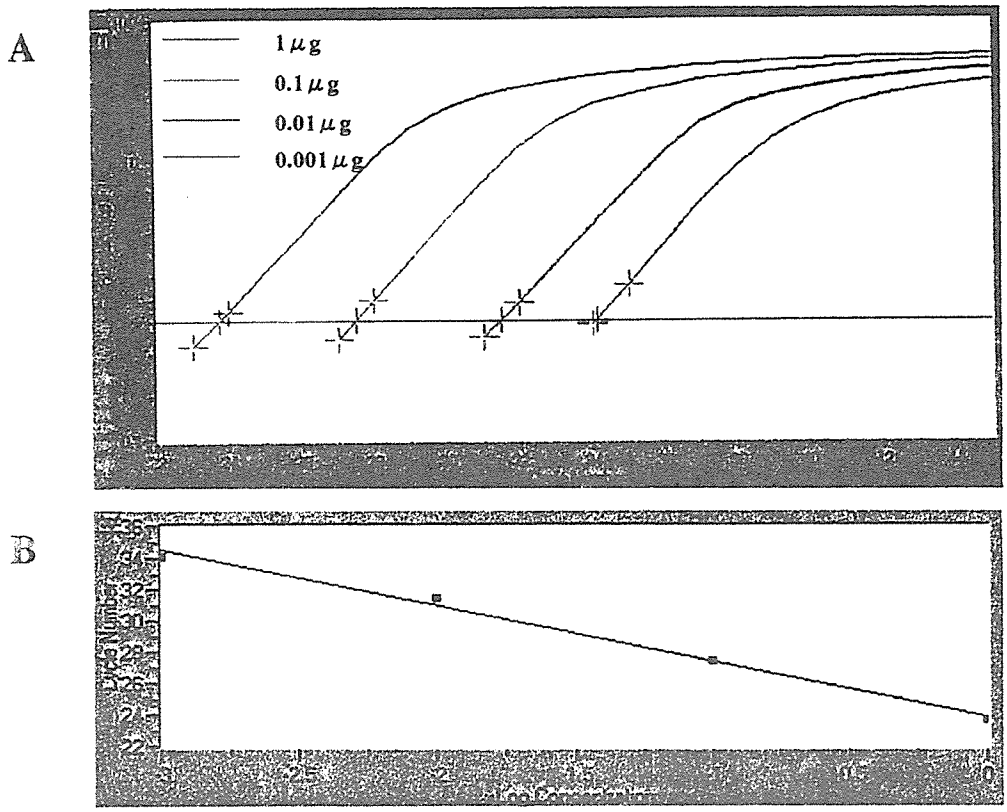

Figure 1. Methylation-specific PCR (MSP) using serial dilutions of methylation control DNA. (A), MSP was performed using 10-fold serial dilutions of methylation control genomic DNA from 2 ng to 2 μg. The PCR products were monitored by the fluorescence of dsDNA-specific dye SYBR Green I. (B), Based on 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):

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

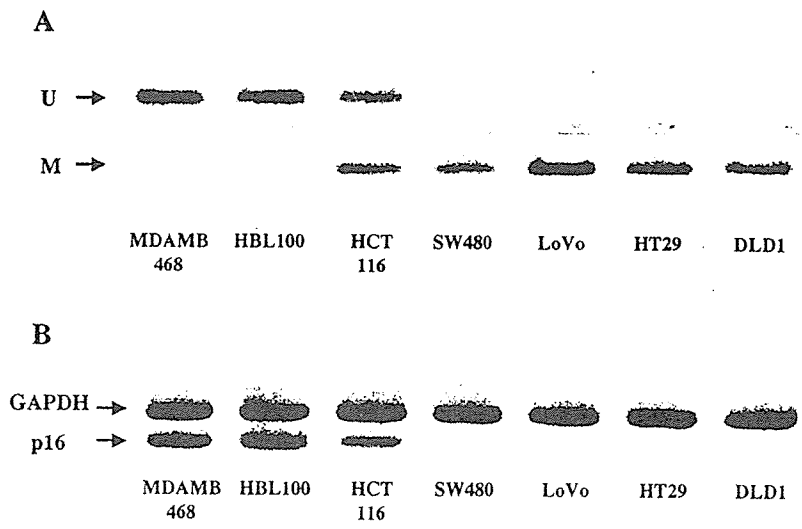

Figure 2. (A), Methylation of the p16 promoter region in cell lines. The breast cancer cell line MDAMB468 and the HBL100 breast epithelial cell line displayed a band when amplified with primers for unmethylated DNA (lanes 1 and 2). The HCT116 CRC cell line displayed bands of both unmethylated DNA and 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.

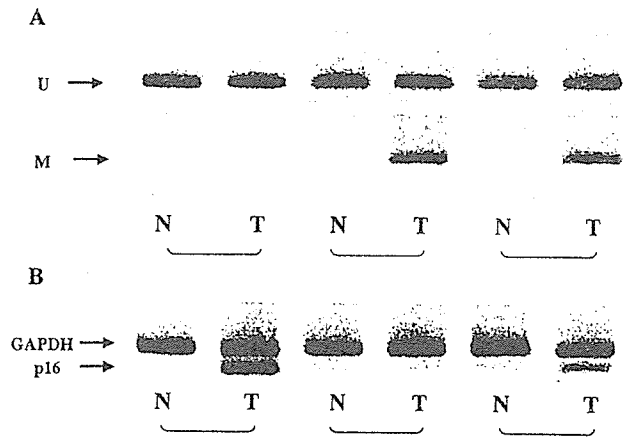

Figure 3. (A), Methylation of the p16 promoter region in surgical specimens. All surgical specimens displayed a band of unmethylated DNA. A band of methylated DNA was found in 2 of 21 normal mucosa samples (9.5%) and 9 of 21 cancer tissues (42.9%). Three representative pairs of normal and cancer tissues 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).

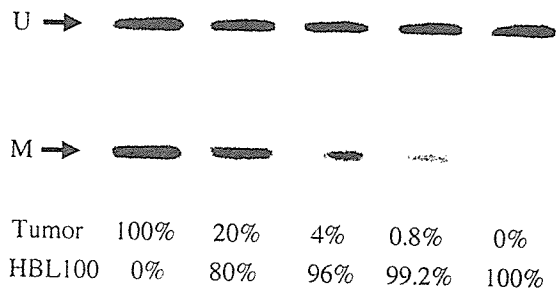

Figure 4. Mixing experiment. Prior to bisulfite treatment, a cancer DNA sample (1 μg) displaying both methylated and unmethylated bands (lane 1) was diluted serially (every 5-fold, lanes 2-4) with increasing amounts of unmethylated DNA from HBL100 cells. Then, PCR was performed. Unmethylated DNA remained 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.

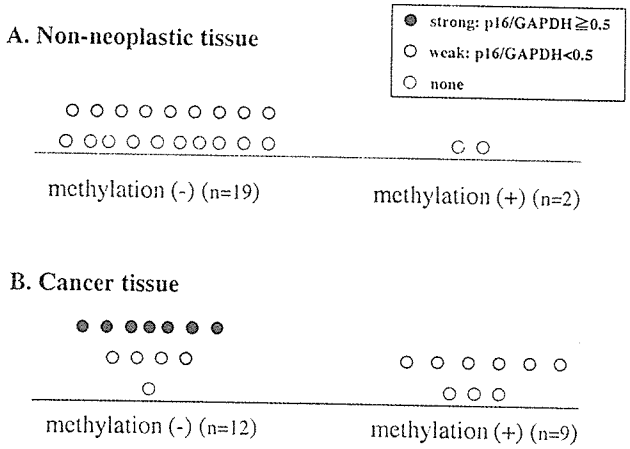

Figure 5. p16 mRNA expression stratified by the presence of methylation in non-neoplastic tissue (A) and cancer tissue (B). When high expression of p16 mRNA was defined as an intensity ratio of the p16 mRNA band to the GAPDH mRNA band of ≥0.5, 7 CRC tissues without methylation belonged to this category (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).

