も，化学療法や放射線療法の早期開始により，QOLを保ちながら生存期間の延長が可能となることも目的のひとつである。

とくに，近年，切除不能再発大腸癌に対して，高い奏効率が報告されているirinotecan，oxaliplatinなどの新規抗癌剤により，切除不能再発症例においても，治療によりある程度の延命効果が期待できる。

一方，直腸癌手術の場合，手術による骨盤内操作による影響や，直腸切除，人工肛門造設による手術侵襲により，術後の排尿・排便・性機能障害のコントロールのために，定期的な外来診察や適切な指導，適切な投薬指導は重要であり，このことは患者の術後のQOL維持に大きく関与する。

各種フォローアップ方法の実際と意義

直腸癌術後フォローアップの検査項目は，問診・診察（直腸診），血液検査（腫瘍マーカー），胸部単純X線検査，腹部超音波検査，CT，MRI，PET，大腸内視鏡検査があげられる。

1. 臨床症状，診察

直腸癌術後再発を疑わせる臨床症状には，腹膜播種再発による腸閉塞症状，吻合部再発による排便習慣の変化や血便，骨盤内再発による会陰部や殿部痛，坐骨神経痛などがある。肝転移，肺転移などの遠隔転移に関連する症状には，黄疸や咳嗽などがあるが，これらの症状が出現したときは，治癒切除術が困難な場合が多い。

直腸指診は，外来で簡単に行うことのできる診察法であり，直腸癌術後の吻合部再発，局所再発，腹膜播種再発でのDouglas窩腫瘍の診断に有効である。また，低位前方切除術後の吻合部の状態を把握し，finger bougieによって吻合部狭窄の診断・予防にもなる。

2. 腫瘍マーカー

直腸癌の腫瘍マーカーとしてもっとも汎用されているのは，現在Carcinoembryonic Antigen（CEA）である。Grahamらは，治療可能な再発巣を発見する方法としてCEA測定がもっともcost-effectiveであると報告している[4]。

3. 胸部単純X線検査

直腸癌の肺再発は，結腸癌と比較して高頻度に認める。今回の検討でも，結腸癌では，肺再発率は2.8%であったが，直腸癌では7.6%であった。しかし，フォローアップにおける胸部X線検査の意義は，controversialである。胸部X線検査により切除可能な肺転移病変を発見できる率は0.9%という報告もあり[4]，欧米では，定期的なフォローアップ検査に推奨しない傾向がある[5]。一方，Ikeらは，2カ月ごとのCEA測定と6カ月ごとの胸部X線検査で発見された肺転移症例中，切除可能症例42例の検討では，5年生存率が63.7%であり，胸部X線検査を含むintensiveフォローアップの有効性を報告している[6]。

4. 腹部超音波検査

腹部超音波検査は，検査費用も高くはなく，非侵襲的な検査であり，大腸癌術後肝転移のスクリーニング検査としてもっとも行われている。Makelaらは，6カ月ごとの超音波検査が肝転移の早期発見に有用であると報告している[7]。

5. CT

CTは，ヘリカルCTやマルチスライスCTにより，高速かつ非常に繊細な撮像や3次元表示が可能となった。マルチスライスCTでは高解像度の多方向表示が可能となり，MRI画像と遜色のないものになってきている。しかし，CTはX線透過性を唯一のパラメーターとするため，軟部組織の鑑別能が低く，直腸癌局所再発の診断方法としては限界がある。造影CTでも瘢痕組織と腫瘍の造影効果の差は少ない。

6. MRI

MRIは組織コントラストが得られやすく，軟部組織の分解能が高い点から，直腸癌局所再発の診断において，CT検査よりも優れている。一般的には，再発巣はT1強調像で低信号，T2強調像では間質量の差などによりさまざまな信号強度を示す。一方，線維性の瘢痕組織はT1，T2ともに低信号を呈することが多く鑑別できるとされている。しかし，実際の症例では瘢痕組織もさまざまな信号強度を示し，癌と線維性瘢痕組織が混在するような症例では鑑別が困難である[8]。

7. PET (positron emission tomography)

大腸癌の再発診断におけるFDG-PETの高い診断能については多くの報告例があり，肺転移，肝転移，また局所再発の診断に有効性が明らかになってい

表7　推奨される直腸癌術後基本的フォローアップシステム

	術後3年間	3～5年間	5年以降
診　察	3カ月	6カ月	12カ月
直腸指診	6カ月	6カ月	12カ月
腫瘍マーカー（CEA）	3カ月	6カ月	12カ月
胸部X線検査	6カ月	12カ月	12カ月
腹部超音波検査	3カ月	6カ月	12カ月
骨盤CT検査	6カ月	12カ月	12カ月
大腸内視鏡検査	12カ月	24カ月	24カ月

る[9)10)]。とくに，CT，MRIでは従来困難とされていた術後の瘢痕や肉芽組織と局所再発巣の鑑別に有用で，その診断能の高さが期待できる。Huebnerらの報告では，FDG-PET検査の直腸癌術後局所再発の感度は94.5%，特異度97.7%，正診率95.9%としている[11)]。

直腸癌術後の局所再発に対するFDG-PET検査の特徴は，①他の検査法では解剖構造の消失，変形や瘢痕組織の増生により診断が困難な病変に対しての確定診断（存在診断，質的診断，病変の進展範囲の診断）が可能である。②この検査で，他臓器転移や多重癌のスクリーニングができる。③FDGの集積度は定量解析が可能なため，化学療法や放射線療法の効果判定が可能となる。しかし，現在のところ，保険適応疾患が限られていて，まだ検査費用が高価であることから，一般的な術後のフォローアップのための検査としては，汎用は困難であり，CT，MRIでは診断が困難な症例に限って使用されるべきであろう。

8. 大腸内視鏡検査

直腸癌術後は，結腸癌に比較して吻合部再発が多いことから，術後フォローアップとしての大腸内視鏡検査が必要である[12)]。

推奨される直腸癌術後フォローアップ法

表7に直腸癌術後フォローアップ法として，intensive follow-upの1例を示す。直腸癌の場合，局所再発の早期診断が重要である。診察で直腸指診を併用することは重要であり，無症状な症例でも早期に再発を発見できる可能性がある。また，局所再発の多いRb癌やstageⅢa，Ⅲb症例は，積極的に骨盤CTを行い，局所再発疑診例にはさらにMRIやPETを併用して，局所再発をなるべく早期，治療可能な時期に発見することが重要なことである。

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学会案内・お知らせ　information

第10回 臨床解剖研究会のご案内と演題募集のお知らせ

期　日：2006年6月17日(土)
会　場：東京コンファランスセンター飯田橋
〒102-8112　東京都千代田区飯田橋3-13-1　大和ハウス工業東京ビル2F
電話 03-5214-2020　FAX 03-5214-2021
当番世話人：衣袋健司（三井記念病院・放射線科部長）
演題募集要項：一般演題を募集いたします。皆さまからの多数のご応募をお待ちしております。

演題募集締切：2006年4月17日（月曜日）正午必着

1．演題申込先
演題の申し込みは，「2．演題登録内容」を演題申込専用のe-mailアドレスにお送りください。

演題申込専用アドレス：clinical-anatomy@mitsuihosp.or.jp

2．演題登録内容
演題名，演者，共同演者，所属，住所，電話番号，FAX番号，e-mailアドレスおよび抄録原稿（抄録本文は全角800字以内）のすべてを入力してください。

3．演題受取通知
演題をe-mailで受信後，e-mailにて返信いたします。1週間以内に返信のない場合は下記事務局までお問い合わせ下さい。

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Expression of Dihydropyrimidine Dehydrogenase, Thymidylate Synthase, p53 and p21 in Metastatic Liver Tumor from Colorectal Cancer after 5-Fluorouracil-based Chemotherapy

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Abstract. *Background: The expression of genes thought to be related to 5-FU chemosensitivity has been extensively investigated. However, little data is available on the expression patterns of these genes after chemotherapy. Patients and Methods: We investigated the expression of four genes, DPD, TS, p53 and p21, in the metastatic liver lesions obtained from colorectal cancer patients who had been treated with hepatic arterial infusions of 5-fluorouracil(5-FU)-based chemotherapy. Results: Expression of DPD, TS and p53 in the metastatic liver lesions was significantly higher in the chemotherapy-response group than in the no response group. In the response group, viable cancer cell nests were seen in confined spaces surrounded by fibrous tissue. It was of interest that these cancer cells in the response group showed conspicuous immunoreactivity of DPD, TS and p53. Conclusion: An analysis of genes involved in 5-FU sensitivity revealed that surviving tumor cells exhibited resistance characteristics, indicating that the chemotherapy regimen should be altered, even in partially responding cases, unless the response is pathologically complete.*

Pharmacogenetic markers of tumor cells have been intensively investigated using molecular biology technologies to predict chemosensitivity to 5-fluorouracil (5-FU) in colorectal cancer patients. Among them, *dihydropyrimidine dehydrogenase* (DPD) and *thymidylate synthase* (TS) are the most promising genes that have been used clinically in gastrointestinal cancer treatment. However, none of these genes are absolute predictors of 5-FU sensitivity.

Neoadjuvant chemotherapy has been proposed as an alternative approach to conventional surgery as an initial management strategy with the aim of improving the outcome of cancer patients. Strategies aimed at downstaging large or multifocal tumors and the control of micrometastases to enable curative resection by neoadjuvant chemotherapy have attracted much attention. Recently, in the field of breast cancer research, a sub-analysis of the National Surgical Adjuvant Breast and Bowel Project B-18 (NSABP B-18) trial revealed that a pathological complete response was the only reliable marker for selecting cases that were sensitive to a specific drug, resulting in an improved survival period (1).

In our institution, the resection of liver metastases for colorectal cancer has been actively performed after 5-FU-based chemotherapy *via* hepatic artery infusion. Although the survival advantage of hepatic arterial infusion over systemic therapy has been debated, the efficacy of this treatment with regard to tumor reduction was shown to be advantageous. We have used this procedure to treat patients with primarily unresectable metastases confined to the liver.

