following irradiation. However, a non-cancerous tissue, that is, a normal rectal epithelial cell, does not inherently produce HIF and VEGF, and thus non-cancerous radiation proctitis tissue did not show VEGF expression.

In radiation proctitis, angiogenesis is induced abnormally in the superficial layer of the lamina propria but not in the submucosal layer, as would occur in normal tissues, and bleeding occurs easily from these abnormal vessels. However, the mechanism accounting for angiogenesis in the superficial mucosal layer remains unclear. It was reported that TGF-β overexpression promotes widespread fibrosis in the submucosal layer in acute radiation injury within 3 months after irradiation [23–25]. This fibrosis already present in the submucosal layer may cause abnormal angiogenesis in the superficial layer of the lamina propria in the late stages of radiation injury. The predominant environment of MMP rather than TIMP may also promote angiogenesis in the mucosal layer.

The mechanism responsible for the induction of angiogenin and FGF1 overexpression in this study remains elusive. Although there has been one report of angiogenin induction under hypoxic conditions [26], the precise mechanism is unclear. Moreover, the mechanism for induction of FGF1 remains to be elucidated. To clarify the mechanism by which these angiogenic factors are induced, further study is warranted.

Radiation proctitis is a refractory disease without effective treatment methods. However, the inhibitors of major angiogenic factors observed in this study, such as angiogenin and FGF1, may be effective for the treatment of radiation proctitis. Moreover, they also may be effective for other forms of radiation injury such as radiation pneumonitis, dermatitis, pleuritis, pericarditis and myelitis.

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Clinical Benefit of High-Sensitivity KRAS Mutation Testing in Metastatic Colorectal Cancer Treated with Anti-EGFR Antibody Therapy

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Key Words
Colorectal cancer · Cetuximab · KRAS mutation testing

Abstract
Objective: We compared high-sensitivity KRAS mutation testing with direct sequencing for predicting the efficacy of antiepidermal growth factor receptor antibodies in patients with metastatic colorectal cancer (mCRC). ***Methods:*** We analyzed the KRAS status in 61 tumors from cetuximab-treated mCRC patients by both direct sequencing and a high-sensitivity method: 2-step PCR restriction fragmentation length polymorphism (RFLP). Therapeutic effects in each mutational status were evaluated. ***Results:*** The incidences of KRAS mutations determined by direct sequencing and 2-step PCR RFLP were 34.4 and 52.5%, respectively ($p = 0.02$). Patients were categorized into 3 groups [W/W, wild-type by both methods (n = 29); W/M, wild-type by direct sequencing, detected mutation by 2-step PCR RFLP (n = 11); M/M, mutant-type by both methods (n = 21)]. The response rate for cetuximab in the W/M group (0%) was the same as that in the M/M group, and was significantly lower than in the W/W group (41.4%) ($p < 0.001$). Progression-free survival in the W/M group (11.0 weeks) was similar to that in the M/M group (8.0 weeks), and was significantly shorter than in the W/W group (18.0 weeks) ($p < 0.002$). ***Conclusion:*** High-sensitivity KRAS mutation testing is useful for selecting true responders to cetuximab.

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Introduction

The recent development of antibody drugs that target molecules such as vascular endothelial growth factor (VEGF) or epidermal growth factor receptor (EGFR) has remarkably improved treatment outcomes of metastatic colorectal cancer (mCRC). They have been widely used as first- to third-line therapy for mCRC [1–3]. However, retrospective subset analyses and prospective randomized phase III trials have revealed that an anti-EGFR antibody agent was effective only for mCRC with wild-type KRAS but not for that with a KRAS mutation [3–7]. Inappropriate characterization of mCRC patients leads to a delay of the administration of appropriate therapy resulting in a poor outcome for patients and unnecessarily high medi-

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Table 1. Patient characteristics

Total number of patients		61
Median age (range)		66.5 (33–84)
Sex	Male	39
	Female	22
Performance status	0	23
	1	28
	2	10
Primary tumor	colon	23
	rectum	38
Differentiation	well	24
	moderately	32
	poorly	5
Metastatic site	liver only	15
	lung only	8
	peritoneum only	5
	≥2 sites	33
Number of previous chemotherapies	1	10
	2	34
	≥3	17
Chemotherapy regimen	cetuximab + FOLFIRI	5
	cetuximab + CPT11	24
	cetuximab alone	22
	others	10

cal expenses which affect the health care system. Ideally, cost-effective and evidence-based treatment should be developed in the field of chemotherapy. When we treat patients with mCRC, it is very important to accurately determine the presence or absence of a KRAS mutation, and anti-EGFR antibodies should not be administered in those cases with a KRAS mutation.