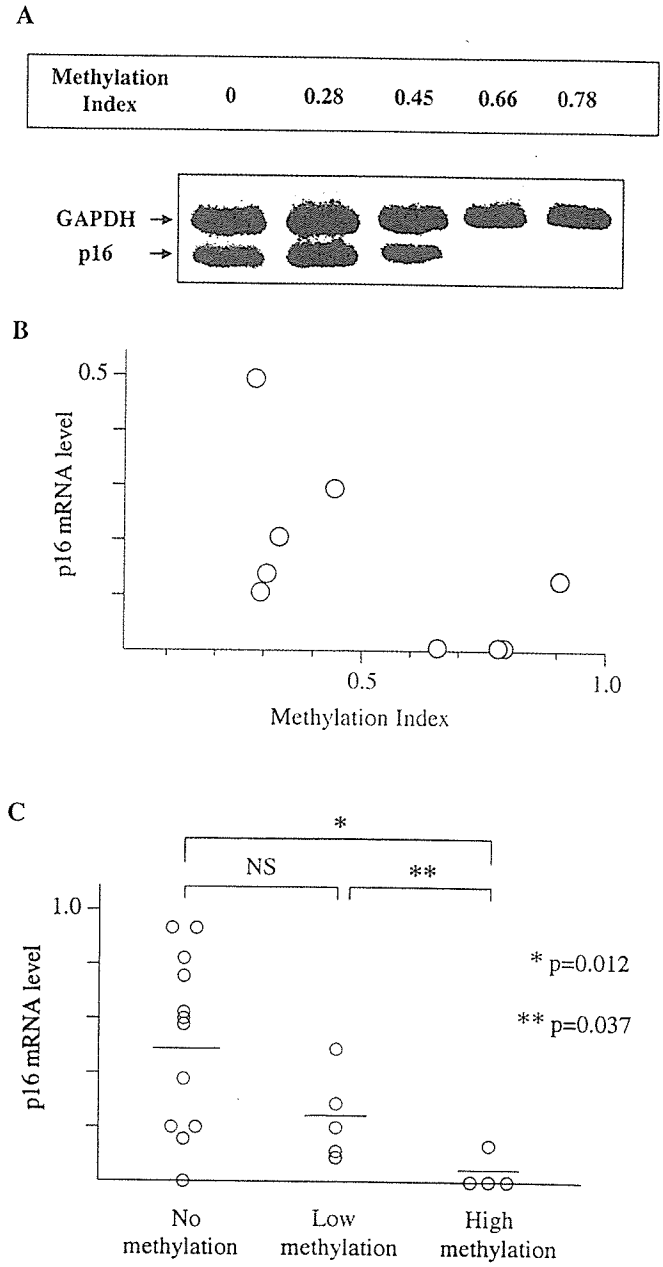

Figure 6. (A), Representative results of duplex RT-PCR for p16 mRNA expression and methylation index (MI). Methylation-specific real-time PCR was performed using methylation-positive CRC tissues. The MI varied as follows: 0.28, 0.29, 0.30, 0.32, 0.45, 0.66, 0.78, 0.79 and 0.91. Methylation-negative CRC 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

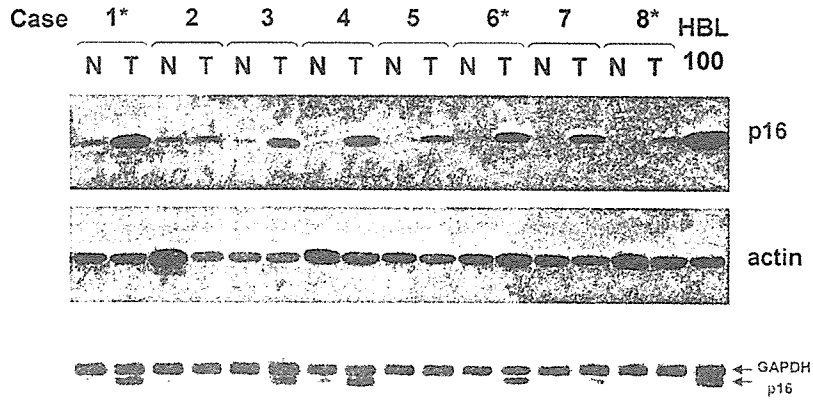

Figure 7. Western blot and RT-PCR analysis. The positive control HBL100 cells displayed a band for the p16 protein. Cancer tissue expressed a clear band for p16 to various extents, while non-neoplastic tissue generally expressed none or scarce band (upper panel). Actin served as loading control of equal 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.

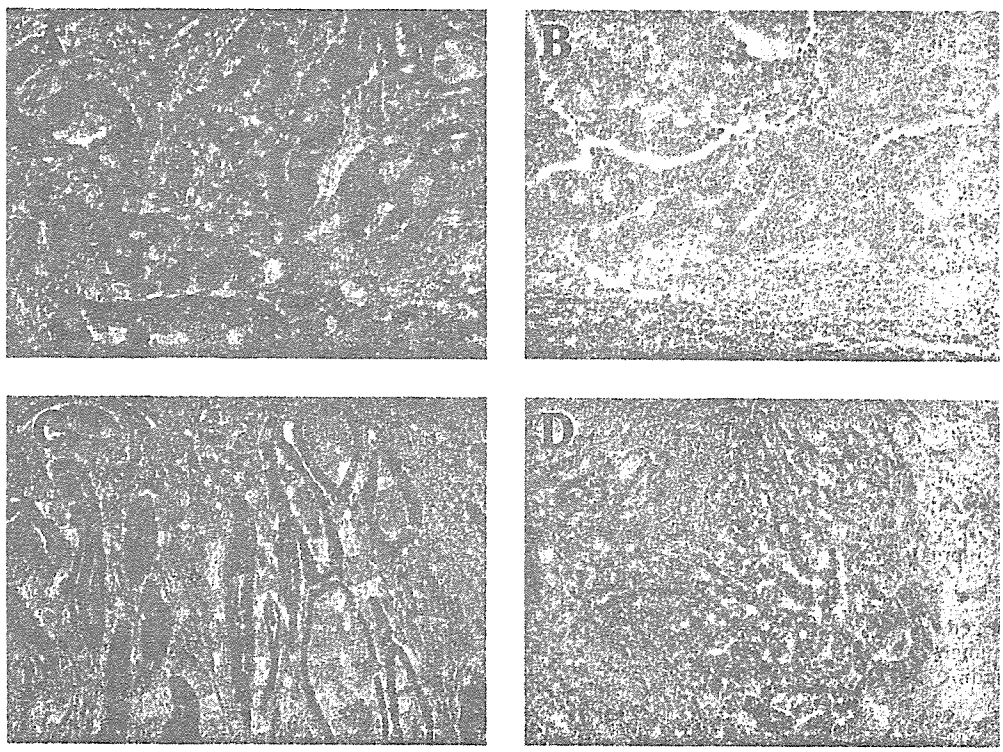

Figure 8. Immunostaining of p16 in colon cancer. In a colon cancer tissue the p16 protein was expressed in the nuclei and cytoplasm. Representative samples of methylation-minus and plus cancer tissues are shown. Methylation-minus groups: (A), high expression; (B), scarce expression. Methylation-plus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

Table I. Relationship between p16 expression and clinicopathologic parameters in colorectal cancer.

Clinicopathologic parameter	Labelling index of p16 staining (%)		
	Group A[a]	Group B[b]	Group C[c]
Gender			
Male	5	27	1
Female	5	16	1
Site			
Colon	6	23	1
Rectum	4	20	1
Dukes'			
A, B	8	21	1
C, D	2[d]	22	1
Histological type			
Well	6	22	0
Moderate/poor	4	21	2
Nodal involvement[e]			
Negative	10	21	1
Positive	0	22	1
Depth of invasion			
~mp	4	14	0
ss~	6	29	2
Distant metastasis			
Negative	8	41	2
Positive	2	2	0
Age (years)	56.7±13.1	62.6±10.3	60.5±20.5
Size (cm)[f]	3.5±1.8	4.8±2.2	8.1±1.6

[a]More than 50% of the cancer cells positive for p16. [b]Ten to 50% of the cells positive. [c]Less than 10% of the cells positive. [d]These 2 cases had no lymph node metastasis but displayed distant metastasis. [e]p=0.003. [f]p=0.048. mp, muscularis propria; ss, subserosa.

yielded no staining in the samples and the HBL100 cells displayed a positive staining and the T98G cells had no staining (data not shown).

In total, 55 paired samples were immunohistochemically stained with the anti-p16 antibody. In the normal colonic mucosa, clear nuclear p16 was detected only occasionally and the LI was <5% in all 55 cases tested (data not shown). In contrast, p16 was abundantly expressed in the majority of CRC tissues, and was localized in the nuclei and cytoplasm. When CRC cases were classified into three groups according to their LI, 10 cases (19%) were classified into group A (LI >50%), 43 (77%) into group B (LI 10-50%) and 2 (4%) into group C (LI <10%). In at least 53 cases (96%), overexpression of p16 was noted in CRC tissue when compared to the corresponding non-neoplastic mucosa. In the 21 CRC tissues utilized for methylation assay, the results of p16 staining correlated well with p16 mRNA expression (data not shown).

Relationship between p16 ^{INK4} expression and clinicopathologic parameters. The relationship between the level of staining of p16 in CRC samples and several clinicopathologic parameters was examined. These parameters included gender, tumor site, disease stage, histological grade, lymph node metastasis, depth of invasion, distant metastasis, age and size (Table I). High p16 expression level was associated with a low incidence of lymph node metastasis (group A vs. group B and C, p=0.003), and p16 expression was associated with small tumor size (p=0.048). There was no correlation between p16 expression and other clinicopathologic parameters.