In this study, we examined the expression of *DPD, TS, p53* and *p21* in surgical specimens of liver metastases obtained from patients after chemotherapy.

Patients and Methods

Samples. Surgical specimens of synchronous or metachronous bilobular multiple liver metastatic tumors from 12 patients were obtained at Yokohama City University, Japan. The patients comprised 5 males and 7 females with a median age of 57.8 years (range, 41-74 years) (Table I). Since none of the 12 patients exhibited metastases at sites other than the liver, surgical resection was performed after 5-FU-based chemotherapy *via* hepatic arterial infusion. The treatment regimen was as follows: an infusion of 5-FU (500 mg/body) or 5-FU (500 mg/ body) + l-Leucovorin (150 mg/

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Key Words: Colorectal cancer, liver metastasis, chemotherapy, gene.

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Table I. Patient characteristics.

Primary tumor	Age	Gender	Response	Depth	n	DPD pri	DPD meta	TS pri	TS meta	p53 pri	p53 meta	p21 pri	p21 meta
rectum	59	F	PD+NC	se	1	2	0	0	0	0	0	1	0
rectum	41	F	PD+NC	ss	4	2	0	0	0	4	0	0	0
colon	55	M	PD+NC	se	1	0	2	0	0	0	0	0	0
colon	45	M	PD+NC	se	1	2	0	2	0	6	0	0	0
colon	51	F	PD+NC	mp	1	-	3	-	1	-	3	-	0
colon	74	M	PD+NC	si	0	1	3	1	1	0	0	0	0
colon	67	F	PR	ss	2	2	6	1	4	0	0	2	2
colon	65	F	PR	se	4	0	4	0	2	12	8	0	0
colon	54	F	PR	ss	2	1	4	1	1	9	9	2	1
rectum	69	M	PR	ss	0	0	4	2	6	9	12	6	1
colon	59	M	PR	ss	2	0	9	1	1	9	6	1	0
colon	55	F	PR	ss	1	4	4	0	3	0	0	1	0

pri: primary lesion
meta: metastatic lesion

body) + Cisplatinum (10 mg/ body) was administered every day for 5 days, and this cycle was repeated every 2 weeks for up to 4 cycles. None of the patients had received any other chemotherapy or radiotherapy treatments prior to the hepatic arterial infusions of 5-FU. Paraffin-embedded archival samples of their primary colorectal lesions were also examined. The study was approved by the institutional review board of the Yokohama City University School of Medicine, Japan.

Clinical evaluation. The chemotherapy response was determined by comparing the volume of the liver metastases before and after chemotherapy. A CT scan was performed after the 4 treatment cycles had been completed, and the results were evaluated using the World Health Organization (WHO) criteria (2). A complete response (CR) was defined as the complete disappearance of all intrahepatic tumor formation, and a partial response (PR) was defined as a reduction in the tumor volume by 50% or more, measured as the sum of the products of the two largest perpendicular diameters of all visible lesions. No change (NC) was defined as a reduction in tumor volume of less than 50% or an increase of less than 25%. An increase in tumor volume of 25% or more or the appearance of new liver lesions was defined as progressive disease (PD).

Antibodies. Rabbit anti-recombinant human *DPD* polyclonal antibody (dilution=1:500; The Second Cancer Laboratory, Taiho Pharmaceutical Co, Saitama, Japan.), *TS* polyclonal antibody (RTSSA, dilution=1:800; The Second Cancer Laboratory, Taiho Pharmaceutical Co.), mouse monoclonal antibody against *p53* protein (DO-7, dilution = 1:100; DAKO, Glostrup, Denmark) and *p21* protein (OP64, dilution=1:100; Oncogene Research Products, Cambridge MA, USA) were used as the primary antibodies for the immunohistochemical staining.

Immunohistochemistry

(i) DPD. Tissue sections (4 μm thick) were cut from each block, deparaffinized in xylene, rehydrated with graded ethanol and immersed in TBS. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in distilled water for 15 minutes. *DPD* protein expression was evaluated using the avidin-biotin complex immunohistochemical technique and a rabbit polyclonal antibody to recombinant human *DPD*. To block the nonspecific binding of the primary antibody, a normal rabbit serum (DAKO X901) dilution in TBS was used for 20 minutes. After removing the blocking solution, the *DPD* antibody (2 mg/ml) was applied for 60 minutes in a humidified chamber at room temperature. The sections were then incubated with biotin-conjugated swine anti-rabbit immunoglobulins for 20 minutes (DAKO-E353), followed by avidin-biotinylated peroxidase complex for 30 minutes. After developing the color reaction product with a freshly prepared 3,3'-diaminobenzidine chromogen solution for 5 minutes, the sections were counterstained with light hematoxylin for 10 seconds, dehydrated in a series of ethanols, cleared in xylene, mounted and covered with glass coverslips. Positive and negative controls were included in each experiment.

(ii) TS, p53 and p21. Tissue specimens (4 μm thick) were fixed in formalin and embedded in paraffin wax. After dewaxing, the sections were treated with 3% hydrogen peroxidase solution in methanol for 20 minutes to block endogenous peroxidase activity. The sections were then heated in a 0.01 M citrate buffer (pH 6.0) for 3-minute periods in a microwave oven for antigen retrieval. Non-specific antibody bindings were blocked using 10% normal bovine serum in PBS at 37°C for 15 minutes for the *p53* and *p21* staining procedures and normal goat serum for the *TS* staining procedure. The sections were then incubated with the primary antibodies described in the previous section. The sections were incubated at room temperature for 10 minutes with a byotinylated anti-mouse IgG + IgA + IgM (for monoclonal primary antibody) for *p53* and *p21* staining, and with a biotinylated anti-rabbit IgG for *TS* staining. The sections were then incubated at room temperature with peroxidase conjugated streptavidin and Elite ABC solution, respectively. The peroxidase reaction was developed using a 3,3'-deaminobenzidine tetrahydrochloride solution (Sigma Chemical Co, St. Louis, MO, USA) and 0.03% hydrogen peroxide. The sections were counterstained with hematoxylin, dehydrated and mounted in a routine fashion. Positive controls and negative controls were always included in all experiments.

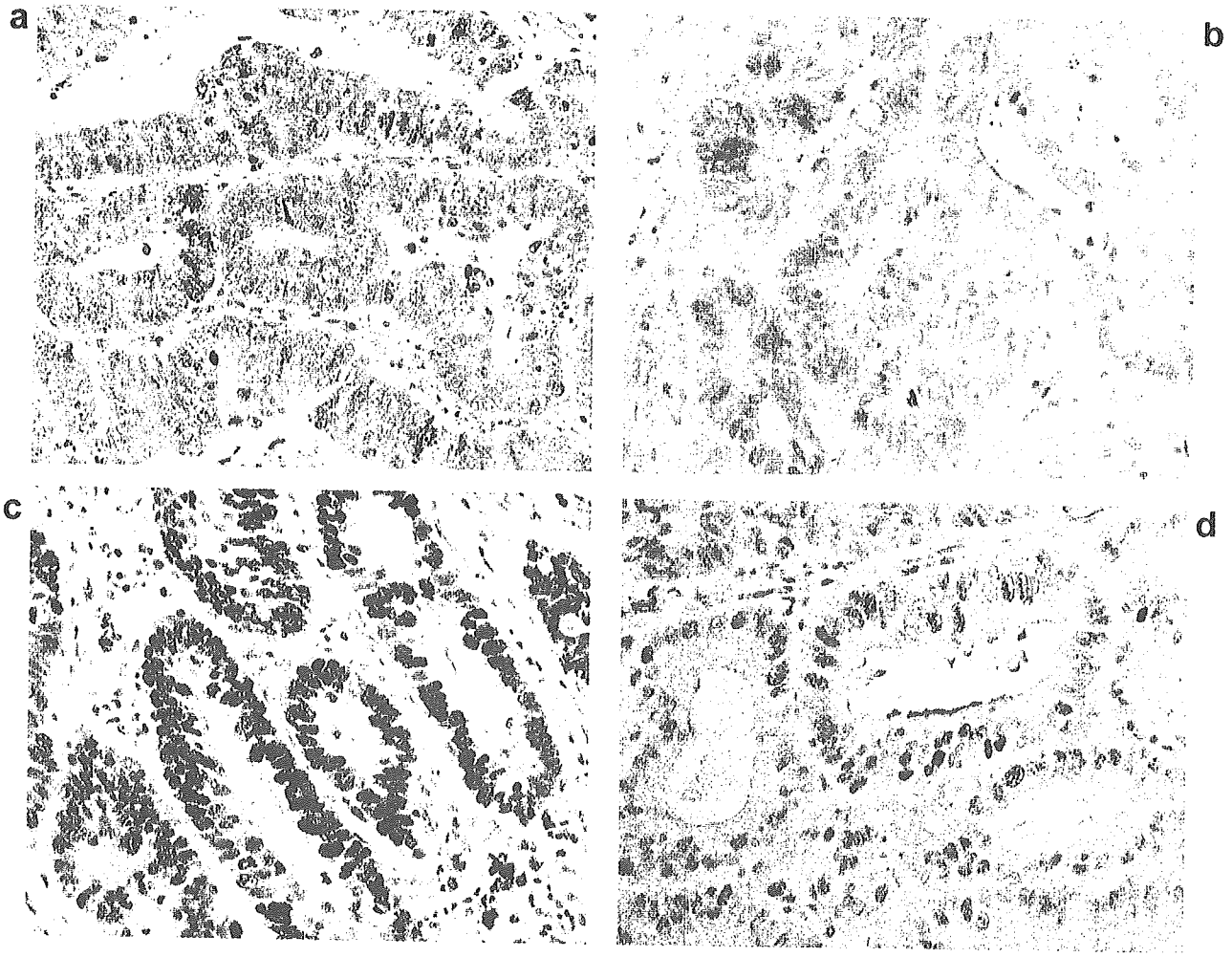

Figure 1. *Typical expression of DPD, TS, p53 and p21 genes after chemotherapy in surgical specimens of liver metastases. A: Expression of DPD, B: Expression of TS, C: Expression of p53, D: Expression of p21 (magnification, x400).*

(iii) Quantitation. Three representative fields were examined, more than 1000 tumor cells were randomly selected and the number of positive cells was counted at magnification x200. The expression of these proteins were evaluated according to the method described by Sinicrope *et al.* (3). In brief, positive-staining tumor cells were expressed as a percentage of the total number of tumor cells and assigned to one of the following five categories: class 0, ≤5%; class 1, 5% to 25%; class 2, 25% to 50%; class 3, 50% to 75%; and class 4, ≥75%. The intensity of the immunostaining was scored as follows: 1, weak; 2, moderate; 3, intense. These two scores were then multiplied. When heterogeneous levels of protein expression were found within a tumor (in multiple sections from different paraffin-embedded blocks of the same tumor), the highest protein expression score obtained for that lesion was used.