KRAS protein encoded by the KRAS gene is a small G protein and is located downstream of the EGFR-induced cell-signaling pathway [8]. Binding of a ligand, such as EGF or transforming growth factor-α (TGF-α), to EGFR, activates KRAS protein, and the cell proliferation signal is transmitted to the downstream RAF/MEK/ERK pathway. In colorectal cancer with a KRAS mutation, despite blockage of EGFR with an anti-EGFR antibody agent upstream of KRAS, cell proliferation signals from the mutated KRAS protein are constitutively transmitted downstream, resulting in treatment failure. Mutation in the KRAS gene occurs as a point mutation in codon 12 or 13 in 90% or more cases, and it is detected in 30–40% of cases of colorectal cancer [9]. Currently, direct sequencing is widely used for KRAS mutation analysis; however, several critical disadvantages of direct sequencing for diagnosis have been indicated. One is its low sensitivity; it cannot detect mutations accurately unless there are at least 20–50% cells with a KRAS mutation in the tissue sample. On the other hand, restriction fragment length polymorphism (RFLP) and the amplification refractory mutation system (ARMS) have been developed as high-sensitivity methods to detect KRAS mutations [10]. With these methods, the KRAS mutation is detected when cells with the mutation account for at least 0.1 and 1% of cells in the sample, respectively. In particular, 2-step PCR RFLP (mutant-enriched PCR RFLP) has made it possible to analyze the KRAS mutation at high sensitivity with a formalin-fixed paraffin-embedded block sample. Although differences in sensitivity and specificity between tests for the KRAS mutation have been noted, few studies have investigated the clinical impact of the accuracy of KRAS mutation testing on treatment with an anti-EGFR agent. In this study, we assessed the KRAS status by both 2-step PCR RFLP and direct sequencing in mCRC treated with cetuximab and evaluated the relationship between the mutation status and the efficacy of cetuximab.

Patients and Methods

Patient Characteristics

We retrospectively assessed 68 patients with mCRC who were treated by cetuximab monotherapy or by combination therapy using cetuximab plus a cytotoxic agent as second- or third-line treatments in three hospitals (Tokushima University Hospital, Sapporo Medical Center Tonan Hospital, and Kochi Health Sciences Center) from September 2008 to August 2009. Among these patients, 61 had measurable lesions detected by computed tomography (CT) scan. All of these patients had histologically proven colorectal adenocarcinoma. All tumor samples were obtained from the primary colorectal tumor by biopsy (n = 5) or by surgery (n = 56). The patients' clinical and pathologic characteristics are listed in table 1. Almost all patients (92%, 56/61) had been refractory to oxaliplatin and irinotecan administration. Tumor response was evaluated by CT scan according to the Response Evaluation Criteria in Solid Tumors (RECIST) [11]. Objective tumor response was classified as complete response, partial response, stable disease or progressive disease. Patients with complete response or partial response were defined as responders.

This retrospective study was conducted with the approval of the institutional review board.

DNA Extraction

Ten slices of a 10-μm tissue section were cut from each of the formalin-fixed paraffin-embedded tumor blocks. Serial sections were stained with hematoxylin and eosin to confirm the presence of carcinoma tissue. Genomic DNA was extracted using a QIAmp DNA FFPE tissue kit (Qiagen GmbH, Germany) and quantified by spectrophotometry.

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Table 2. Correlation between 2-step PCR RFLP and direct sequencing for the detection of KRAS mutations

2-Step PCR RFLP	Direct sequencing		
	wild-type	mutant-type	
Wild-type	29 (47.5%)	0	29 (47.5%)
Mutant-type	11 (18.0%)	21 (34.4%)	32 (52.5%)
Total	40 (65.6%)	21 (34.4%)	

Direct Sequencing of KRAS Gene

Reactions were set up in 50-μl volumes as follows: 1.25 unit of Taq polymerase (Takara, Japan), 0.5 μmol/l of each primer (forward primer 5′-ACTGAATATAAACTTGTGGTAGTTGG-AGCT-3′ and reverse primer 5′-TCAAAGAATGGTCCTGCA-CC-3′), 0.2 mmol/l of deoxyribonucleoside triphosphates, 10 ! PCR buffer and 2 μg genomic DNA. PCR conditions comprised an initial denaturation step at 94°C for 3 min, followed by 35 cycles at 95°C for 30 s, 54°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min. The PCR products were sequenced after purification using an ABI PRISMh 3100 Genetic Analyzer (Applied Biosystems-HITACHI, Japan).