Discussion

Dysregulation of CKIs, negative regulators of the cell cycle, is often found in human malignancies. The expression of $p21^{Waf1}$ is reduced in aberrant crypt foci with dysplasia and carcinomas of the colon and its high expression is related to a better prognosis in gastric cancer (39-41). A decrease in $p27^{Kip1}$ is associated with poor prognosis or high grade tumors in various types of human cancer (29,42). Loss or decreased expression of p16 is found in a subset of primary cancers (8-12). These lines of evidence indicate that inactivation of CKI may play a role in carcinogenesis and/or tumor progression of various types of human cancers. However, it appears to be unlikely that loss of p16 plays a central role in tumorigenesis of the colorectum *in vivo*. In this study we found that p16 gene expression was often upregulated in primary CRC tissues compared with non-neoplastic mucosa at both the mRNA and protein levels, though p16 mRNA was lost in most CRC cell lines. These findings are consistent with previous reports (15,24,25) and suggest that the regulatory mechanism of p16 gene expression may be quite distinct between cell lines and *in vivo* tissues of the colorectum.

Loss of p16 mRNA in CRC cell lines is understandable since a band corresponding to the methylated p16 gene was generally present in all CRC cell lines, and hypermethylation, as well as gene alterations, is known to be a major mechanism for inactivation of p16 gene transcription (13,14). On the other hand, a methylated p16 band was detected in 42.9% (9/21) of surgically resected CRC tissues, consistent with other reports that p16 methylation is present in 29-55% of CRC tissues (18-22). However, unlike cell lines, such methylation-positive CRC tissues frequently still expressed p16 mRNA. One clue to explain the differential regulatory mechanisms between CRC cell lines and CRC tissues may be methylation density in the p16 promoter region, rather than the presence of methylation *per se* (26,27). In support of this hypothesis on regulation of the p16 transcript, even cell lines exhibited varying extent of p16 methylation, which could affect p16 gene expression (38,43). We also confirmed that the HCT116 cell line whose one allele of p16 is methylated and silenced and the other is mutated and unmethylated (44), did display both a methylated and an unmethylated p16 band, leading to only modest expression of p16 mRNA (Fig. 2). Other CRC cell lines displaying only the methylated p16 band (MI: 100%) exhibited no p16 mRNA expression. Although methylation density of the p16 gene has been measured in several tumor cell lines, the extent of methylation in CRC tissues has yet not been clarified.

We performed quantitative MSP, using CRC tissues that exhibited p16 gene methylation. Our findings showed that there was a significant decrease in p16 mRNA level in the high-methylation group compared to the no-methylation or low-methylation groups (Fig. 6C). These findings suggest that a hyper-methylation density would facilitate transcriptional repression of the p16 gene in CRC tissues, but that low-methylation may only partially repress p16 gene expression. It was of interest that MI was variable (range: 0.28-0.91) and this could be a characteristic of *in vivo* p16 gene methylation. Concordant p16 expression at protein and RNA level (Fig. 7) suggest that p16 expression may be regulated at transcriptional level. Immunohistochemistry revealed that low level of the p16 protein represented reduced expression within neoplastic epithelium, but not in stromal cells (Fig. 8). In the no-methylation group, CRC tissues exhibited a wide range of p16 mRNA levels (Fig. 6C). This suggests that the basal p16 mRNA level could be also variable in the methylation-positive group, and therefore p16 expression level may not be determined by methylation extent alone. This might account for a few exceptional CRC samples that expressed some p16 mRNA even with the highest MI (0.91) or displayed relatively low p16 mRNA expression despite a low MI (0.29, 0.3) (Fig. 6B).

It remains controversial whether p16 expression is predictive of clinical events. Inactivation of the p16 gene is associated with aggressive disease phenotype including lymph node metastasis, shorter survival in esophageal cancer and advanced stage of hepatocellular carcinomas (45,46). Conversely, it has been reported that high levels of p16 are associated with poor prognosis in carcinomas of the ovary, prostate, and breast (47-49). In the latter tumor types, increased p16 expression might in turn reflect upregulated activity of the cell cycle positive regulators. Our immunohistochemical analysis showed that low p16 expression in CRC tissues correlated with larger tumor size and lymph node metastasis. Other investigators have also shown that p16 expression in CRC tissues was associated with low proliferative activity (24,25) and hypermethylation of the p16 gene was associated with advanced tumor stage and shorter survival (20,22). Taken together, these findings suggest that p16, despite its upregulation, may act as a tumor suppressor gene in CRC tissues, and it is likely that methylation would at least in part contribute to reduction in p16 expression, resulting in facilitation of tumor expansion and enlargement of CRC.

Overexpression of the p16 tumor suppressor gene in CRC tissues appeared to be paradoxical and we are not yet certain of the reason for this. However, overexpression of p16 is not limited to CRC, but is also seen in other gastrointestinal tumors, such as gastric and intestinal cancers (24,25,30,50). Moreover, studies have shown that several human cancers display increased expression of negative cell cycle regulators, although no mutations of the $p27^{Kip1}$ and $p21^{Waf1}$ genes have been reported for human cancer tissues. High levels of $p21^{Waf1}$ expression are seen in glial tumors, non-small cell lung cancer, and breast cancer (51-53). $p27^{Kip1}$ expression is increased in a subset of cancers, including breast and colon cancers (29,54). The precise reason for why negative regulators of the cell cycle often display increased expression is unknown at present. One explanation proposed by Weinstein is that multistage carcinogenesis is not simply a summation of the individual effects of oncogene activation and tumor suppressor gene inactivation, but that the evolving cancer cell must maintain a state of homeostasis between positive- and negative-acting factors in order to maintain structural integrity, viability, and normal replication (55). In this context, we speculate that p16 gene methylation may break the homeostatic balance, thereby tipping the balance towards positive regulators.

In conclusion, our study demonstrated that CRC tissues exhibited a variable methylation density of the CpG island in the p16 gene promoter, suggesting that p16 expression may be repressed in a methylation-dependent manner. Our data suggest that the majority of CRC tissues overexpressed p16 protein, and that low p16 expression among CRC tissues, probably via hypermethylation, may contribute to tumor enlargement and lymph node metastasis.

Acknowledgements

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Effects of p21$^{cip1/waf1}$ overexpression on growth, apoptosis and differentiation in human colon carcinoma cells

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Abstract. The cyclin-dependent kinase inhibitor p21$^{cip1/waf1}$ negatively regulates the progression of cell cycle and the potential usefulness of *p21$^{cip1/waf1}$* gene is proposed in gene therapy. However, studies have demonstrated a protective role of p21$^{cip1/waf1}$ against apoptosis and little is known about effects of ectopic expression of p21$^{cip1/waf1}$ on differentiation of colon cancer cells. In the present study, we found diffuse p21$^{cip1/waf1}$ expression in only a few clinical samples of colorectal cancer with wild-type *p53* gene. To explore the role of p21$^{cip1/waf1}$ in cell growth, apoptosis and differentiation, we constitutively overexpressed p21$^{cip1/waf1}$ in HT29 colon carcinoma cells. Ectopic overexpression of p21$^{cip1/waf1}$ was associated with inhibition of CDK2-associated kinase activity, indicating the functionality of the introduced *p21$^{cip1/waf1}$* gene. Overexpression of p21$^{cip1/waf1}$ caused an appreciable growth inhibition in monolayer and soft agar cultures and it significantly reduced sodium butyrate- but not 5-fluorouracil-induced apoptosis. p21$^{cip1/waf1}$ overexpressing cells exhibited marked decrease of intestinal differentiation when assayed with intestinal alkaline phosphatase. Our findings suggest that introduction of *p21$^{cip1/waf1}$* gene into colon cancer cells may be useful for inhibiting cell growth but caution should be taken regarding the increased resistance to certain apoptosis-inducing agents and dysregulation of endogenous p21$^{cip1/waf1}$-mediated differentiation process.

Introduction

Progression of cells through the cell cycle is under positive control by a series of specific cyclin/cyclin-dependent kinase (CDK) complexes and it is negatively controlled by specific CDK inhibitors (1). The latter includes the Cip/Kip family, which consists of several members such as p21$^{cip1/waf1}$ (designated p21 hereafter), p27^{kip1} and p57^{kip2}. These proteins share a conserved N-terminal domain and inhibit a broad range of CDKs by binding to several cyclin/CDK complexes, including cyclin D/CDK4 (or CDK6), cyclin E/CDK2 and cyclin A/CDK2.