Statistical analyses. Dr. SPSS software for Windows was used for the statistical analyses; statistical significance was defined as $p<0.05$.

Results

Chemotherapy *via* hepatic arterial infusion was successfully performed in all 12 cases with no severe complications. The total 5-FU dosage was 3200±1500 mg (mean±SD). Clinical evaluations revealed that the liver tumors in 6 of the 12 patients partially responded to the treatment, although no complete responses occurred. In the remaining 6 cases, the tumors did not change in size or progressed after treatment (Table I).

Immunohistochemistry for the 4 genes was successfully performed in all 12 cases. *DPD* and *TS* were clearly observed in the cytoplasm of the cancer cells, while *p53* and *p21* were observed in the nuclei of the cancer cells. Representative cases are shown in Figure 1. Since half of the patients exhibited partial responses to the treatment,

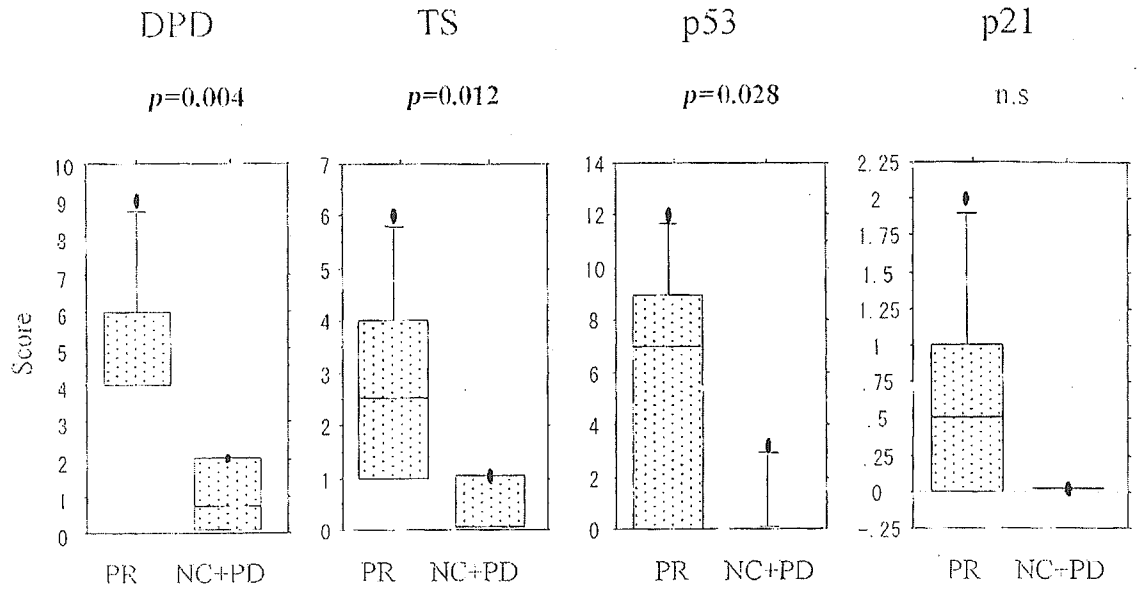

Figure 2. *Comparison of metastatic liver lesions in the PR group and the NC+PD group. The expression levels of DPD, TS and p53 were significantly higher in the PR group than in the NC+PD group ($p<0.05$).*

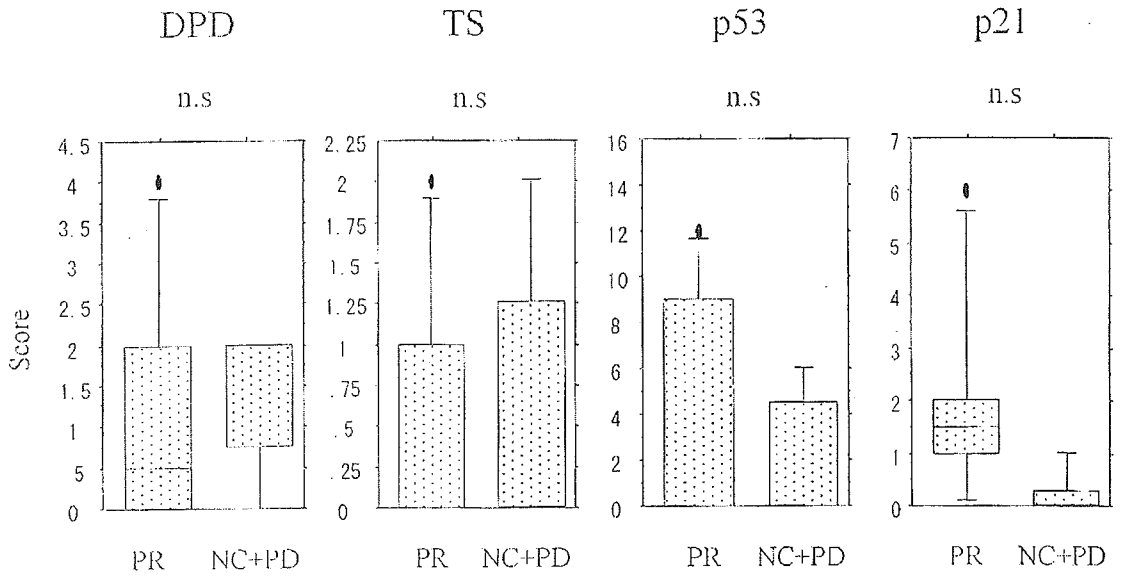

Figure 3. *Comparison of primary lesions in the PR and NC+PD groups. No significant differences in the expression levels of the 4 genes were seen between the PR and NC+PD groups.*

we compared the expression levels of these 4 genes in the chemotherapy response and no response groups. The mean scores for *DPD, TS* and *p53* immunoreactivity were significantly higher in the response group than in the no response group. All of the differences were statistically significant, as shown in Figure 2. However, the scores for *p21* were not significantly different between the two groups.

In the response group, viable cancer cell nests were seen in confined spaces surrounded by fibrous tissue. It was of interest that these cancer cells in the response group showed conspicuous immunoreactivity of *DPD, TS* and *p53*.

When the primary colorectal lesions from the archival samples were examined, no significant differences in the scores for any of the 4 genes were found between the response and no response groups (Figure 3).

Discussion

The expression of genes thought to be related to 5-FU chemosensitivity has been extensively investigated in the hope that methods for predicting 5-FU sensitivity might be established. However, little data is available on the expression patterns of these genes after chemotherapy. In our institute, the hepatic arterial infusion of 5-FU-based chemotherapy is routinely performed in patients with multiple liver metastases to improve the curative resection rate. Using surgical specimens, we examined the expression of 4 genes in liver tumors obtained from patients after chemotherapy. Although the number of cases in this study was limited, the expression rates of *DPD, TS* and *p53* were significantly higher in the response group than in the no response group. These findings suggest that the surviving tumor cells have both malignant and 5-FU-resistant characteristics.

DPD is the initial, rate-limiting enzyme in the catabolism of fluoropyrimidines, through which more than 80% of administered 5-FU is eliminated. Thus, the activity of this enzyme limits the efficacy of 5-FU treatment and is associated with tumor resistance to 5-FU (4,5). The intratumoral expression level of *TS* is considered a prognostic factor for survival in patients with colorectal cancer (6-8), although the ability of this marker to predict 5-FU chemosensitivity is controversial (9). The main pathway by which anticancer drugs induce apoptosis is a *p53*-dependent pathway (10,11). Normal *p53* protein has tumor-suppressing properties, and mutations in the *p53* gene result in the disruption of critical growth-regulating mechanisms (12-14). *p53* is also related to the malignancy of tumors and/or tumor resistance to chemotherapy. We previously reported that *p21* expression was correlated with the inhibiting activity of 5-FU (15), suggesting that *p21* may be a marker of 5-FU sensitivity.

These findings suggest two hypotheses: i) cells that are sensitive to 5-FU undergo apoptosis, but those that are resistant survive after chemotherapy, and ii) 5-FU exposure induces a mechanism that leads to drug resistance. The first hypothesis is feasible, but no direct evidence has been obtained to support this idea. However, Michael *et al.* examined *TS* expression in colorectal liver metastases after chemotherapy and found that previous fluorouracil exposure seemed to increase the resistance of the tumor cells to regional floxuridine *via TS* up-regulation (16). Nishiyama *et al.* performed an *in vitro* study on 5-FU exposure to examine changes in the expression of various genes, including *TS, DPD* and *MRP*. Although the results were very complicated, making their interpretation difficult, *DPD* and *TS* expression tended to increase in 5-FU-resistant cell lines after exposure to 5-FU (17). The mechanism of 5-FU chemoresistance is impossible to explain using the results of the present study alone.

However, the present findings may support the data obtained by the NSABP B-16 study on preoperative chemotherapy in patients with breast cancer (1). The outcome of chemotherapy was better in women whose tumors showed a pathological complete response than in women whose tumors exhibited a clinical partial response or a clinical no-response (relapse-free survival rates, 85.7%, 76.9% and 63.9%, respectively). Unless the cancer cells are totally killed by the drugs, remnant tumor cells survive and the prognosis of the patient does not improve. These findings strongly suggest that the initial chemotherapy treatment should be changed to one with a different mechanism, such as switching anthracycline to taxane, in patients with breast cancer who exhibit anything but a pathological complete response.