2-Step PCR RFLP for KRAS Mutations

To detect KRAS codon 12 and 13 mutations, the PCR products were amplified using mismatch primers, which were designed to introduce an *Mva*I restriction site for codon 12 and a *Bgl*I restriction site for codon 13 of the wild allele, and analyzed by the RFLP method, as described by Kahn et al. [12]. In brief, the mismatched nucleotide primers used for the enzymatic amplification of KRAS sequences were as follows (nucleotide substitutions are underlined): 12&13F: 5′-ACTGAATATAAACTTGTGGTAGTTGG-ACCT-3′, wtR: 5′-TCAAAGAATGGTCCTGCACC-3′, 12mtR: 5′-TCAAAGAATGGTCCTGGACC-3′, and 13mtR: 5′-AACAAG-ATTTGCCTCTATGGCTGGATCA-3′. The 1st step in PCR amplification was performed on 2 μg of genomic DNA in a final reaction volume of 50 μl, which contained 0.2 mmol/l of deoxyribonucleoside triphosphate, 10 ! PCR buffer, 0.5 μmol/l of 12 and 13F primer and wtR primer, and 1.25 units of Taq polymerase. The PCR condition was as follows: 94°C for 3 min and 30 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 45 s and finally 5 min at 72°C. Aliquots (2 μl) of the first PCR product were then digested with 20 units of *Mva*I (Takara) and 10 units of *Bgl*I (Takara) in a final volume of 20 μl at 37°C for 2 h under conditions recommended by the supplier. Ten-microliter aliquots of these *Mva*I and *Bgl*I digests were used in a 2nd-step PCR for mutations in codons 12 and 13, respectively. These aliquots were diluted to a final volume of 50 μl as described above. The PCR for codon 12 was performed with the 12&13F and 12mtR primers, whereas the PCR for codon 13 was performed with the 12&13F and 13mtR primers. The 2nd-step PCR conditions were as described above. Aliquots (25 μl) of KRAS codon 12 and 13 products obtained after 2nd-step PCR were then digested with either *Mva*I (for codon 12) or *Bgl*I (for codon 13) at 37°C for more than 2 h and were then electrophoresed through a polyacrylamide gel. This RFLP analysis by *Mva*I digestion for KRAS codon 12 mutations generated 114-bp, 29-bp, and 14-bp fragments if there was no mutation and 143-bp and 14-bp fragments if there was a mutation. Similarly, the RFLP analysis by *Bgl*I digestion for codon 13 mutations generated 74-bp, 32-bp, and 14-bp fragments if there was no mutation and 106-bp and 14-bp fragments if there was a mutation.

Statistical Analysis

All data were analyzed by using the Statistical Package for the Social Sciences (SPSS) statistics 18 package software (SPSS Inc., Japan). The positive rates of KRAS mutations with the 2-step PCR RFLP and direct sequencing were compared by the χ^2 test. Progression free survival (PFS) was estimated by the Kaplan-Meier method. Comparison between each group was performed by the log-rank test. $p < 0.05$ was considered statistically significant.

Results

Prevalence of KRAS Mutations by Direct Sequencing and 2-Step PCR RFLP

A total of 61 specimens of colorectal cancer tissues were examined for KRAS codon 12 and 13 mutations by both direct sequencing and 2-step PCR RFLP, and positive rates by the 2 methods were compared. KRAS mutations were detected in 34.4% (21/61) of specimens by direct sequencing and in 52.5% (32/61) of specimens by 2-step PCR RFLP. The positive rate of KRAS mutations by 2-step PCR RFLP was significantly higher than that by direct sequencing (p = 0.02). Table 2 shows the correlation between KRAS wild-type and mutant-types by direct sequencing and 2-step PCR RFLP. All of the 21 mutant-type patients identified by direct sequencing were also determined to have the mutant-type by 2-step PCR RFLP. Of the 40 wild-type patients identified by direct sequencing, 11 (18.0%) were determined to be the mutant-type by 2-step PCR RFLP. Figure 1 shows results of the KRAS analysis in all 11 patients with discordant findings. In the analysis of direct sequencing, no abnormal signals were observed in the nucleotide sequence of codon 12 or 13 in any of those 11 patients (fig. 1a). In the analysis of 2-step PCR RFLP, however, 143-bp bands (which represent a mutation of codon 12) or 106-bp bands (fig. 1b) (which represent a mutation of codon 13) were observed in all 11 patients.

Response Rate according to KRAS Mutation Status

In order to examine the relationship between KRAS mutation status and the response to cetuximab, we investigated the response rate for each KRAS mutation status by the 2 different methods. The response rate in KRAS wild-type patients determined by direct sequencing was 30.0% (12/40), whereas that determined by 2-step PCR RFLP was 41.4% (12/29) (table 3), with the latter being higher. None of

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those patients with a KRAS mutation determined by either direct sequencing or 2-step PCR RFLP were responders.

We then categorized the patients into 3 groups as follows: W/W – wild-type by both methods, W/M – wild-type by direct sequencing but mutant-type by 2-step PCR-RFLP and M/M – mutant-type by both methods. When we compared baseline characteristics among the 3 groups, we found no statistically significant differences (suppl. table, www.karger.com/doi/10.1159/000336792). The response rate was compared among the 3 groups. The response rate in the W/M group was 0% (0/11), which was the same as in the M/M group (0/21); these rates were significantly lower than that in the W/W group (41.4%, 12/29) ($p < 0.001$). This indicates that patients with the mutant-type identified by 2-step PCR RFLP, irrespective of being positive or negative by direct sequencing, would have no response to cetuximab.