The expression of *p21* gene is regulated through either p53-dependent or -independent pathway (2). The p21 protein can inhibit CDK/cyclin activities and overexpression of p21 causes G1 arrest (3). p21 also physically interacts with proliferative cell nuclear antigen (PCNA) and blocks DNA replication (3). Therefore, p21 is a strong negative regulator of cell proliferation. Dysregulation of the cell cycle components including p21 has been shown in many types of human cancer. Accumulating evidence indicates low levels of p21 in colonic dysplastic aberrant crypt foci and carcinoma of the colon (4,5), although mutation of *p21* gene is very rare in human cancers (6). Based on these findings, it was proposed that ectopic overexpression of p21 might be useful in the treatment of colon cancer (5,7). At present, gene therapy utilizing a wild-type *p53*, an inducer of p21, is being tested in various human cancers. However, the significance of p21 molecule in gene therapy is not fully determined.

p21 was reported to promote as well as inhibit apoptosis (8-12), and to have no effect on this process (13,14). Thus, the exact role of p21 in apoptosis remains controversial. p21 is also involved in the process of cell differentiation. *In vitro*, p21 is coupled to differentiate in myocytes, hepatoma, hematopoietic and neuroblastoma cells by various types of differentiation inducers [including phorbol ester, vitamin D3, tumor necrosis factor (TNF)-α, MyoD and nerve growth factor plus aphidicolin] (15-19). In gut differentiation, p21 level increases during the differentiation of Caco-2 colon carcinoma cells (20,21), and sodium butyrate, an inducer of differentiation, up-regulates p21 in HT29 colon carcinoma cells (22). Considering the dynamic change of p21 in the differentiation processes, ectopic expression of p21 appears to exert a favorable effect, i.e., enhancement of cell differentiation.

The overall theme of the present study was to evaluate the efficacy of *p21* gene therapy. Specifically, we examined p21 expression in clinical samples of multistage carcinogenesis of the colorectum. Based on the observation that abundant p21 expression was noted only in a few clinical samples of colo-

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rectal cancer (CRC) with wild-type of *p53* gene, we introduced *p21* cDNA into p53 mutant HT29 colon cancer cells and examined its diverse effects on cell growth, apoptosis and cell differentiation.

Materials and methods

Cell lines and tissues. The HCT116, SW480 and HT29 human colon carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). The HCT116 cell line was grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37°C. We also used 53 colorectal carcinomas and paired adjacent normal mucosa samples, 40 samples of adenomatous colorectal polyps (30 tubular and 10 tubulo-villous adenomas) and 19 polyps containing focal carcinomas. All human samples were collected during surgery or during endoscopic polypectomy in our department. The samples were fixed in buffered formalin at 4°C overnight and embedded in paraffin. For Western blot analyses, a piece of tissue was immediately frozen in liquid nitrogen and stored at -80°C.

Immunostaining. For immunocytostaining, the cells were fixed on the slide in 10% buffered formalin for 10 min. Immunostaining was performed by an avidin-biotin complex method. Anti-human p21 monoclonal antibody (mAb, Oncogene Research Products, Calbiochem, San Diego, CA) and anti-p53 mAb (Novocastra, Newcastle, UK) were applied to the sections at a dilution of 1:50 for both. A negative control section, to which normal mouse serum had been applied, was included in each staining procedure.

Mutation analysis. For detection of mutations in *p53* gene, the polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) analysis was employed. First, exons 5-8 of the *p53* gene were amplified by PCR using the primers described previously (23). Then, 1.5 μl of 1/10 (v/v) of PCR-amplified products were added to 1.5 μl of a mixture of deoxynucleotide triphosphate (60 μM), ^{32}P-end-labeled primers (0.1 μM each), $MgCl_2$ (1.5 mM), Tris-HCl (pH 8.3, 10 mM), KCl (50 mM) and 0.1 units of Tag polymerase. PCR amplification was then performed for 20 cycles. The annealing temperatures were 60, 64, 62 and 62°C for exons 5, 6, 7 and 8, respectively. The SSCP analysis was performed with the following modifications. Briefly, the PCR product (3 μl) was diluted 10-fold with stop solution [20 mM ethylenediaminetetraacetic acid (EDTA), 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol] and heat-denatured at 98°C for 5 min. One μl of this mixture was loaded onto an 8% non-denaturing acrylamide gel (acrylamide:methylene-bis-acrylamide, 80:1) and run at a constant voltage of 400 V at room temperature for 12-16 h. The gel was then vacuum-dried and exposed to Kodak X-OMAT film at room temperature for 1-2 days.

Flow cytometric analysis. The cells were trypsinized, collected and washed twice with PBS, and then flow cytometric analysis was performed. Briefly, after fixation in 70% cold ethanol, the cell pellets were resuspended in 400 μl of 0.2 mg/ml propidium iodide (PI) containing 0.6% Nonidet P-40 (NP-40) plus the same volume of 1 mg/ml RNAse (Sigma, St. Louis, MO) and then incubated in the dark at room temperature for 30 min. The cell suspension was then filtered through a 60 μm Spectra mesh filter and analyzed with a flow cytometer. The percentage of cells in different phases of the cell cycle was determined with a ModFit 5.2 software.

Apoptosis. For the detection of apoptotic cells, both adherent and floating cells were pooled, fixed in 10% buffered formalin and stained with DAPI (4',6-diamidino-2-phenylindole, Boehringer Mannheim, Indianapolis, IN) solution on the glass slides. The cells were incubated with DAPI solution at a concentration of 1.5 μg/ml in PBS for 30 min and then washed in PBS for 2 h. For quantitation of apoptotic cells, 10 microscopic fields were randomly chosen at x25 magnification, and pictures were taken under light (for whole cells) or fluorescence (for DAPI-positive cells) microscopy. The total number of the cells and the number of apoptotic cells (those with nuclear fragmentation or condensation) were counted in each field. Over 700 total cells were counted in each sample, and the percentage of the apoptotic cells was calculated. The examiner who counted the number of apoptotic cells was blinded to the histopathological diagnosis in the case of human tissues.

Western blot analysis. The cell pellets were resuspended in lysis buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 μg/ml aprotinin and 10 μg/ml leupeptin] and the lysates were clarified by centrifugation at 15,000 x g for 5 min at 4°C. Total cellular protein was determined with Bradford protein assay (Bio-Rad, Hercules, CA). The protein samples (100 μg) were subjected to SDS-polyacrylamide gel electrophoresis as described previously (24). The mouse mAb to p21 and rabbit polyclonal antibody to actin (Sigma) were used at the concentrations recommended by the manufacturers.

Monolayer cell growth curves. The cells were plated in triplicate at a density of $2x10^4$ per well in 35-mm well dishes with 3 ml of DMEM medium plus 10% FBS. Cells were re-fed with fresh medium every 2 days. The number of cells was counted every 2 days for the subsequent days.

Soft agar assay. Assays for growth in 0.33% Noble agar (Difco Laboratories, Detroit, MI) were performed. In brief, $5x10^4$ cells were suspended in DMEM with 10% FBS, containing 0.33% agar, and plated in triplicate in 35-mm well plates. After 2 weeks, the number colonies with a diameter of >2 mm were counted by microscopy.

Assays for induction of alkaline phosphatase activity. Intestinal alkaline phosphatase (Sigma) is a well-established marker of differentiation in HT29 cells and other colon cancer cell lines (22,25). Sodium butyrate (Sigma), a short-chain fatty acid, induces the differentiation of various cell types (22). Cells were seeded in duplicate at a density of $5x10^4$ per well in 24-well plates in complete medium containing 10% FBS. Two days later, the cells were re-fed with 1 ml of fresh medium containing sodium butyrate at a concentration of 5 mM, and the cells were grown for an additional 3 days. Briefly, the cells

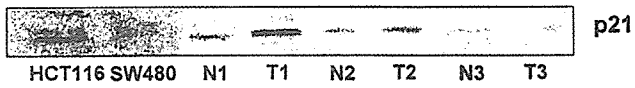

Figure 1. Western blot analysis for detection of p21 protein. Two cell lines, HCT116 (p53 wild-type) and SW480 (p53 mutant), and three colorectal carcinomas (T) and paired adjacent normal mucosa (N) samples were blotted using anti-p21 antibody.

were washed twice with PBS, fixed with citrate buffered acetone for 30 sec and then incubated with an alkaline dye mixture (fast violet B salt capsule, 2 ml of naphthol AS-MX phosphatase alkaline solution and 48 ml of distilled water) for 30 min, in the dark at room temperature. After washing with water, the cells were overlayed with PBS.

In vitro assay for cyclin E, CDK2-associated kinase activity. *In vitro* CDK2-associated kinase assays were performed as described previously (26), with minor modifications. After releasing from serum starvation, re-feeding with 10% FBS, the cells were collected at 6 and 12 h and sonicated in kinase buffer (in lysis buffer described above plus 10 mM ß-glycerophosphate, 1 mM NaF and 0.1 mM sodium orthovanadate). After centrifugation, the supernatant fraction was collected. Immunoprecipitation with 1 µg of the CDK2 polyclonal antibody was carried out as described above and the beads were washed four times with kinase buffer, twice with reaction buffer [50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 2.5 mM ethylene glycol bis (ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10 mM ß-glycerophosphate, 1 mM NaF and 0.1 mM sodium orthovanadate]. The final pellets were resuspended in 45 µl of reaction buffer containing 2 µg of Histone H1 (Sigma) and 5 µCi of γ-^{32}P-ATP, and incubated for 30 min at 30°C. The reaction mixture was then subjected to SDS-PAGE, and the intensity of phosphorylation of the Histone H1 substrate was determined by autoradiography.