We also examined the expression of the 4 genes in the primary colorectal cancers obtained from this patient series, because liver metastases specimens obtained before chemotherapy were not available. No significant differences in the immunoreactivity of the 4 genes were seen between the response and the no response groups. Some differences in gene expression between the primary colorectal tumor cells and the metastatic liver tumor cells may exist. Therefore, it is not clear whether the 5-FU administration altered the expression of the 4 genes in the metastatic liver tumors after chemotherapy in the 2 groups.

Cancer chemotherapy has gradually improved with the production of new drugs exhibiting unique mechanisms and modifications. Hepatic resection after chemotherapy provides useful information enabling second-line chemotherapy treatments to be optimized. The results of this study suggest that a partial response may not be sufficient to improve the prognosis of colorectal cancer. Single-drug use limits the efficacy of treatment, while combination or sequential usage, like modified 5-FU, camptothecin and taxane regimens, may improve the prognosis of colorectal cancer patients.

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Feasibility of autonomic nerve-preserving surgery for advanced rectal cancer based on analysis of micrometastases

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Background: Autonomic nerve preservation has been advocated as a means of preserving urinary and sexual function after surgery for rectal cancer, but may compromise tumour clearance. The aim of this study was to determine the incidence of micrometastasis in the connective tissues surrounding the pelvic plexus.

Methods: The study included 20 consecutive patients who underwent rectal surgery with bilateral lymph node dissection for advanced cancer. A total of 78 connective tissues medial and lateral to the pelvic plexus and 387 lymph nodes were sampled during surgery. All connective tissue samples and 260 lymph nodes were examined for micrometastases by reverse transcriptase–polymerase chain reaction (RT–PCR) after operation. All patients were followed prospectively for a median of 36·0 months.

Results: Of 245 histologically negative lymph nodes, 38 (15·5 per cent) were shown by RT–PCR to harbour micrometastases. However, micrometastases to tissues surrounding the pelvic plexus were detected in only two (3 per cent) of 78 tissues, that is in two of 20 patients. Clinical follow-up showed that the two patients had a poor prognosis owing to distant metastases.

Conclusion: Autonomic nerve-preserving surgery may be feasible for advanced rectal cancer, but study of more patients positive for micrometastases is required.

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Introduction

The complex flow patterns of lymphatic fluid around the rectum were investigated in 1951 by Sauer and Bacon[1]. They regarded the lateral ligament as the tissue in which the inflow of lymphatic fluid from the rectum first occurs *en route* to the internal iliac artery. However, the high incidence of urinary and sexual dysfunction after rectal cancer surgery gradually led surgeons to realize that the lateral ligament plays an important role in postoperative urinary and sexual function[2]. The pelvic autonomic nerve system is comprised of the superior hypogastric plexus, hypogastric nerves, pelvic plexus (inferior hypogastric plexus) and pelvic splanchnic nerves[3]. The lateral ligament of the rectum consists of a neurovascular bundle derived from the combined redistributing sympathetic and parasympathetic nerves of the system, and dissection injury to these nerves may cause problems.

Inclusion of total mesorectal excision (TME) in rectal cancer surgery can improve the rate of cure[4]. Dissection of the rectum at the layer of TME allows preservation of the pelvic autonomic nerves, reducing postoperative urinary and sexual morbidity[5–7]. Extensive pelvic lymphadenectomy involving resection of both pelvic autonomic nerves and lateral lymph nodes was employed in Japan from the 1970s to the early 1980s with the aim of reducing local recurrence of rectal cancer[8]. However, postoperative urinary and sexual dysfunction invariably ensued, leading to marked reduction in quality of life. More recently, efforts have been made to preserve urinary and sexual function and to achieve local control of cancer, by a combination of autonomic nerve-preserving surgery and lateral lymph node dissection[9–12].

Recent pathological studies have warned against autonomic nerve preservation because histologically proven cancer foci might exist in the autonomic nerves

and surrounding connective tissues, and lead to local recurrence[13,14]. To aim of the present study was to examine the incidence of micrometastases in the region of the pelvic plexus by reverse transcriptase–polymerase chain reaction (RT–PCR) analysis, which is more sensitive than conventional histological diagnosis[15,16].

Patients and methods

The study included 20 consecutive patients who underwent rectal cancer surgery with bilateral lymph node dissection at the Graduate School of Medicine, Osaka University between October 1999 and May 2001. Demographic data are shown in *Table 1*. Tumours were located 0–10 (median 6·5) cm above the anal verge and diagnosed before surgery as advanced cancer (T2,T3 and T4)[17]. The operative procedures are summarized in *Table 1*. Curative resection was performed in all patients. Tumour stages determined by postoperative histological examination are shown in *Table 1*[17]. Two patients with lateral node metastasis without distant organ metastasis were included in stage III (*Table 2*). Nine patients with stage III or IV tumours were treated with 5-fluorouracil-based chemotherapy but none had radiation therapy. The patients were reviewed at least every 3 months after operation with blood tests such as measurement of carcinoembryonic antigen (CEA), and underwent computed tomography (CT) or magnetic resonance imaging (MRI) every 6 months. The study protocol was approved by the Human Ethics Review Committee of Osaka University Graduate School of Medicine and a signed consent form for the study was obtained from each patient.

Table 1 Clinical characteristics of patients

Median (range) age (years)	63·5 (35–92)
Sex ratio (M : F)	15 : 5
Surgical procedure	
Low anterior resection	9
Abdominoperineal resection	7
Hartmann resection	3
Total pelvic exenteration	1
Autonomic nerve preservation	
Bilateral	18
Unilateral	2
TNM stage	
I	4
II	7
III	6
IV	3
Histological type	
Well differentiated	1
Moderately differentiated	16
Poorly differentiated	2
Mucinous	1
Tumour location (cm)*	
< 6	9
≥ 6	11

*Distance from anal verge. TNM, tumour node metastasis.

All patients underwent radical resection with autonomic nerve preservation and lymph node dissection[11]. The following is a brief description of the surgical procedures, with schematic presentation in *Fig. 1*, focusing on the collection of specimens. After isolating the sigmoid colon, the mesorectum was dissected from the parietal fascia of the sacrum at the layer of TME to preserve the hypogastric nerves, and the medial side of the pelvic plexus was exposed. At this stage, the connective tissues of the medial and lateral sides of the pelvic plexus were meticulously sampled taking care not to injure the plexus, with four specimens being obtained from each patient. However, if the tumour had macroscopically infiltrated the pelvic plexus, only tissue from the lateral side was collected because the infiltrated pelvic plexus was dissected *en bloc* with the tumour. The specimens were promptly frozen in liquid nitrogen and stored at −70°C pending RNA extraction. A total of 78 connective tissues were obtained from 20 patients (*Table 2*).

After removing the tumour and performing lateral node dissection, the lymph nodes were sampled from the mesorectum and the lateral area along the internal iliac artery and the obturator nerve outside the boundaries of TME (*Fig. 1*). In 12 patients, each of the 260 lymph

Table 2 Metastasis to lymph nodes and tissues surrounding pelvic plexus

		Site of metastasis			Survival at 1 year	
TNM stage	No. of patients	Upper lymph nodes (HE)	Lateral lymph nodes (HE)	Tissues surrounding pelvic plexus (micrometastases)	Relapse free	Overall
I	4	0	0	0 (16)	4	4
II	7	0	0	0 (28)	6	7
III	6	6	2	0 (24)	5	6
IV	3	3	3	2 (10)	0	0

Values in parentheses are number of tissues; HE metastasis detected by histological examination with haematoxylin and eosin staining. TNM, tumour node metastasis.

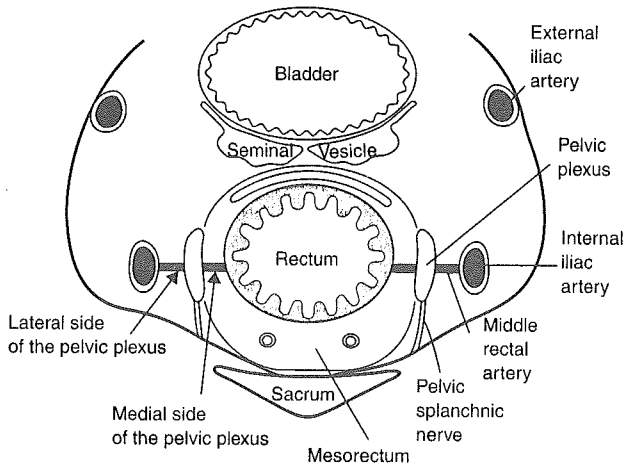

Fig. 1 Schematic presentation of the pelvic autonomic nerve system and connective tissues surrounding the pelvic plexus. The connective tissues comprise fat and lymphatic vessels including the middle rectal artery. The medial tissue is equivalent to the lateral ligament and the lateral tissue is located outside the boundaries of total mesorectal excision, between the plexus and internal iliac artery

node samples was halved, with one part being subjected to conventional histological diagnosis and the other being frozen in liquid nitrogen and stored at −70°C until extraction of RNA. In the other eight patients, 127 samples were collected and subjected to histological diagnosis only.

Total RNA was extracted by a single-step method as described previously[18]. Complementary DNA was generated with avian myeloblastosis virus RT using the procedure outlined by the supplier (Promega, Madison, Wisconsin, USA). *CEA* and *cytokeratin 20* (*CK-20*) transcripts were used as sensitive markers for micrometastases, and *phorphobilinogen deaminase* (*PBGD*) transcript was used to check for the presence of mRNA in samples[15]. PCR products were analysed by electrophoresis on 2 per cent agarose gels stained with ethidium bromide. The reproducibility of cDNA products was checked by repeated RT–PCR and gel electrophoresis. The sensitivity of PCR was determined by detecting *CEA* and *CK-20* transcripts in serial dilutions of a human colonic cancer cell line (HT29) mixed with human lymphocytes; the detection sensitivity was 100 HT29 cells among 10^6 lymphocytes.