PFS in Patients with KRAS Wild- and Mutant-Types

The relationship between PFS and KRAS mutation status by the 2 methods was analyzed. Kaplan-Meyer curves for PFS in KRAS wild- and mutant-types by direct sequencing and 2-step PCR RFLP are shown in figure 2a, b. The PFS in the KRAS wild group determined by direct sequencing was significantly longer than in the KRAS mutant group with a p value of 0.01. The PFS in the KRAS wild group determined by 2-step PCR RFLP was even longer with greater significance in the KRAS mutant group with a p value of less than 0.001. The PFS in the KRAS wild group identified by 2-step PCR RFLP was longer than that by direct sequencing although there was no statistical significance. Figure 2c shows Kaplan-Meyer curves for the W/W, W/M and M/M groups. The median PFSs were 8.0 weeks (95% CI 6.9–9.1) for the M/M group, 11.0 weeks (95% CI 6.5–15.5) for the W/M group and 18.0 weeks (95% CI 13.5–22.5) for the W/W group. The PFS in the W/M group was almost the same as that in the M/M group, and was significantly shorter than that in the W/W group ($p = 0.002$). Thus, the detection of KRAS mutations by 2-step PCR RFLP reflected a poor prognosis regardless of the results by direct sequencing, and this method could be a significant predictor of appropriate candidates for cetuximab therapy.

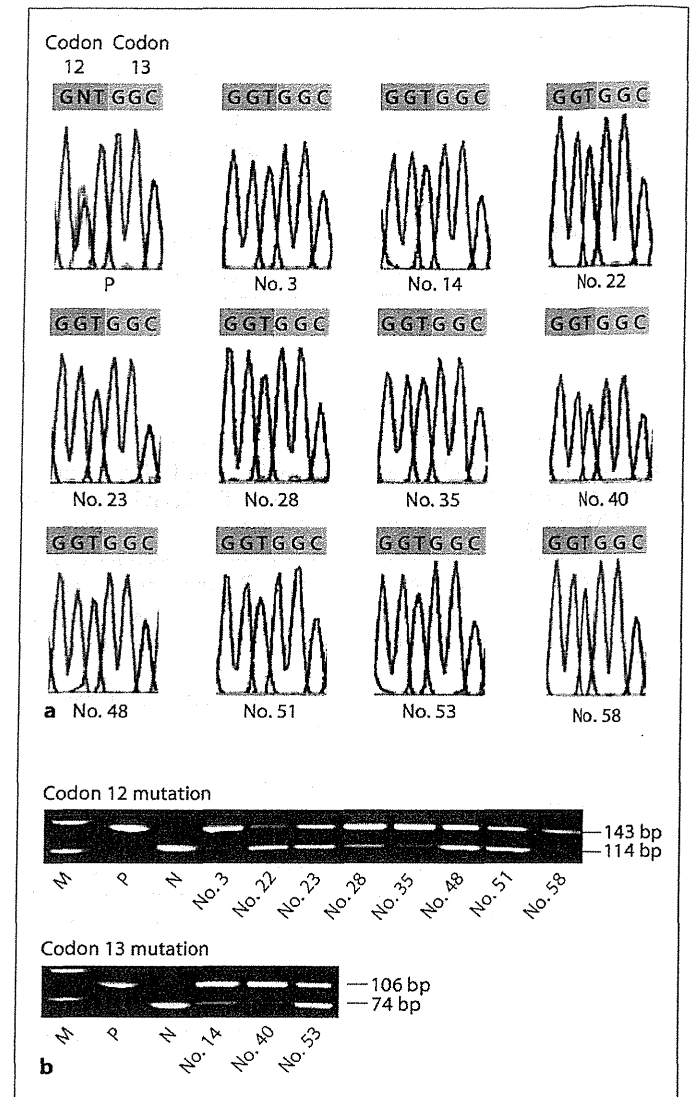

Fig. 1. Data on 11 patients with discordant KRAS mutation status between direct sequencing and 2-step PCR RFLP. **a** In the analysis by direct sequencing, no abnormal signals were found in the nucleotide sequence of codons 12 and 13 in any of the 11 patients. **b** In the analysis by 2-step PCR RFLP, however, 143-bp bands (which represent a mutation of codon 12) or 106-bp bands (which represent a mutation of codon 13) were observed in all 11 patients. ASPC-1, which is known to have a KRAS codon 12-point mutation, and HCT116, which has a KRAS codon 13 mutation, were used as positive controls. P = Positive control; M = marker; N = normal mucosa.