Statistical analysis. All data were expressed as mean ± SD. Differences between groups were examined for statistical significance by the Mann-Whitney test using the StatView

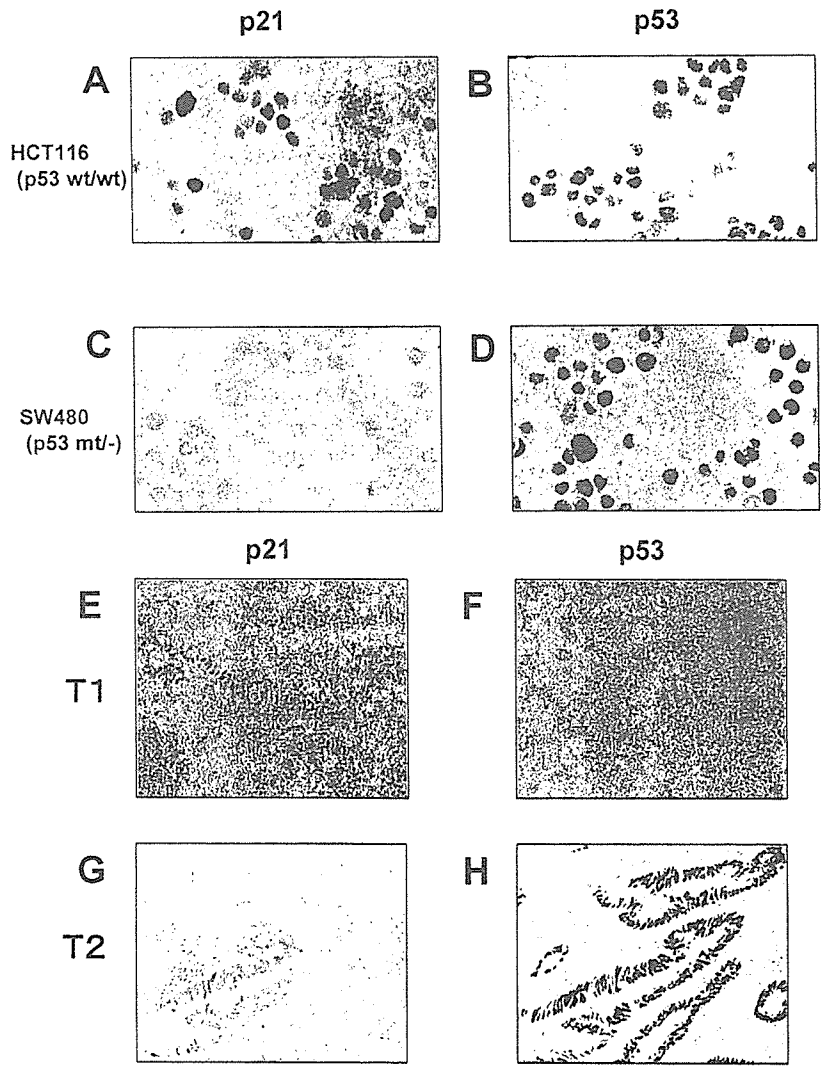

Figure 2. Immunostaining for p21 and p53. Expression of p21 and p53 in cell lines (A-D) and colorectal carcinomas (E-H). Nuclear staining for p21 was observed in HCT116 cells (A) and T1 tumor tissue (E), but not in SW480 cells (C), T2 (G) or T3 tumors (data not shown). Weak nuclear p53 was found in a small cell fraction of HCT116 (B), but strong staining was generally noted in SW480 (D). T1 tumor did not express p53 protein (F), while T2 tumor expressed the protein (H). When mouse non-immunized IgG was applied instead of primary antibody, no staining was obtained.

Table I. p21 expression in multistage carcinogenesis.

	Diffuse	Focal	Scarce	Negative
Adenomatous polyp (n=40)	0	0	12	28
Focal carcinoma in adenomatous polyp (n=19)	2	0	8	9
Advanced colorectal cancer (n=53)	3	3	6	41

J-5.0 program (SAS Institute Inc., Cary, NC). A p-value <0.05 denoted the presence of a statistically significant difference.

Results

Western blot analysis and immunostaining. Western blot analysis was performed using anti-p21 antibody. The p21 protein was detected in p53 wild-type HCT116 cells, whereas p21 expression was only negligible in p53 mutant type SW480 cell line (Fig. 1). In three paired non-tumor and tumor tissues, only little p21 expression was noted except for one tumor tissue (T1). Nuclear p21 expression was observed in HCT116 cells (Fig. 2A) and T1 tumor tissue (Fig. 2E), but not in SW480 cells (Fig. 2C), T2 tumor tissue (Fig. 2G) or T3 tumor tissue (data not shown). When mouse non-immunized IgG was applied instead of the primary antibody, no staining was obtained. These findings indicate that the antibody specifically reacted with the p21 protein.

Expression of p21 in polyps and cancer tissues. p21 was expressed in the top part of the normal epithelium. Of 40 adenomatous polyps 12 samples occasionally displayed p21 expression in cells at the surface area of the polyps. In 19 focal carcinoma in adenomatous polyps, diffuse p21 expression was identified in 2 focal carcinomas and only a small number of cancer cells expressed p21 in 8 focal carcinomas. Of 53 advanced colorectal cancers, diffuse p21 expression was found in 3 cases, and focal expression (in 3 cases) or only an occasional expression (in 6 cases) was noted. These results are summarized in Table I.

Relationship between p21 and p53 expression and mutation status. Since it is postulated that p53 is one of the inducers of p21 expression, we examined p53 expression in colon cancer cell lines and in a series of samples. A weak nuclear p53 expression was found in HCT116, but strong staining was generally noted in SW480 (Fig. 2B and D). Immunoreactivity for p53 was not observed in adenomatous polyps. T1 tumor did not express p53 protein, while T2 tumor expressed the protein (Fig. 2F and H). The relationship between p53 and p21 expression in focal carcinoma and advanced colorectal cancer tissue is shown in Table II, and no significant correlation was found. No *p53* gene mutations were detected in three advanced cancers with diffuse p21 expression (Fig. 3A-C). On the other hand, mutation of *p53* gene in exon 7 was detected in the T2 tumor (p21-negative) (Fig. 3D).

Table II. Relationship between p53 and p21 expression in focal carcinoma in adenomatous polyps and advanced colorectal cancers.

	p53	
Focal carcinoma in adenomatous polyp (n=19)	+	
p21		
+	5	5 (2)[a]
-	6	3
Advanced colorectal cancer (n=53)		
p21		
+	1	11 (3)[a]
-	28	13

[a]Diffuse expression.

Inhibition of CDK2 kinase in p21-overexpressing clones. Western blot analysis identified two clones that overexpressed p21 protein (#C1, #C2), compared with two vector control derivatives, Vp (a pool of vector control clones) and V1 (a single vector control clone) (Fig. 4A). Equal protein loading of each lane was confirmed by the blot for actin. *In vitro* CDK2-associated kinase assay indicated that the two p21-overexpressing clones displayed reduced kinase activity compared with the control cells (Fig. 4B). These results indicated that the overexpressed p21 protein was functional. Negative control studies showed that the use of non-immune rabbit serum for the immunoprecipitation resulted in negligible activities in the CDK2-kinase assays.

Growth properties of p21-overexpressing HT29 derivatives. In the next step, we generated growth curves using the two p21-overexpressing HT29 derivatives. Comparison with the vector control cells showed slow growth of the p21- over-expressers (Fig. 5). Cell cycle analyses showed increased G0/G1 population in p21 derivatives compared with the three control cells (Table III, p=0.002). When grown in agar in complete medium containing 10% FBS, the two control cell lines showed a colony forming efficiency of 26.7 and 27.0%, respectively. In contrast, the p21-overexpressing clones showed a decrease in colony forming efficiencies of 16.0 and 17.0%. These differences between two cell types were significant (p=0.031) and were also seen in two additional experiments.