Results

The presence of *PBGD* products was confirmed in all 78 connective tissue specimens and 260 lymph nodes examined for micrometastases by RT–PCR. The diagnosis was positive if bands specific for *CEA* or *CK-20* were present[15].

The 260 lymph nodes were collected from 12 patients (median 22 per patient). The appearance of both *CEA* and *CK-20* mRNAs was verified in all 15 histologically positive lymph nodes. Of the 245 histologically negative lymph nodes, 38 (15·5 per cent) harboured micrometastases (*Table 3*).

Metastases were identified histologically in the upper lymph nodes within the mesorectum in nine of 20 patients, and in the lateral lymph nodes along the internal iliac artery and the obturator nerve in five of 20, suggesting that many patients had very advanced cancer. Direct tumour invasion into the right or left pelvic plexus was observed macroscopically in two patients with stage IV disease. The pelvic plexus on the affected side was therefore resected *en bloc* with the tumour, whereas that on the other side was preserved.

Eighteen of 20 patients had neither *CEA* nor *CK20* mRNA in the four connective tissues surrounding the bilateral pelvic plexus. Two of three patients with stage IV disease were positive for both *CEA* and *CK20* mRNA in the lateral tissue from the resected pelvic plexus, but all other tissues surrounding the preserved pelvic plexus were negative. Micrometastases were identified in two (3 per cent) of 78 samples of connective tissue surrounding the pelvic plexus, that is in two of 20 patients (*Table 2*). All five patients with lateral node metastasis had metastasis to the upper lymph nodes, but only two had micrometastases to the connective tissues surrounding the pelvic plexus.

Median follow-up after surgery was 36·0 months. By 1 year after surgery, five of 20 patients had developed local recurrence or distant metastases, despite undergoing curative surgery and postoperative chemotherapy, and three had died from cancer (*Table 2*). Of three patients with pelvic recurrence, one patient showed relapse in the lateral area of the pelvis despite lateral node dissection, and two patients developed recurrent tumour in the rectum after a Hartmann's procedure. The autonomic

Table 3 Lymph node metastases detected by reverse transcriptase–polymerase chain reaction and histological examination with haematoxylin and eosin staining

	RT–PCR		
	Positive	Negative	Total
Haematoxylin and eosin			
Positive	15	0	15
Negative	38	207	245

RT–PCR, reverse transcriptase–polymerase chain reaction.

nerve system was preserved completely in 18 patients and unilaterally in two; no recurrence was found in this region by repeated follow-up CT and MRI. The overall survival rate, estimated by the Kaplan–Meier method, of the 18 patients without micrometastases surrounding the pelvic plexus was 94 per cent at 1 year and 88 per cent at 3 years. Neither of the patients with micrometastases was alive at 1 year after surgery.

Discussion

The lateral ligament is still regarded as a pathway of lymphatic vessels (middle lymphatic flow) from the lower rectum towards the lateral lymph nodes[1,19]. However, in the present study micrometastases to the connective tissues, including the lateral ligament, were identified by highly sensitive RT–PCR analysis in only two patients with distant metastases. Three of five patients with both upper and lateral lymph node metastases had no micrometastases in the connective tissues. A partial explanation for the discrepancy between the presence of lymph node metastases and the very low incidence of micrometastases to the connective tissues might be that lateral lymph node metastases developed via lower lymphatic flow rather than via middle lymphatic flow through the lateral ligament[19].

The autonomic nerve system was completely preserved in all but two patients in the present study, However, no local recurrence in the region of the preserved nerve system was observed by CT and MRI during follow-up. Contrary to expectation, micrometastases to the connective tissue surrounding the pelvic plexus were rare, verifying the feasibility of nerve preservation without oncological compromise in most patients.

Neither patient with micrometastases in the tissues surrounding the pelvic plexus survived for 1 year after surgery. Ueno *et al.*[14] performed complete dissection of the autonomic nerve system and pelvic lymph nodes with the aim of achieving local control in 61 patients with rectal cancer. They reported spread of cancer cells to the autonomic nerves in nine patients (15 per cent), six with Dukes' C and three with Dukes' 'D' lesions. The patients with Dukes' C tumours underwent curative radical resection, but all developed recurrence within 1 year and none survived for 4 years. The circumferential resection margin for TME is located inside the pelvic plexus whereas the pelvic nerve plexus and the lateral tissue are situated outside the margin. It has also been documented that TME in patients with tumour involvement of circumferential resection margin is associated with a poor prognosis[20].

The present results indicate that any patients with micrometastases in the preserved pelvic plexus already have advanced cancer, so their prognosis is unlikely to be affected by local recurrence that might develop if the autonomic nerves are preserved. Management of such patients should focus on maximizing the quality of remaining life.

The follow-up period in the present study was relatively short (median 36·0 months). However, some 50–80 per cent of local recurrences occur within 2 years after rectal cancer surgery, with a peak at 6–12 months[21]. The follow-up period should therefore have been sufficient for the analysis.

Based on examination of micrometastases, these results suggest that the autonomic nerve system should be preserved wherever possible, even in surgery for advanced rectal cancer. However, study of more patients positive for micrometastases is needed.

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Methylation and expression of p16^{INK4} tumor suppressor gene in primary colorectal cancer tissues

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Abstract. It is known that p16^{INK4} tumor suppressor gene expression in colon cancer cells is repressed by methylation at the CpG island of promoter, but *in vivo* silencing of p16 gene is not fully understood. Some studies showed that primary colorectal cancer (CRC) tissues often overexpress the p16 protein, while others showed the high incidence of p16 methylation. The aim of this study was to clarify p16 gene regulation *in vivo*. We used real-time methylation-specific PCR (MSP) to examine density of p16 methylation, and immunohistochemistry, Western blot analysis to determine p16 protein expression. Methylation was detected in 5 CRC cell lines tested and 9 of 21 (42.9%) CRCs. Four of 5 CRC cell lines did not express p16 mRNA, but 6 of 9 CRCs did express p16 mRNA even with methylation. Real-time MSP showed that CRC tissues had a wide variety in methylation density (methylation index: 0.28-0.91) and that highly methylated CRC tissues displayed significantly lower p16 mRNA expression than those with no-methylation or low-methylation. Immunohistochemistry showed that the majority of CRCs (53 of 55: 96.4%) overexpressed the p16 protein. Low p16 expression was associated with lymph node metastasis (p=0.003) and large tumor size (p=0.048). Western blot in a subset of non-tumor and tumor samples showed a consistent overexpression of the p16 protein. These results showed that CRC tissues displayed variable methylation density, which may be characteristics of p16 gene methylation *in vivo*. Our data suggest that a low p16 expression due to methylation may contribute to tumor enlargement and expansion of CRC.

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Key words: p16^{INK4}, colorectal cancer, methylation density, methylation-specific PCR

Introduction

The progression of the cell cycle is controlled by the cyclin-dependent kinase (CDK)/cyclin complex countered by CDK inhibitors (CKIs) (1,2). Cyclin D1/CDK4, CDK6 and cyclin E/CDK2 control the progression from the G1 to S phase of the cell cycle. Based on their structural and functional characteristics, CKIs are classified into two groups, the p21^{Waf1} family, which includes p27^{Kip1} and p57^{Kip2}, and another group, which consists of p16^{INK4a} (hereafter designated p16), p15^{INK4b}, p18^{INK4c} and p19^{INK4d}. The p16 gene is localized on chromosome 9p21 and the p16 family can form complexes with CDK4, CDK6 and D-type cyclins (3-5). Overexpression of INK4 proteins can arrest cells in the G1 phase through inhibition of cyclin D/CDK activity (6). On the other hand, p16-deficient mice develop spontaneous tumors at an early stage and are highly sensitive to carcinogens, suggesting that p16 is a tumor suppressor gene (7). Indeed, deletions and mutations of the p16 gene are frequently present in primary cancers of the brain, biliary tract, lung, pancreas and esophagus (8-12), although a panel of cancer cell lines tends to retain p16 gene alterations more frequently (3). There is also evidence that p16 expression is down-regulated by *de novo* methylation of 5' CpG islands in the p16 promoter region (13,14).

In colorectal cancer (CRC) cell lines, expression of the p16 protein was reported to be undetectable and inactivation of p16 is thought to be a common alteration in CRC (15). It appears that deletion or loss of the p16 gene is rare in CRC (13,16,17) and the alternative pathway for inactivation of the p16 gene is thought to be methylation of its promoter (13,14). In CRC tissues, there is evidence that the p16 gene promoter is methylated in 29-55% of primary cancer tissues (13,18-22), whereas in non-neoplastic colonic mucosa the p16 promoter is hardly methylated (18,23). These findings invoke the notion that p16 gene inactivation via methylation may be involved in carcinogenesis of the colorectum.

However, recent immunohistochemical studies have shown that the majority of CRC tissues (64-82%) expressed the p16 protein, while normal mucosa displayed negative or very low expression (24,25). This overexpression of p16 in CRC tissues

is somewhat paradoxical in view of the possible role of p16 as a tumor suppressor gene, indicating that the above scenario on human CRC tumorigenesis via p16 inactivation may not be quite as simple *in vivo*. The question then arises as to how methylation and overexpression of the p16 gene can coexist in a significant fraction of CRC tissues? One clue to this puzzle might be the difference in methylation density of the CpG island in the p16 promoter between cell culture systems and primary CRC tissues, since it is reported that the level of transcriptional repression is dependent on methylation density (26,27). Although many studies have examined the incidence of p16 methylation alone or p16 expression alone *in vivo* (18-23), no study has yet simultaneously examined the degree of methylation and p16 gene expression in CRC tissues. To address this question, we examined p16 methylation and p16 gene expression in CRC tissue samples together with control experiments using CRC cell lines. Real-time methylation-specific PCR (MSP) was also performed in methylation-positive CRC tissue samples. An extended immunohistochemical analysis was conducted to elucidate the functional significance of the p16 protein in CRC tissues. Our data provide a rationale for methylation-associated regulation of p16 expression in primary CRC tissues.