Discussion

In this study, the incidences of KRAS codon 12 or 13 mutations in mCRC by direct sequencing and 2-step PCR RFLP were 34.4 and 52.5%, respectively, with the latter being significantly higher than the former ($p = 0.02$). The response rate (41.4%) in patients with wild-type by 2-step PCR RFLP was higher than that by direct sequencing (30.0%), suggesting that 2-step PCR RFLP is useful for selecting true responders to cetuximab, excluding the pa-

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Table 3. Correlation between KRAS status and response to cetuximab

	Number	CR	PR	SD	PD	RR	p value
Direct sequencing							
KRAS wild	40	1	11	17	11	30% (12/40)	<0.001
KRAS mutant	21	2	0	10	11	0% (0/21)	
2-step RFLP							
KRAS wild	29	1	11	12	5	41.4% (12/29)	<0.001
KRAS mutant	32	0	0	15	17	0% (0/32)	
Direct sequencing/2-step PCR RFLP							
W/W	29	1	11	12	5	41.4% (12/29)	<0.001
W/M	11	0	0	5	6	0% (0/11)	
M/M	21	0	0	10	11	0% (0/21)	

CR = Complete response; PD = progressive disease; PR = partial response; RR = response rate; SD = stable disease.

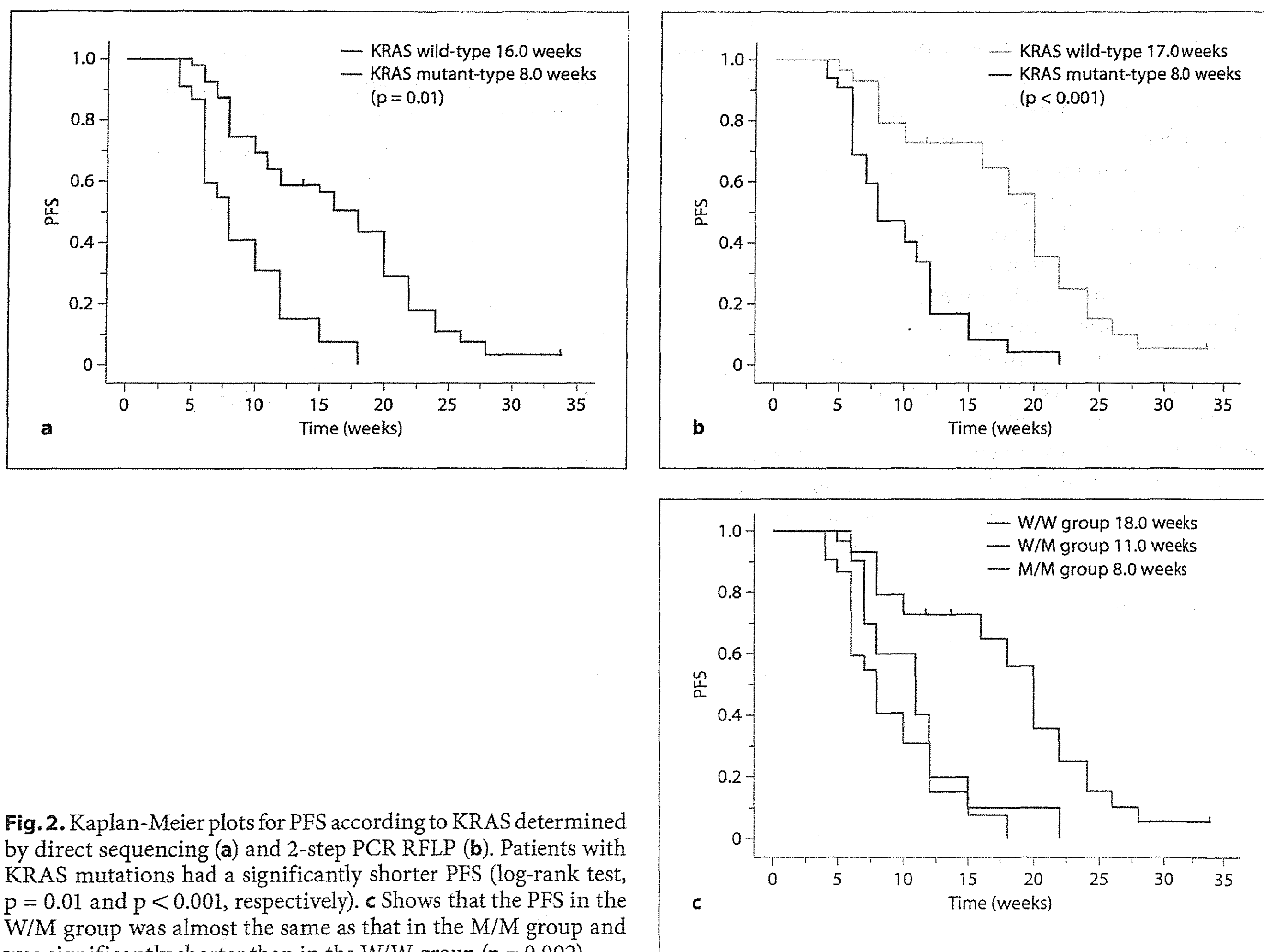

Fig. 2. Kaplan-Meier plots for PFS according to KRAS determined by direct sequencing (**a**) and 2-step PCR RFLP (**b**). Patients with KRAS mutations had a significantly shorter PFS (log-rank test, p = 0.01 and p < 0.001, respectively). **c** Shows that the PFS in the W/M group was almost the same as that in the M/M group and was significantly shorter than in the W/W group (p = 0.002).