Differentiation of p21-overexpressing HT29 derivatives. Histochemical analysis showed that alkaline phosphatase-positive cells were only rarely detected in the vector control cells and the p21-overexpressers. After induction of intestinal

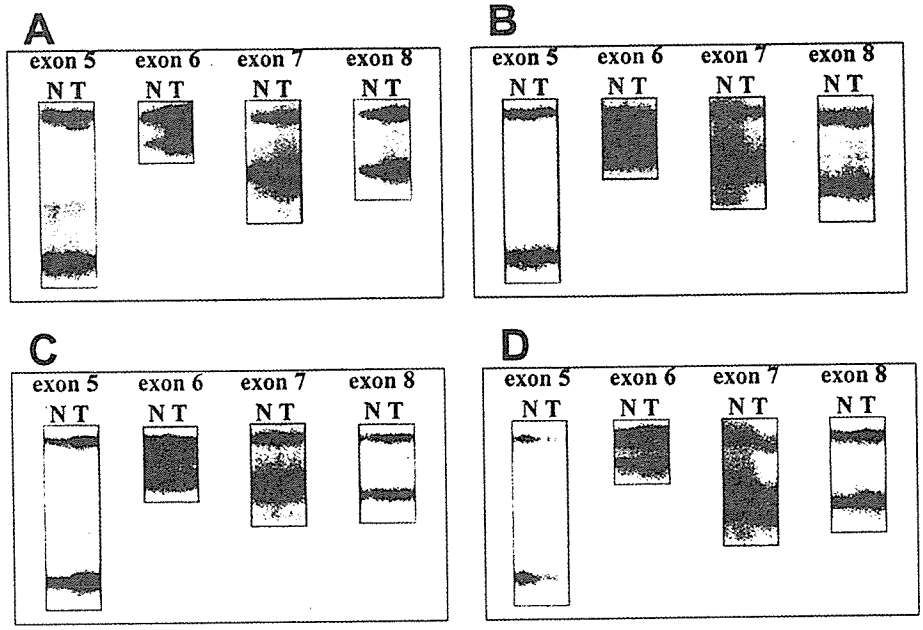

Figure 3. Mutation analysis. The PCR-SSCP analysis was employed for the detection of *p53* gene mutations. No *p53* gene mutations were found in 3 advanced cancers that displayed a diffuse p21 expression (A-C). However, T2 tumor (p21-negative) had *p53* gene mutation in exon 7 (D).

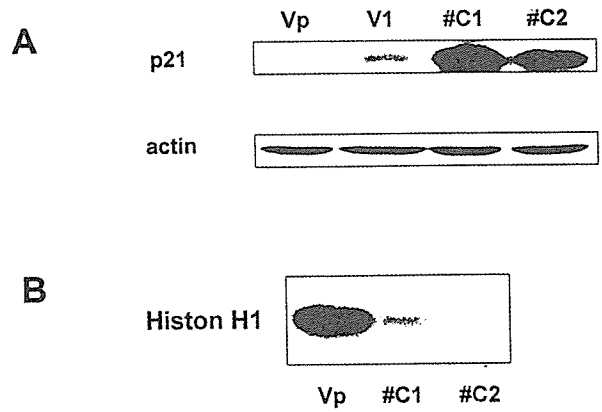

Figure 4. Inhibition of CDK2 kinase in p21 overexpressing cells. (A), We identified two clones that overexpressed p21 protein (#C1, #C2), compared with two vector control derivatives, Vp (a pool of vector control clones) and V1 (a single vector control clone). Equal protein loading of each lane was confirmed by the blot for actin. (B), *In vitro* CDK2-associated kinase assay indicated that the two p21-overexpressing clones displayed low kinase activity compared with the control cells.

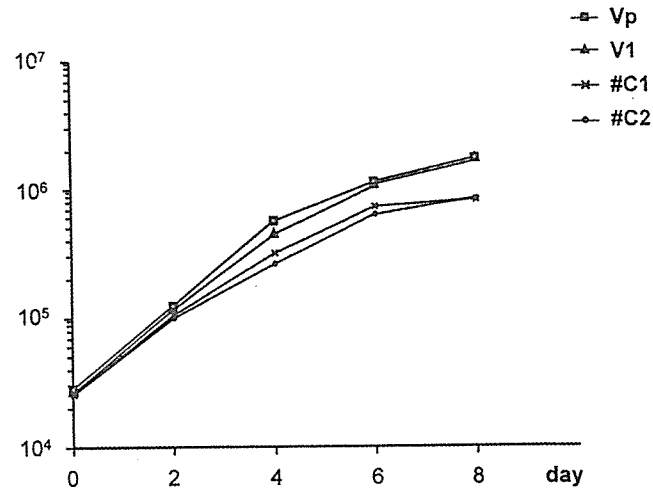

Figure 5. Growth properties of HT29 derivatives overexpressing p21. Using the two HT29 derivatives that overexpress p21, growth curves were drawn over eight days. Note the apparent slow growth of p21 overexpressers compared with the vector control cells.

differentiation, the subconfluent cultures of control cells displayed abundant alkaline phosphatase-positive cells, whereas the subconfluent cultures of the p21 overexpressers showed only a faint alkaline phosphatase expression (Fig. 6).

Effects of p21 on sodium butyrate-induced apoptosis. Since sodium butyrate is also a well-known apoptosis-inducing agent in various cell culture systems (27), we examined the effects of p21 on sodium butyrate-induced apoptosis. Sodium butyrate at 5 mM induced marked apoptosis in two vector control cell lines (26.5±2.2% and 26.0±1.3% of total cells, respectively). On the other hand, p21 overexpressers induced a milder degree of apoptosis (15.2±1.6% and 18.3±1.7%,

Table III. Cell cycle distribution of p21 derivatives.

Cell type	Vp	V1	V2	#C1	#C2
Cell cycle distribution (%)					
G0/G1[a]	54.7	53.4	54.0	66.0	63.6
S	31.4	32.5	31.9	24.2	25.9
G2/M	13.8	14.1	14.1	9.8	10.5

[a]Values were significantly higher in p21 derivatives #C1 and #C2 when compared to the control cells (p=0.002).

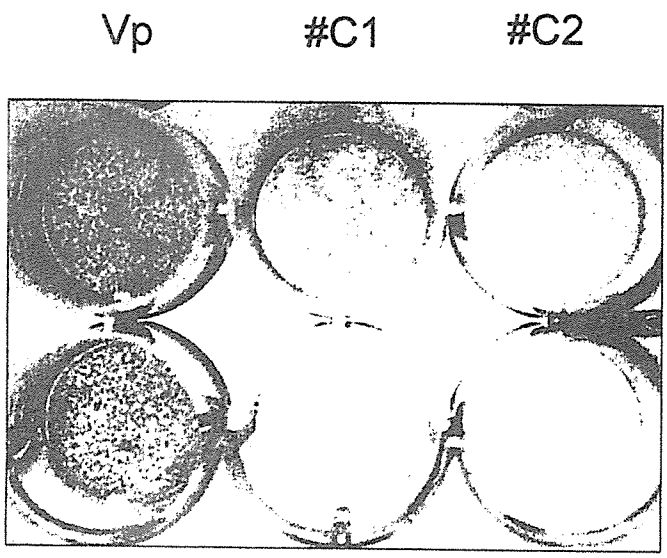

Figure 6. Differentiation of p21-overexpressing HT29 derivatives. Histochemical analyses showed that alkaline phosphatase-positive cells were rarely detected in the vector control cells and p21 overexpressers. After induction of intestinal differentiation, the subconfluent cultures of control cells displayed intense and frequent alkaline phosphatase-positive cells, whereas subconfluent cultures of the p21 overexpressers showed a faint alkaline phosphatase expression.

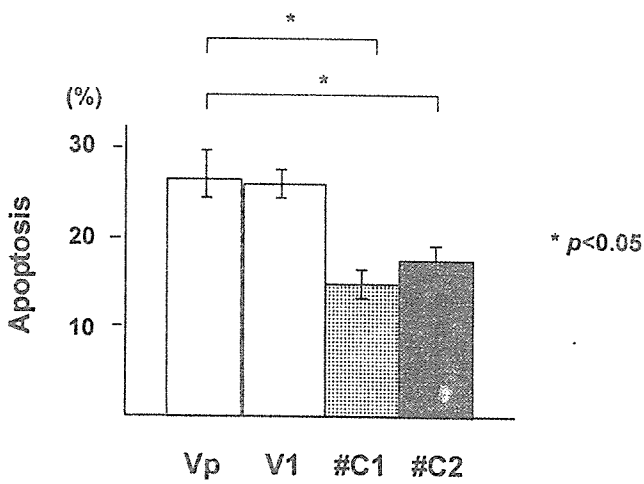

Figure 7. Effects of p21 on sodium butyrate-induced apoptosis. Apoptosis induced by 5 mM sodium butyrate was significantly more evident in the two vector control cell lines than the p21 overexpressers. Data are mean ± SD of three independent experiments. *p<0.05 by the Mann-Whitney test.

respectively) (Fig. 7). The difference between the vector pool and p21 derivatives was significant ($p<0.05$ for both). When 5-FU was used at IC_{50}, no difference in apoptotic cells was found between p21 overexpressers and the control cells (data not shown).

Discussion

In the present study, we found high p21 expression in a subset of CRC and a generally low p21 level in the majority of CRC. Especially, diffuse expression of p21 was noted only in CRC with wild-type *p53* gene (7,28). Several studies showed a higher p21 expression in CRC compared to that of the present study. The different results may be due to differences in the antibody used or the criterion used for positivity (i.e., cut-off point), but at least our data indicate that p21 is not up-regulated during adenoma carcinoma sequence and that p21 is unlikely to be expressed abundantly in CRC tissues.

