged that this particular patient was unsuitable for enrollment into the trial. According to the recommendation of the committee, part of the study protocol was modified to clarify some requirements regarding the postoperative condition of the patients. No serious complications were encountered after the modification was carried out.

On the other hand, this trial failed to show any significant benefit of adjuvant chemotherapy in terms of either survival or recurrence, even though the absolute values of the survival rate and recurrence rate at 5 years were slightly better in the patients to whom adjuvant chemotherapy was administered. It is possible that a larger number of patients must be examined to appreciate the statistical significance of the treatment effect. However, a significant influence of the typical prognostic factors on survival was confirmed in the present trial. It is possible that the influence of adjuvant chemotherapy on survival is much weaker than that of these prognostic factors. Another possibility is that further courses of chemotherapy might reinforce the effectiveness of the treatment and allow it to become evident. However, it must also be considered that the life expectancy of patients with pancreatic cancer is extremely short. Adjuvant treatment for pancreatic cancer would be practical only when its beneficial effect can compensate for the compromised quality of life of the patient resulting from the treatment. Therefore, a distinct effect with a short treatment period, besides minimum toxicity, would seem to be the essential prerequisite of effective adjuvant chemotherapy. Otherwise, the lifetime spent with low quality of life can cancel out or even reverse the potentially beneficial effects of adjuvant treatment.

To conclude, the present trial did not prove that the regimen can be recommended as adjuvant treatment for pancreatic cancer.

Contributors

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(The affiliation of each member is the affiliation at the time of the patient recruitment.)

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