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tients with a false negative. Moreover, the response rate and PFS in the 11 cases in the W/M group were almost the same as those in the M/M group and were significantly lower and shorter, respectively, than those in the W/W group. These results indicated that the KRAS mutation status determined by 2-step PCR RFLP is more precisely predictive of the response to cetuximab than that by direct sequencing.

According to The Catalogue of Somatic Mutations in Cancer (COSMIC), a public mutation database [13], the incidence of a KRAS mutation in codons 12 and 13 in colorectal cancer was 31.3% (5,111/16,345) based on the 368 papers reviewed between 1992 and 2008. The mutation rate by direct sequencing in the current study was compatible with that in the COSMIC database. This is reasonable because the direct sequencing method has been employed as a gold standard in most previous studies on the KRAS mutation. However, it does not provide satisfactory sensitivity, as DNA with the KRAS mutation must be present in 20–50% of the sample. On the other hand, many sensitive methods have been reported previously. Bando et al. [14] reported that KRAS mutations detected by ARMS assay improved prediction of cetuximab efficacy. Two-step PCR RFLP, employed in this study, has the highest sensitivity and requires only 0.1% of mutated DNA at codon 12 or 13 [10, 12]. This high sensitivity is achieved by selective PCR amplification of mutant KRAS gene sequences employing a 2-step PCR in combination with restriction enzyme digestion.

The significant advantage of a high-sensitivity method might be explained by the following potential scenarios. First, a higher sensitivity method can detect fewer numbers of KRAS mutations in a sample that is contaminated with other noncancerous cells. In general, formalin-fixed paraffin-embedded specimens include normal colorectal cells, inflammatory cells such as lymphocytes, neutrophils and macrophages and other noncancerous cells such as fibroblasts. When a tissue sample is contaminated with more than a certain amount of these noncancerous cells, failure to detect the KRAS mutation in DNA from cancer cells is possible. Second, intratumoral heterogeneity of the KRAS mutation in colorectal cancer tissue is plausible. To date, several studies have reported heterogeneity of the KRAS mutation in colorectal cancer tissue [15–17]. Losi et al. [16] reported that heterogeneity of the KRAS mutation was observed within the tumor tissue in about 20% of advanced colorectal cancer cases. Therefore, unless a high-sensitivity method is employed, a KRAS mutation that only partly exists in colorectal cancer tissue may not be detectable.

Although involvement of BRAF mutations, PI3KCA mutations and PTEN loss of expression have been suggested in resistance to cetuximab [18–20], KRAS is the only target gene that is clinically used to date. In our study, it was possible to select cetuximab responders efficiently by employing a high-sensitivity method to analyze the KRAS mutation. Although macro- or micro-dissection and preparation of fresh-frozen tissue samples have been used to try to improve the sensitivity of direct sequencing, these procedures are hard to introduce to clinical practice due to high cost and inconvenience. In this respect, a high-sensitivity method using paraffin-embedded tissues provides a simple, accurate and clinically useful way for analysis of the KRAS mutation. The 2-step PCR RFLP method may take somewhat longer in comparison with the conventional direct sequencing method. However, the examination times needed are at most 3–5 days, which seems to be an acceptable range. Moreover, the method does not require any special and expensive equipment, and therefore is very economical. In view of these points, it appears that the 2-step PCR RFLP method can be easily applied to clinical use.

Analyses of the cell proliferation signaling for the KRAS wild and mutant samples could provide additional valuable information on how the mutant KRAS escapes cetuximab the therapy. Further investigations using pre- and post-therapeutic samples are warranted in the future.

In conclusion, 2-step PCR RFLP is very useful for selecting true responders to cetuximab, in comparison with direct sequencing. This highly sensitive KRAS mutation detection system should be applied in the clinical setting for efficient chemotherapy.

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Suppressive effect of RAS inhibitor manumycin A on aberrant crypt foci formation in the azoxymethane-induced rat colorectal carcinogenesis model

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Key words
aberrant crypt foci, colorectal cancer, manumycin A cancer prevention, RAS inhibitor.