Using clinical samples, previous studies reported that p21 expression is associated with better prognosis in several types of human malignancies including carcinomas of the stomach and pancreas (29,30). Considering the known role of p21 in regulation of proliferation based on CDK inhibitory function as well as suppression of PCNA, the strategy of *p21* gene transduction appears to be reasonable. In particular, CRC with mutant *p53* gene may be an appropriate candidate and this category is estimated at approximately 50% CRC (31). Indeed, ectopic expression of p21 seems to be functional even in malignant cells because it inhibits growth and tumorigenicity in human brain tumor cells, prostate cancer cells, HeLa cervical carcinoma cells and rat glioma cells (32-35). In the present study, we constitutively overexpressed p21 in p53-mutant HT29 colon carcinoma cells and found that p21 overexpressing cells grew slower in monolayer cultures. Growth suppression in 3-D soft agar cultures was also evident. Since the latter assay is thought to reflect *in vivo* growth, the effect of growth suppression is promising. However, previous studies showed that p21 is involved not only in cell growth, but also in other aspects of cellular process including apoptosis and cellular differentiation (as mentioned in Introduction).

Several studies showed the coupling of marked up-regulation of endogenous p21 with apoptosis in various cell types in response to certain agents including taxol, okadaic acid, etoposide, IFN-γ (36-39), suggesting that p21 may be a crucial factor that modulates cell viability. In the present study, we found that overexpression of p21 inhibited sodium butyrate-induced apoptosis rather than enhanced it. This finding is consistent with the cumulative evidence on the protective role of p21 in apoptosis. Cyclopentenone prostaglandin A2 (PGA2)-mediated cell death is promoted by antisense p21 in MCF-7 breast cancer cells and it is diminished by ectopic expression of p21 in RKO colon carcinoma cells (12,40). The loss of p21 sensitizes the HCT116 colon carcinoma cells to killing by UV-irradiation, γ-irradiation, cisplatin and nitrogen mustard (41,42). On the other hand, induction of p21 with an inducible expression system did not affect adriamycin-induced apoptosis of DLD1 colon carcinoma cells (43) and p21 antisense resulted in equal sensitivity to AraC-induced apoptosis of HL60 (44), being consistent with our results of 5-FU treatment. Furthermore, overexpression of p21 led to increased sensitivity to doxorubicin and methotrexate in an osteosarcoma cell line (9) and enhanced radiosensitivity in rat glioma cells (10). Taken together, it is possible that p21 can act as either a reducer or enhancer of apoptosis depending on the type of agents used and cell types.

p21 is thought to play a role in differentiation in several leukemia cells and tumor cells (18,32). In normal epithelia of various organs, p21 expression is associated with terminal differentiation (20). p21 plays a role in differentiation in leukemia cells, neuroblastoma cells and mice keratinocytes

(14,16-18). Thus antisense to p21 decreased monocytic differentiation of HL60 (44) and p21-deficient keratinocytes underwent down-modulated differentiation (45). Conversely, there is direct evidence that overexpression of p21 induces differentiation in the myelomonocytic cell line and megakaryoblastic leukemia cell line (46,47). These findings strongly suggest that p21 is an indispensable molecule acting during differentiation of cells of various origins. However, Di Cunto *et al* (48) surprisingly demonstrated that overexpression of p21 blocked the differentiation of keratinocytes. In our study, we found that p21 overexpression inhibited intestinal cell differentiation. Taken together, ectopic overexpression of p21 may not always enhance cellular differentiation, rather it can perturb the normal process of cellular differentiation in certain conditions.

There is no doubt, based on numerous studies, that p21 can inhibit cell growth. Therefore, p21 is one of the appropriate choices against human carcinomas such as hepatocellular carcinoma, in which cell proliferation positively correlates with prognosis (49), or pancreatic carcinoma, which progress rapidly. However, it is possible that the unfavorable outcome might be produced with regard to differentiation and apoptosis, depending on the cancer cell type. Thus, the diverse effects of forced expression of p21, especially the influence on chemotherapy, should be carefully evaluated in the application of p21 molecule in human gene therapy.

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Genetic Polymorphism in Cytochrome *P*450 7A1 and Risk of Colorectal Cancer: The Fukuoka Colorectal Cancer Study

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Abstract

Bile acids have long been implicated in the etiology of colorectal cancer, but epidemiologic evidence remains elusive. Cholesterol 7α-hydroxylase (*CYP7A1*) is the rate-limiting enzyme in the synthesis of bile acids from cholesterol in the liver, and thus may be an important determinant of bile acid production. We examined the association between the *CYP7A1 A-203C* polymorphism and colorectal cancer. The *CYP7A1 A-203C* polymorphism was determined by the PCR-RFLP method in 685 incident cases of colorectal cancer and 778 controls randomly selected from a community in the Fukuoka area, Japan. The *CC* genotype was slightly less frequent in the case group, and the adjusted odds ratio for the *CC* versus *AA* genotype was 0.88 (95% confidence interval, 0.65-1.20). In the analysis by subsite of the colorectum, a decreased risk associated with the *CYP7A1 CC* genotype was observed for proximal colon cancer, but not for either distal colon or rectal cancer. The adjusted odds ratios (95% confidence intervals) of proximal colon cancer for the *CC* genotype were 0.63 (0.36-1.10) compared with the *AA* genotype, and 0.59 (0.37-0.96) compared with the *AA* and *AC* genotypes combined. A decreased risk of proximal colon cancer in relation to the *CC* genotype of *CYP7A1 A-203C*, which probably renders less activity of the enzyme converting cholesterol to bile acids, is new evidence for the role of bile acids in colorectal carcinogenesis. (Cancer Res 2005; 65(7): 2979-82)

Introduction

Colorectal cancer is one of the most common cancers in the world, accounting for nearly 10% of all incident cases of cancer (1). Japan has experienced a rapid increase in mortality from colorectal cancer in the past 50 years (2), and is currently among the countries with the highest incidence rates worldwide (3). Bile acids have long been implicated in the etiology of colorectal cancer. Primary bile acids such as cholic and chenodeoxycholic acids are excreted in the liver, and are degraded to secondary bile acids, mainly deoxycholic and lithocholic acids, by bacteria in the intestinal lumen. Animal studies showed that secondary bile acids promoted chemically induced colorectal cancer (4, 5), and recent *in vitro* studies have identified several molecular mechanisms of deoxycholic acid promoting colorectal carcinogenesis (6, 7).

Despite these experimental observations, epidemiologic evidence remains elusive regarding the role of bile acids in colorectal carcinogenesis. Fecal levels of secondary bile acids as well as of total bile acids are higher in populations at high risk of colorectal cancer (8, 9). Several case-control studies reported higher levels of secondary bile acids in the feces or sera in patients with colorectal cancer or adenomas as compared with those without these lesions (10–13), but the findings were not replicated in other studies (14–16). A prospective study reported a suggestive increase in the risk of colorectal cancer associated with a high ratio of serum deoxycholic to cholic acids (17). Another epidemiologic evidence is the increased risk of proximal colon cancer in individuals having the gallbladder removed (18, 19). Cholecystectomy results in increased fecal excretion of secondary bile acids, probably due to increase in the bile acid pool in the enterohepatic circulation and increased degradation of primary bile acids in the gut (20, 21).

Recent studies (22, 23), but not all (24), showed that a common genetic polymorphism of cholesterol 7α-hydroxylase (*CYP7A1 A-203C*) was associated with plasma total and low-density lipoprotein cholesterol concentrations, suggesting lower activity of the enzyme in individuals with the variant C allele. *CYP7A1* is the rate-limiting enzyme in the synthesis of bile acids from cholesterol in the liver, and thus may be an important determinant of not only plasma cholesterol levels but also bile acid production. This article examined the association between the *CYP7A1 A-203C* polymorphism and colorectal cancer in order to further clarify the role of bile acids in colorectal carcinogenesis.

Materials and Methods

A case-control study was designed to examine the relation of lifestyle factors and genetic susceptibility to the risk of colorectal cancer. Cases were recruited from eight large hospitals in the study area (Fukuoka City and three adjacent areas), and controls were randomly selected in the community by frequency-matching to the distribution of incident cases with respect to sex and 10-year age class. The study protocol was approved

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by the ethical committees of the Faculty of Medical Sciences, Kyushu University, and of all but two of the participating hospitals. The two hospitals had no ethical committees at the time of survey. Details of the methods have been described elsewhere (25).

Participants. Cases were a consecutive series of patients with histologically confirmed incident colorectal adenocarcinomas who were admitted to two university hospitals or six affiliated hospitals for surgical treatment during the period October 2000 to December 2003. Other eligibility criteria were: age of 20 to 74 years at the time of diagnosis, residence in the study area, no prior history of partial or total removal of the colorectum, familial adenomatous polyposis or inflammatory bowel disease, mental competence to give informed consent and to complete the interview. Of 1,053 eligible cases, a total of 840 cases (80%) participated in the interview, and 685 out of them gave an informed consent to genotyping.