Materials and methods

Cell lines and tissues. The human breast epithelial cell line HBL100, human CRC cell lines HCT116, SW480, LoVo, HT29, DLD1, human breast cancer cell line MDAMB468, and human glioblastoma cell line T98G were obtained from the Japanese Cancer Research Resources Bank and ATCC (American Type Culture Collection). These cells were cultured in RPMI-1640 (Nissui, Tokyo, Japan) or DMEM medium (Nikken Bio Medical Laboratory, Kyoto, Japan), supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum at 37°C in 5% CO_2.

A total of 55 samples of CRC tissues together with adjacent non-neoplastic mucosa were obtained from patients who underwent surgery at the Department of Surgery and Clinical Oncology, Osaka University, between 2000 and 2001. The age of the patients ranged from 28 to 81 years (mean age: 61 years). The resected surgical specimens were fixed in 10% buffered formalin, dehydrated in graded ethanol, and embedded in paraffin. Tissue sample of sufficient quantity was frozen immediately in liquid nitrogen and stored at -80°C until use for reverse transcription-polymerase chain reaction (RT-PCR), methylation assay or immunoblotting. Samples of non-neoplastic mucosa were excised at least 5 cm lateral to the tumor. The study protocol was approved by the Human Ethics Review Committee of Osaka University, Graduate School of Medicine.

Immunostaining. The tissue specimens fixed in 10% buffered formalin were sliced into 4-µm thick sections, deparaffinized in xylene and rehydrated with graded concentrations of ethanol. Immunostaining was performed using the Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame, CA) after boiling for antigen retrieval, as described in our previous studies (28,29). Anti-$p16^{INK4}$ polyclonal antibody, which was raised against a full-length recombinant GST-p16 fusion protein, was purchased from PharMingen (San Diego, CA). The primary antibody was applied to the sections at a dilution of 1:400. The human breast epithelial cell line HBL100, which expresses a high level of p16, and the glioblastoma cell line T98G in which the p16 gene is inactivated by homozygous deletion, were used as positive and negative controls, respectively (30). For the absorption test, the immunogen was obtained from PharMingen.

Evaluation of immunohistochemistry. Inflammatory cells served as positive internal controls and nuclear staining for p16 was considered positive as reported previously (30-32). Ten fields in each specimen were randomly selected and examined under high power magnification and >500 cells were counted in order to determine the labeling index (LI), which represented the percentage of cells that were p16-positive. The samples were then classified into three groups according to the value of LI: group A consisted of tissue samples that contained >50% p16-positive cells, group B contained 10-50% p16-positive cells, and group C contained <10% p16-positive cells. Staining was repeated at least twice to eliminate possible technical errors, and the results were reproducible.

Western blot analysis. Approximately $5x10^6$ cells or 100 mg of tissue were homogenized and lysed in 1.0 ml of lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 0.5% NP40, 1 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin, and then placed on ice for 10 min. The lysates were clarified by centrifugation at 15,000 x g for 25 min at 4°C. The protein samples were subjected to SDS-PAGE (15% gels) and immunoblotting was performed as described in our previous studies (28-30). The final dilution of the primary antibody was 1:1,000.

RNA extraction and RT-PCR analysis. Total RNA was extracted with a single-step method using TRIzol reagent (Invitrogen Corp., Carlsbad, CA), and cDNA was generated using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). Semi-quantitative analysis for expression of p16 mRNA was performed by the multiplex RT-PCR technique, using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the internal standard (33,34). To minimize the inter-PCR difference, PCR was performed with GAPDH and p16 primers in identical tubes under unsaturated conditions, as described in our previous studies (33,35,36). PCR reactions were performed in a total volume of 25 µl of reaction mixture containing 2 µl of cDNA template, 1X Universal PCR buffer, 2 mM deoxynucleotide triphosphates, 20 pmol of primer for p16, 4 pmol of each primer for GAPDH, and 1 unit of Taq DNA Polymerase (AmpliTaq Gold; Roche Molecular Systems, Inc., Alameda, CA). The primer set for p16 was designed to be localized in exon 1 and exon 2 of the p16 gene, flanking intron 1 and tested to ensure amplification of cDNA only, so that amplification of potentially contaminating genomic DNA could be avoided. The sequences of these PCR primers were as follows: p16 sense primer, 5'-AGC CTT CGG CTG ACT GGC TGG-3'; p16 antisense primer, 5'-CTG CCC ATC ATC ATG ACC TGG A-3'. The primers for GAPDH were synthesized as described previously (34). The sizes of the

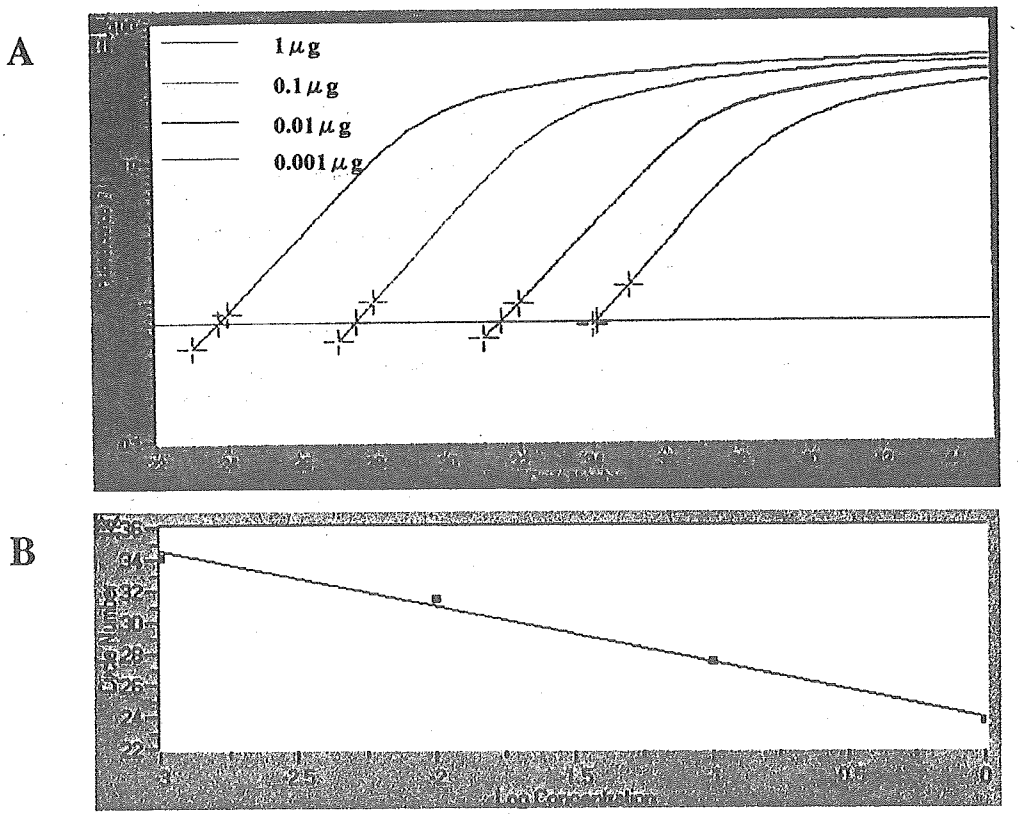

Figure 1. Methylation-specific PCR (MSP) using serial dilutions of methylation control DNA. (A), MSP was performed using 10-fold serial dilutions of methylation control genomic DNA from 2 ng to 2 μg. The PCR products were monitored by the fluorescence of dsDNA-specific dye SYBR Green I. (B), Based on the amplification results of (A), a standard curve was drawn using LightCycler software. Similar curves were drawn for unmethylated p16 DNA products, using bisulfite-converted unmethylated control genomic DNA (data not shown).

amplicons for p16, and GAPDH were 139 and 181 bp, respectively. The PCR conditions were as follows: a) initial denaturing at 95°C for 12 min; b) 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and c) a final extension at 72°C for 10 min. Each PCR product (10 μl) was electrophoresed on 2% agarose gels and stained with ethidium bromide. The PCR products were assayed by densitometry.

Methylation-specific PCR (MSP). To detect methylation at the 5' CpG island in the p16 promoter region, MSP was performed using the CpGenome™ DNA Modification kit (Chemicon International, Inc., Temecula, CA), as recommended by the supplier (30). Briefly, genomic DNA was modified by treatment with sodium bisulfite, which converts all unmethylated cytosines to uracils while 5-methylcytosines remain unaltered. PCR amplification was then carried out using primers specific for either methylated or unmethylated DNA (CpG WIZ™ Amplification Kit, Chemicon International, Inc.). The PCR mixture contained Gene-Amp 10X Universal PCR buffer, 2.5 μl of 2.5 mM dNTPs, 1.0 μl of primer pairs, 2 μl of template DNA, and 0.2 μl of AmpliTaq Gold (Roche Molecular Systems, Inc., Alameda, CA) for a final volume of 25 μl. Amplification was performed in a temperature cycler (Takara, Shiga, Japan) for 35 cycles (45 sec at 95°C, then at annealing temperature for 45 sec, and finally 60 sec at 72°C), followed by a final 5-min extension at 72°C. The PCR samples were loaded onto a 2% agarose gel, stained with ethidium bromide, and directly visualized under UV illumination.

Quantitative real-time MSP using LightCycler™. Quantitative PCR was performed using LightCycler system (Idaho Technology Inc., Salt Lake City, UT), as described in our previous studies (36,37). Briefly, 10 μl PCR reaction contained 0.2 μM of each primer, LightCycler-DNA Master SYBR Green I (Roche Molecular Systems, Inc.), 4 mM $MgCl_2$ and 2 μl of template DNA. PCR conditions were set up as follows: one cycle of denaturing at 95°C for 2 min, followed by 50 cycles of 95°C for 0.1 sec, 62°C for 5 sec and 72°C for 18 sec. Fluorescence was acquired at the end of each 72°C extension phase. The melting curves of the final PCR products were analyzed after 50 cycles of PCR amplification by cooling samples to 65°C, increasing the temperature up to 99°C at a rate of 0.1°C/sec, and monitoring fluorescence at each 0.1°C. Quantification data from each sample were analyzed using LightCycler™ analysis software. Two real-time quantitative PCR reactions were performed for the detection and quantitation of the bisulfite-unconverted methylated version of the p16 gene and the bisulfite-converted unmethylated version of the p16 gene. Serial dilutions of methylated or unmethylated control genomic DNAs (CpG WIZ™ Amplification kit, Chemicon International, Inc.) were used for constructing the standard curves (Fig. 1).