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Abstract

Background and Aim: The chemopreventive effect of RAS inhibitors on colorectal cancer is unknown. Because aberrant crypt foci (ACF), earliest preneoplastic lesions, are highly positive for K-RAS mutation, RAS inhibitors are likely to be effective for chemoprevention. Therefore, in the present study, the suppressive effect of a RAS inhibitor, manumycin A, on ACF formation in an azoxymethane (AOM)-induced rat colorectal carcinogenesis model was investigated.
Methods: Rats injected with AOM were administered manumycin A (30 mg/kg) subcutaneously thrice weekly for 8 weeks or for 4 weeks (latter half), sacrificed at 8 weeks, and examined for ACF in the colorectum. Phosphorylated ERK and Ki-67 expression was evaluated by immunohistochemistry. Apoptosis was assessed by TUNEL staining.
Results: The mean number of ACF in the 8-week manumycin A group (72.9 ± 20.1) was significantly lower than in the vehicle group (155.6 ± 56.7, $P < 0.01$), and it was significantly lower even in the 4-week manumycin A group than in the vehicle group (92.2 ± 13.0 *vs* 222.3 ± 83.3, $P < 0.01$). The positive rate for phosphorylated ERK in the manumycin A group (13.5 ± 19.2%) was significantly lower than in the vehicle group (50.2 ± 19.8%, $P < 0.01$). The positive rate for Ki-67 in the manumycin A group (2.2 ± 3.4%) was significantly lower than in the vehicle group (14.7 ± 8.2%, $P < 0.01$). There were significantly more terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling-positive cells in tissue samples from the manumycin A group *versus* the vehicle group (8.6 ± 9.7% *vs* 2.9 ± 2.0%, $P < 0.05$).
Conclusion: Manumycin A suppressed ACF formation in the AOM-induced colorectal carcinogenesis model, demonstrating that RAS inhibitors may be very effective for chemoprevention of colorectal cancers.

Introduction

Colorectal cancer is the third most common cancer in men and the second most common form in women worldwide.[1] Chemoprevention is an important and effective strategy for reducing the incidence of colorectal cancer. Because it is well accepted that colorectal cancer develops through an adenoma-carcinoma sequence, chemoprevention trials targeting adenoma (polyps) have been performed using several candidate agents. It has been reported that aspirin or non-steroidal anti-inflammatory drugs such as sulindac inhibit the development of adenoma by 20–35%.[2–4] It has also been reported that cyclooxygenase-2 inhibitors inhibit the development of adenoma by 30–35%.[5–7] However, these drugs are associated with gastrointestinal and cardiovascular toxicity and it is therefore not possible to use them continuously for the prevention of colorectal cancer.[7]

It has been reported that K-RAS mutation plays an important role in the adenoma-carcinoma sequence of colorectal carcinogenesis. The positive rates of K-RAS mutations are reported to be 9–58% in adenoma and 47% in cancer.[8–11] Because K-RAS mutations are positive at codons other than the codon 12 and the direct sequencing method routinely employed lacks sufficient sensitivity, it has been suggested that these K-RAS mutation rates underestimate the true incidence of K-RAS mutation.

We have observed aberrant crypt foci (ACF), a minute type of colorectal lesion, using magnifying colonoscopy in normal subjects and patients with adenoma and cancer, suggesting that ACF are precursor lesions of the adenoma-carcinoma sequence. We

and other investigators found that ACF are frequently positive for K-RAS mutation.[10,13] Furthermore, we found that K-RAS mutation induces overexpression of glutathione S-transferase-π (GST-π) via activator protein 1 activation and that GST-π plays a pivotal role in the resistance of apoptosis to bile acids in the colorectum.[14] Therefore, it appears that K-RAS, which exhibits mutations in ACF, the earliest lesion associated with colorectal carcinogenesis, is the most suitable target molecule for chemoprevention.

Manumycin A is a natural product derived from *Streptomyces parvulus* that acts as a potent and selective RAS farnesyltransferase inhibitor.[15] The enzyme farnesyltransferase modifies RAS and other proteins with the farnesyl isoprenoid lipid that is required for their correct cellular localization and biological activity.[16] Recently, the antineoplastic activity of manumycin A has been demonstrated in various experimental systems.[17–20] It has been reported that manumycin A inhibits cell growth of tumor cells by suppression of RAS farnesylation.[21] It has also been reported to induce apoptosis in various cancer cell lines including human colon tumor cells.[22,23] Furthermore, manumycin A induces the death of breast cancer cells via cytoplasmic vacuolation.[24] However, the chemopreventive effect of manumycin A on colorectal cancer has not been studied to date, nor has the chemopreventive effect of RAS inhibitors, such as FTI276 and L744,832, on colorectal cancer been investigated. Therefore, in the present study, we investigated the inhibitory effect of the RAS inhibitor manumycin A in a rat model of azoxymethane (AOM)-induced colorectal carcinogenesis, which mimics human colorectal carcinogenesis with high K-RAS mutation in ACF, as well as adenoma and cancer.