Eligibility criteria for controls were the same as described for cases except for the two items, i.e., having no diagnosis of colorectal cancer and age of 20 to 74 years at the time of selection. A total of 1,500 persons were selected as control candidates by two-stage random sampling. The number of control candidates by sex and 10-year age class were determined in accordance to sex- and age-specific numbers of estimated incident cases of colorectal cancer. The first step was a random selection of 15 small areas out of 178 in total, and then ~100 persons were randomly selected in each small area using the municipal resident registry on the basis of proportions of population in the small areas by sex and 10-year age class. A letter of invitation was sent to each candidate, and at most three additional letters of invitation were mailed to nonrespondents. A total of 833 persons participated in the survey, and 778 gave an informed consent to genotyping. The net participation rate was calculated as 60% (833/1,382), after exclusion of 118 persons for the following reasons: death (n = 7), migration from the study area (n = 22), undelivered mail (n = 44), mental incompetence (n = 19), history of partial or total removal of the colorectum (n = 21), and diagnosis of colorectal cancer after the survey (n = 5).

In both cases and controls, older persons and women were less likely to give consent to genotyping, whereas there was no material difference in residence, smoking habit, and alcohol use between individuals giving consent and those who did not (Table 1).

Procedures. DNA was extracted from the buffy coat by using a commercial kit (Qiagen GmbH, Hilden, Germany). Genotyping was done by one of the authors (T. Hagiwara) using the PCR-RFLP method. The PCR was done in a reaction mixture of 10 μL containing 0.5 units of Taq and 1 μL of template DNA with a concentration of ~50 to 150 ng/μL. The *CYP7A1* genotype was determined, as described by Han et al. (24) using primers 5′-AATGT TTTTC CCAGT TCTCT TTC-3′ (sense) and 5′-AATTA GCCAT TTGTT CATTC TATTA G-3′ (antisense). After the initial denaturation at 94°C for 4 minutes, 30 cycles of PCR were done for 30 seconds at 94°C, for 30 seconds at 53°C, and for 30 seconds at 72°C, with a final extension at 72°C for 7 minutes. The PCR product of 393 bp fragment was digested with 10 units of *BsaI* in a reaction mixture of 20 μL for 3 hours at 50°C. The digestion results in fragments of 300 and 93 bp for the *A* allele, and those of 261, 93, and 39 bp for the *C* allele. The digested PCR products were applied to electrophoresis of 3% agarose gel (NuiSieve GTG, Rockland, ME), and visualized by ethidium bromide.

The polymorphism was referred to as *A-204C* by Couture et al. (23), but the actual site of the polymorphism is located 203 bp upstream of the transcription start site according to the latest report of the sequence http://www.ncbi.nlm.nih.gov/Genomes). This was also confirmed by our sequencing of the relevant fragment.

Statistical analysis. The association of *CYP7A1* genotypes with the risk of colorectal cancer was examined in terms of odds ratio (OR) and 95% confidence intervals (CI). ORs were obtained from multiple logistic regression analysis, including indicator variables for gender, 5-year age class, and resident area (Fukuoka City or suburban area) as covariates. Statistical significance was declared if 95% CI did not include unity. All statistical analyses were done using the SAS version 8.2 (SAS Institute, Inc., Cary, NC).

Results

Proportions of the *AA*, *AC*, and *CC* genotypes in cases of colorectal cancer were 24%, 56%, and 20%, respectively (Table 2). The corresponding proportions in the control group were 25%, 51%, and 24%, respectively. The distribution in the control group was in agreement with the Hardy-Weinberg equilibrium (P = 0.59). The *CC* genotype was slightly less frequent in the case group, and the adjusted OR for the *CC* versus *AA* genotype was slightly lower than unity, with the 95% CI including unity. When the *AA* and *AC* genotypes were combined as the referent, the adjusted OR for the *CC* genotype was 0.81 (95% CI, 0.63-1.04).

The association with *CYP7A1* polymorphism was further examined for cancers of the proximal colon, distal colon, and rectum separately (Table 3). A nearly significant decrease in the OR for the *CC* versus *AA* genotype was observed for proximal colon cancer, but not for the other sites of cancer. When the *AA* and *AC* genotypes were combined as referents, the adjusted ORs of proximal colon cancer for the *CC* genotype was significantly lower than unity.

Discussion

The present study was the first that examined the relation between a functional *CYP7A1* polymorphism (*A-203C*) and

Table 1. Characteristics of cases and controls by consent to genotyping

Variable	Cases			Controls		
	With consent	Without consent	P*	With consent	Without consent	P*
Number	685	155		778	55	
Mean age (y)	60.2	61.9	0.03	58.6	62.9	0.004
Male (%)	62.2	48.4	0.002	63.0	45.5	0.01
Fukuoka City (%)	61.3	57.4	0.37	64.4	67.3	0.67
Ever-smoking (%)†	56.3	53.1	0.22	59.7	41.8	0.18
Alcohol use (%)†,‡	58.9	55.4	0.22	59.3	45.0	0.33

*P values (two-sided) were based on t test or χ^2 test unless otherwise specified.
†Adjusted for sex and 5-year age class by the direct method with total number of cases or controls as standard population. P values were based on the Mantel-Haenszel method.
‡Drinking alcohol at least once per week ~5 years ago.

Table 2. Adjusted ORs and 95% CI of colorectal cancer according to *CYP7A1 A-203C* polymorphisms

CYP7A1 A-203C genotype	Number (%)		Adjusted ORs (95% CI)*
	Cases (n = 685)	Controls (n = 778)	
AA	163 (24)	193 (25)	1.00 (referent)
AC	385 (56)	399 (51)	1.13 (0.88-1.46)
CC	137 (20)	186 (24)	0.88 (0.65-1.20)

*Adjusted for gender, 5-year age class, and resident area.

colorectal cancer, and showed a decreased risk of cancer of the proximal colon, but not of the distal colon and rectum, among individuals having the *CC* genotype. This genotype is probably associated with lowered capability of synthesizing bile acids (22, 23), the findings provide further evidence to the role of bile acids in colorectal carcinogenesis.

An advantage in this large-scale case-control study is that controls were derived from free-living residents in the community. It is also notable that participation rates of eligible cases and controls were fairly high. Genotyping was done in 82% of the cases and 93% of the controls who participated in the survey. It is generally considered that selection and confounding are less likely to occur in studies of genetic polymorphisms (26, 27). It is, however, possible that use of hospital controls may cause selection bias even in the gene-disease association. For instance, individuals with high blood cholesterol levels may have been included or excluded differentially in the controls if selection had occurred in patients with cholesterol-related diseases. The study subjects were an ethnically homogenous population of Japanese, and the concern over population stratification would be negligible (28).

Since the first report by Rose et al. (29), many prospective studies have observed an inverse association between serum total or low-density lipoprotein cholesterol and colon cancer (30). Although this inverse association is generally ascribed to the effect of preclinical cancer existing at the baseline (30), an increased risk of proximal colon cancer associated with low levels of serum total cholesterol persisted 10 to 20 years later in a prospective study in Hawaii (31). Furthermore, a case-control study observed lower levels of total and low-density lipoprotein cholesterol in cases of proximal colon cancer, but not of distal colon cancer, than in controls (32). These findings are congruent with decreased risk of proximal colon cancer associated with the *CYP7A1 CC* genotype.

High-fat diets are shown to increase fecal excretion of secondary bile acids as well as of total bile acids in humans (33), and to enhance chemically induced colon carcinogenesis in animals (34). Although fat intake is strongly positively correlated with colon cancer rates among countries (35), and over time in Japan (36), studies of individuals have consistently failed to find a positive association between fat intake and colon or colorectal cancer (37). The lack of an association with fat in studies of individuals may be due to a limited variation of fat intake within populations. In this regard, the present findings emphasize the usefulness of studying functional genetic polymorphisms when study populations are homogeneous with respect to exposure to environmental factors such as nutrient and food intake.

In conclusion, a large case-control study in Japan showed a decreased risk of proximal colon cancer in individuals having the *CC* genotype of *CYP7A1 A-203C*, which probably renders less activity of the enzyme converting cholesterol to bile acids. The findings add to evidence for the role of bile acids in colorectal carcinogenesis.

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Table 3. Adjusted OR and 95% CI of colorectal cancer according to *CYP7A1 A-203C* genotypes by subsite

CYP7A1 A-203C genotype	Proximal colon (n = 150)		Distal colon (n = 232)		Rectum (n = 290)	
	No.	OR (95% CI)*	No.	OR (95% CI)*	No.	OR (95% CI)*
Model 1						
AA	39	1.00 (referent)	52	1.00 (referent)	69	1.00 (referent)
AC	88	1.09 (0.71-1.65)	129	1.18 (0.81-1.70)	159	1.09 (0.78-1.53)
CC	23	0.63 (0.36-1.10)	51	1.01 (0.65-1.57)	62	0.93 (0.62-1.39)
Model 2						
AA + AC	127	1.00 (referent)	181	1.00 (referent)	228	1.00 (referent)
CC	23	0.59 (0.37-0.96)	51	0.90 (0.63-1.29)	62	0.87 (0.63-1.22)

*Adjusted for gender, 5-year age class, and resident area.

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