The methylation index (MI) in each sample was calculated using the following equation (38):

$$\text{Methylation index} = \frac{M}{M+U} \times 100$$

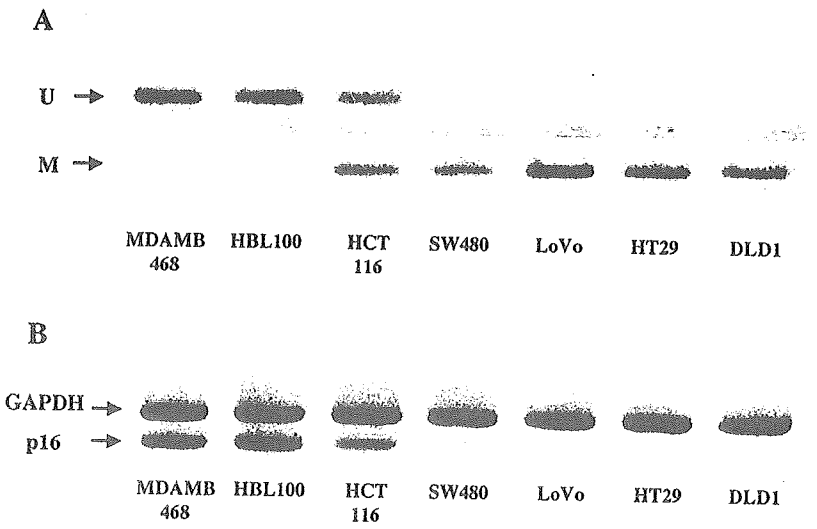

Figure 2. (A), Methylation of the p16 promoter region in cell lines. The breast cancer cell line MDAMB468 and the HBL100 breast epithelial cell line displayed a band when amplified with primers for unmethylated DNA (lanes 1 and 2). The HCT116 CRC cell line displayed bands of both unmethylated DNA and methylated DNA (lane 3). The CRC cell lines, SW480, LoVo, HT29 and DLD1 yielded a band of methylated DNA (lanes 4-7). U, unmethylated DNA; M, methylated DNA. (B), p16 mRNA expression by RT-PCR assay in cell lines. The MDAMB468 and the HBL100 cell lines expressed an intense band for p16 mRNA (lanes 1 and 2). HCT116 cells expressed a modest band for p16 mRNA (lane 3). The SW480, LoVo, HT29 and DLD1 cells lines did not express p16 mRNA (lanes 4-7). GAPDH served as internal control in duplex PCR.

Statistical analysis. Contingency tables were used to determine correlations between the expression of p16 protein and clinicopathologic parameters. Statistical association was determined by Fisher's exact test and mean values were compared by the Mann-Whitney U test using the Statview J-5.0 program (SAS Institute Inc., Cary, NC).

Results

Methylation and p16 gene expression in cell lines. Seven cell lines were examined for the presence of methylation in the p16 promoter region using MSP. The breast cancer cell line MDAMB468, which retains only the unmethylated p16 gene, was used as a negative control, while the SW480 CRC cell line served as a positive control for hypermethylation of the p16 gene, as described previously (30). The HBL100 breast epithelial cell line and the MDAMB468 cell line displayed a band only when amplified with primers for unmethylated DNA (Fig. 2A, lanes 1 and 2). By contrast, like the SW480 cell line, the CRC cell lines, LoVo, HT29 and DLD1 displayed a clear band of methylated DNA but not unmethylated DNA (Fig. 2A, lanes 4-7). The HCT116 cell line alone displayed bands of both methylated DNA and unmethylated DNA (Fig. 2A, lane 3).

The MDAMB468 and the HBL100 cell lines expressed an intense band for p16 mRNA (Fig. 2B, lanes 1 and 2), but CRC cell lines SW480, LoVo, HT29, and DLD1 did not express p16 mRNA (Fig. 2B, lanes 4-7). In HCT116 cells, a modest band for p16 mRNA was detected (Fig. 2B, lane 3).

Methylation and p16 gene expression in surgical specimens. Twenty-one paired normal and cancer tissues were then examined for the presence of p16 gene methylation using MSP. All surgical specimens displayed a band of unmethylated DNA that could be derived from unmethylated DNA of normal colonic cells and cancer cells as well as normal constituents in the stroma such as vascular endothelial cells, smooth muscles, fibroblasts, and inflammatory cells (Fig. 3A). A band of methylated DNA was found in 2 normal mucosa samples (9.5%) and 9 (42.9%) cancer tissues.

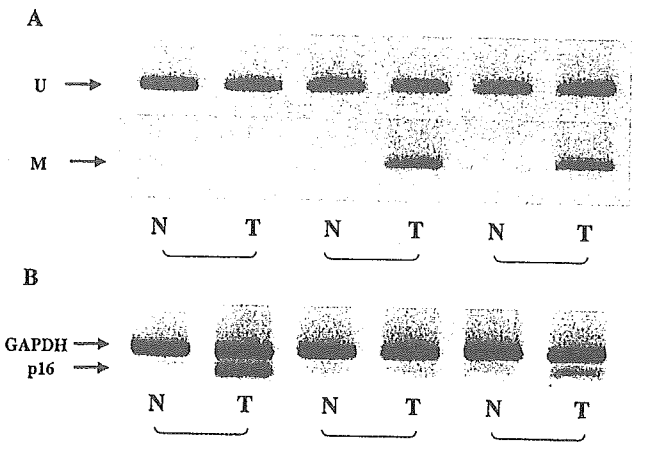

Figure 3. (A), Methylation of the p16 promoter region in surgical specimens. All surgical specimens displayed a band of unmethylated DNA. A band of methylated DNA was found in 2 of 21 normal mucosa samples (9.5%) and 9 of 21 cancer tissues (42.9%). Three representative pairs of normal and cancer tissues are shown here. U, unmethylated DNA; M, methylated DNA. (B), p16 mRNA expression in surgical specimens. p16 mRNA was undetectable in 12 of 21 normal mucosa samples (57.1%) (lanes 1, 3 and 5). On the other hand, p16 mRNA was detected in 10 of 12 methylation-minus CRC tissues (lane 2) and 5 of 9 methylation-plus CRC tissues (lane 6). GAPDH served as an internal control in duplex PCR. N, normal mucosa; T, CRC tumor tissue.

To confirm the conversion efficiency of bisulfite from unmethylated cytosines to uracils, a mixing experiment was performed. Prior to bisulfite treatment, a cancer DNA sample displaying both methylated and unmethylated bands was diluted serially with increasing amounts of unmethylated DNA from HBL100 cells. As expected, methylated band gradually decreased, resulting in an increasing ratio of unmethylated to methylated product in the mixture (Fig. 4).

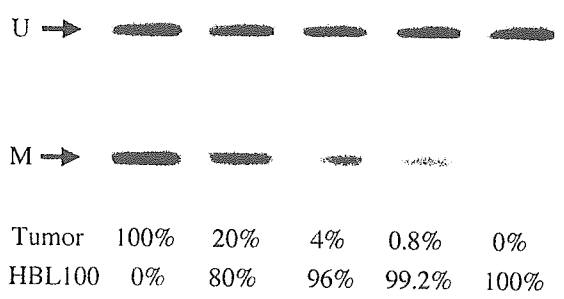

Figure 4. Mixing experiment. Prior to bisulfite treatment, a cancer DNA sample (1 μg) displaying both methylated and unmethylated bands (lane 1) was diluted serially (every 5-fold, lanes 2-4) with increasing amounts of unmethylated DNA from HBL100 cells. Then, PCR was performed. Unmethylated DNA remained unchanged, while methylated DNA product decreased in the mixture, enduring high conversion efficiency of bisfulfite from unmethylated cytosines to uracils. U, unmethylated DNA; M, methylated DNA.

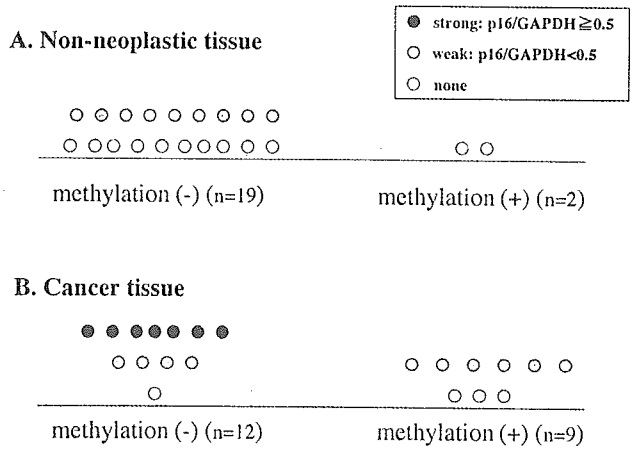

Figure 5. p16 mRNA expression stratified by the presence of methylation in non-neoplastic tissue (A) and cancer tissue (B). When high expression of p16 mRNA was defined as an intensity ratio of the p16 mRNA band to the GAPDH mRNA band of ≥0.5, 7 CRC tissues without methylation belonged to this category (solid circles). There were no high expressors of p16 in non-neoplastic tissues. Nine normal tissues devoid of methylation expressed weak p16 mRNA expression (intensity ratio of p16 mRNA band to GAPDH mRNA band <0.5, grey circles). Open circles indicate samples that did not express p16.

p16 mRNA was undetectable in 12 of 21 normal mucosa samples (57.1%) (Fig. 3B, lanes 1, 3 and 5). p16 expression was detected in 9 normal mucosa samples, all of which were devoid of p16 methylation; the expression level was generally weak (intensity ratio of p16 band to GAPDH band <0.5). On the other hand, p16 mRNA was detected in 11 of 12 methylation-negative CRC tissues (Fig. 3B, lane 2) and 6 of 9 methylation-positive CRC tissues (Fig. 3B, lane 6). Strong p16 mRNA expression (intensity ratio ≥0.5) was detected in 7 CRC tissues only in the methylation-negative group (results summarized in Fig. 5). When expression of p16 mRNA was compared between paired normal mucosa and tumor samples, only 1 of 21 cases (4.7%) showed a decrease in p16 mRNA expression during carcinogenesis (from normal mucosa to cancer tissue).

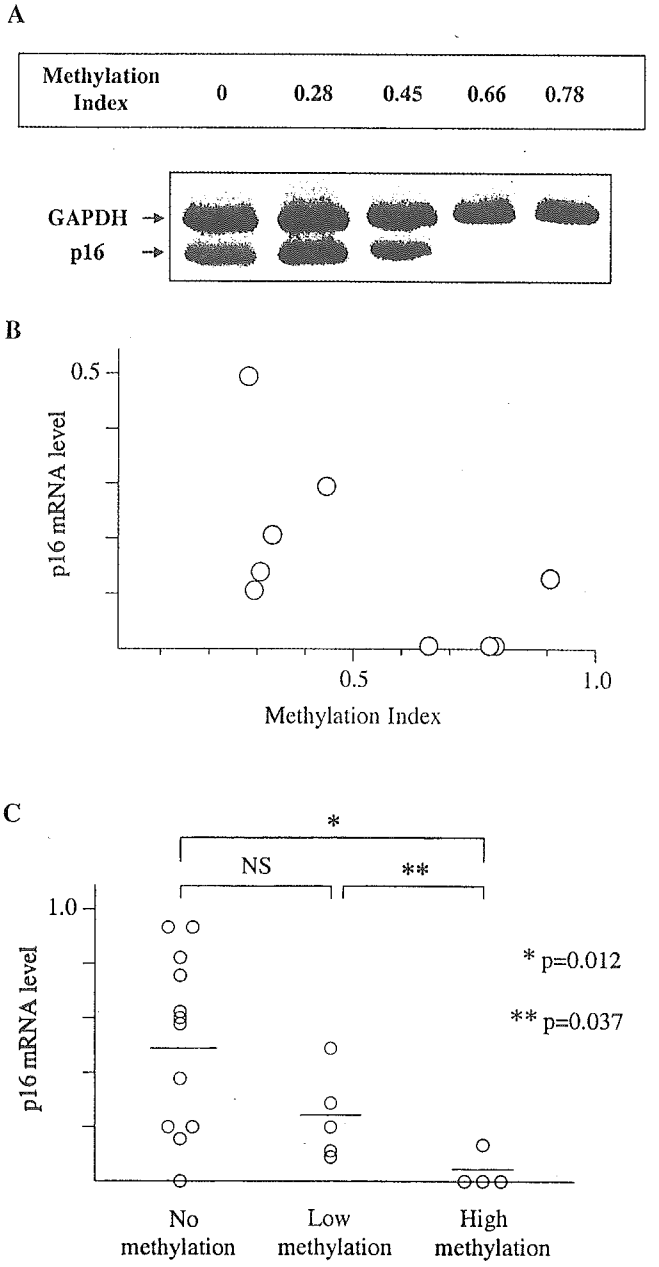

Figure 6. (A), Representative results of duplex RT-PCR for p16 mRNA expression and methylation index (MI). Methylation-specific real-time PCR was performed using methylation-positive CRC tissues. The MI varied as follows: 0.28, 0.29, 0.30, 0.32, 0.45, 0.66, 0.78, 0.79 and 0.91. Methylation-negative CRC tissues were regarded as MI 0. Representative CRC samples are shown. (B), Relationship between p16 mRNA level and MI. The MI value and level of p16 mRNA expression of methylation-positive CRC samples were plotted. (C), Association of p16 mRNA level and methylation extent. CRC cases were classified into three categories; no-methylation (MI ≥0), low-methylation (0< MI <0.5), high-methylation (MI ≥0.5). The mean expression levels of p16 mRNA were 0.518±0.322, 0.25±0.15, and 0.037±0.075, respectively. NS, not significant. The units ascribed to p16 mRNA level represent the ratio of the p16 to GAPDH RT-PCR products.

Methylation-specific real-time PCR. We then measured the extent of p16 methylation in methylation-positive CRC tissues (n=9) by a methylation-specific real-time PCR method. The MI varied widely as follows: 0.28, 0.29, 0.30, 0.32, 0.45, 0.66, 0.78, 0.79, and 0.91. The MI values were then compared with p16 mRNA expression (Fig. 6A) and plotted (Fig. 6B). CRC tissues with high methylation had relatively low p16

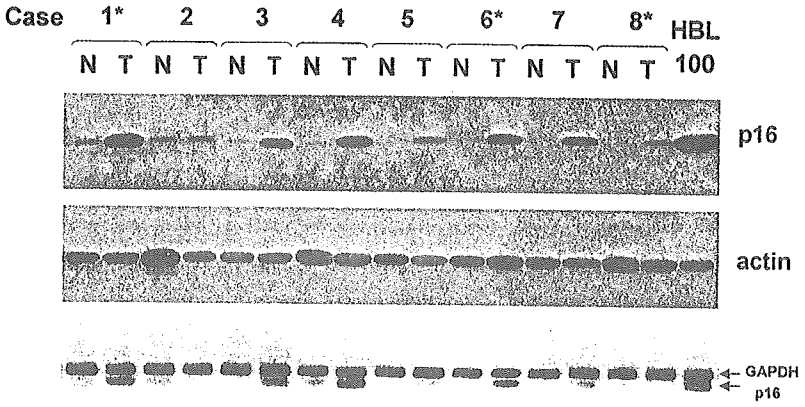

Figure 7. Western blot and RT-PCR analysis. The positive control HBL100 cells displayed a band for the p16 protein. Cancer tissue expressed a clear band for p16 to various extents, while non-neoplastic tissue generally expressed none or scarce band (upper panel). Actin served as loading control of equal amount of the protein (middle panel). RT-PCR assay for p16 mRNA expression in the same series of tissue sample (lower panel). Asterisk indicates cases with p16 methylation in cancer tissue. N, non-neoplastic tissue; T, tumor tissue.

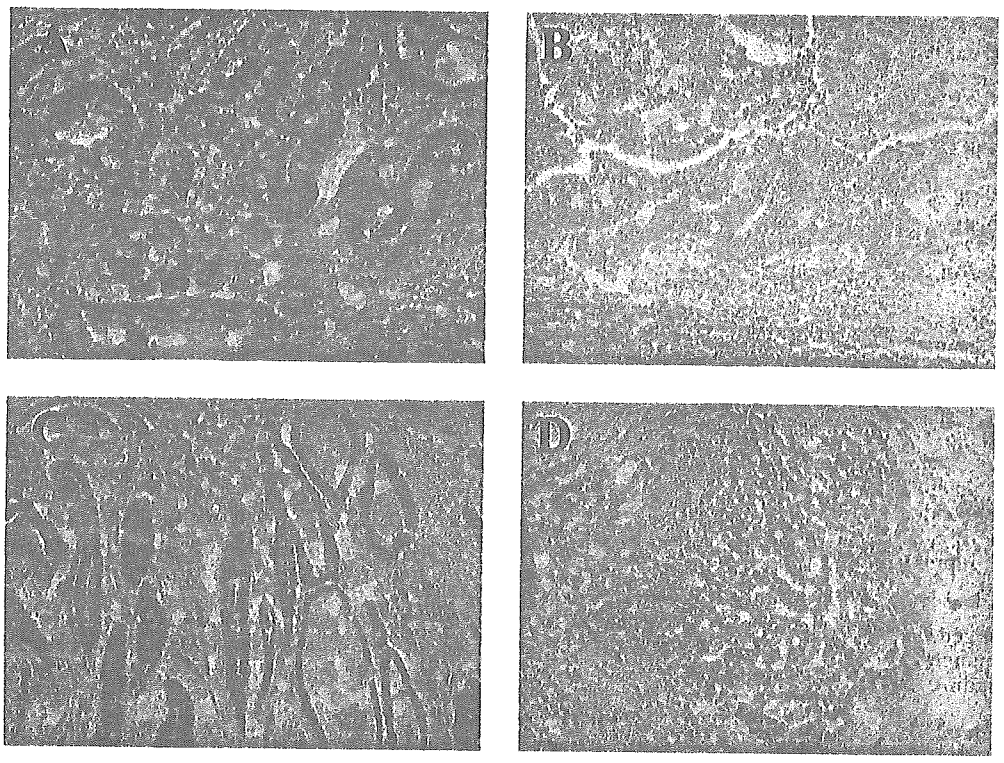

Figure 8. Immunostaining of p16 in colon cancer. In a colon cancer tissue the p16 protein was expressed in the nuclei and cytoplasm. Representative samples of methylation-minus and plus cancer tissues are shown. Methylation-minus groups: (A), high expression; (B), scarce expression. Methylation-plus groups: (C), high expression; (D), faint expression. Magnification, x150.

expression. When CRC cases were classified into three categories according to MI; no-methylation (MI = 0), low-methylation (0< MI <0.5), high-methylation (MI ≥0.5), there was a significant difference in p16 mRNA expression between the high-methylation group and the no-methylation group or low-methylation group (p=0.012, p=0.037, respectively, Fig. 6C).

Expression of p16 ^{INK4} protein in surgical specimens. Western blotting of 8 pairs of the non-neoplastic colonic mucosa and their corresponding CRC tissues showed that the intensity of the p16 band was scarcely detected in the non-neoplastic mucosa and an increased level of p16 expression was noted to various extent in cancer tissues (Fig. 7). RT-PCR indicated that p16 mRNA expression correlated well with p16 protein expression (Fig. 7). Immunostaining of the corresponding tissue samples showed that p16 quantity determined by Western blot was correlated with p16 protein expression in cancer tissues, but not in stromal cells (Fig. 8). The non-immune rabbit serum and antibodies with preabsorbed immunogen