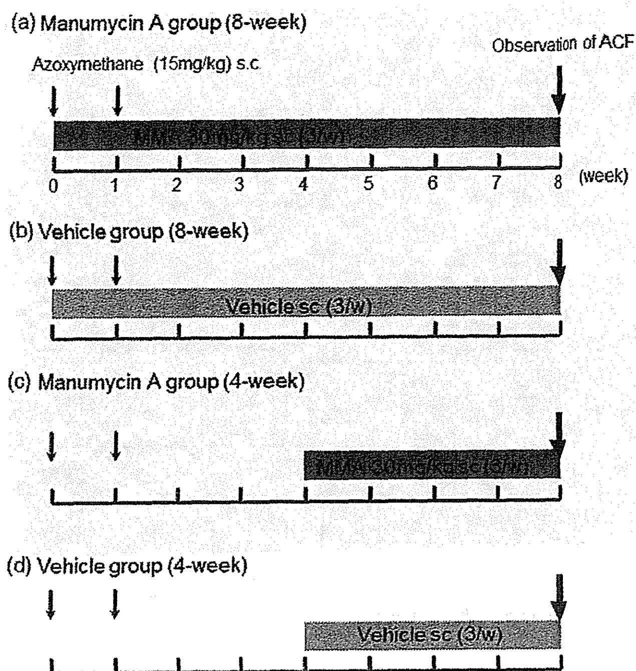

Figure 1 Experimental protocol. Five-week old rats were administered azoxymethane at a dose of 15 mg/kg (once a week for 2 weeks). (a) Manumycin A (MMA) was administered to rats at a dose of 30 mg/kg (three times per week) for 8 weeks. (b) Only vehicle was administered to rats for 8 weeks. (c) Manumycin A was administered to rats at a dose of 30 mg/kg (three times per week) only during the latter 4 weeks of the study (weeks 5–8). (d) Only vehicle was administered to rats for the latter 4 weeks of the study (weeks 5–8). ACF, aberrant crypt foci.

Materials and methods

Animals and treatment protocol. All animal experiments were approved by the University of Tokushima Graduate School. Male F344 rats (age 5 weeks) were purchased from Charles River Japan, Inc. (Tokyo, Japan) and maintained in the animal housing facility at the University of Tokushima Graduate School. Manumycin A was obtained from Enzo Life Sciences (Tokyo, Japan). AOM was purchased from Sigma-Aldrich Co. (St Louis, MO). Animals were maintained on a 12-h light/dark cycle with free access to water and food. The experimental protocol for the present study is shown in Figure 1. AOM was administered to 28 rats subcutaneously at a dose of 15 mg/kg once a week for 2 weeks, and the animals were assigned to four groups (Fig. 1). Manumycin A was dissolved in dimethyl sulfoxide (DMSO), further diluted in phosphate buffered saline (PBS), and administered subcutaneously to rats at dose of 30 mg/kg thrice weekly for 8 weeks or for the latter 4 weeks of the study, according to the method of Jamroz-Wiśniewska *et al.*[25] Control rats received vehicle only for either 8 weeks or for the latter 4 weeks of the study.

ACF examination. Entire colorectums were carefully removed, immersed in 10% neutral buffered formalin, opened longitudinally from the cecum to the anus, and placed between filter paper. Subsequently, the tissues were fixed in 10% neutral buffered formalin for 24 h. They were then stained with 0.2% methylene blue in saline and placed, mucosal side up, on microscope slides; ACF was then examined under a stereoscopic microscope. Lastly, the colorectal mucosa including ACF was excised and paraffin-embedded to prepare cross sections (4 μm thickness) for hematoxylin and eosin staining and for immunohistochemistry.

Immunohistochemistry. Immunohistochemical staining was performed using the streptavidin-biotin peroxidase method with labeled streptavidin-biotin (Dako, Tokyo, Japan), as we described previously.[26] Briefly, formalin-fixed paraffin-embedded sections were cut 3 μm thick, deparaffinized in xylene, and then rehydrated in graded ethanol solutions and PBS. The slides were autoclaved for 15 min at 121°C in target retrieval solution (pH 9.0) (Dako). They were then blocked with protein block (Dako) and incubated with rabbit antihuman Ki-67 polyclonal antibody or mouse antihuman phosphorylated ERK (p-ERK) monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) as the primary antibody. After washing with PBS, the slides were incubated with biotinylated secondary antibody followed by incubation with horseradish-streptavidin and visualization with 3,3-diaminobenzidine tetrahydrochloride (DAB, Dako). Finally, the sections were counterstained with Mayer's hematoxylin.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling staining. An Apoptosis in situ Detection Kit (Wako, Osaka, Japan) was used to label apoptosis-induced DNA strand breaks by

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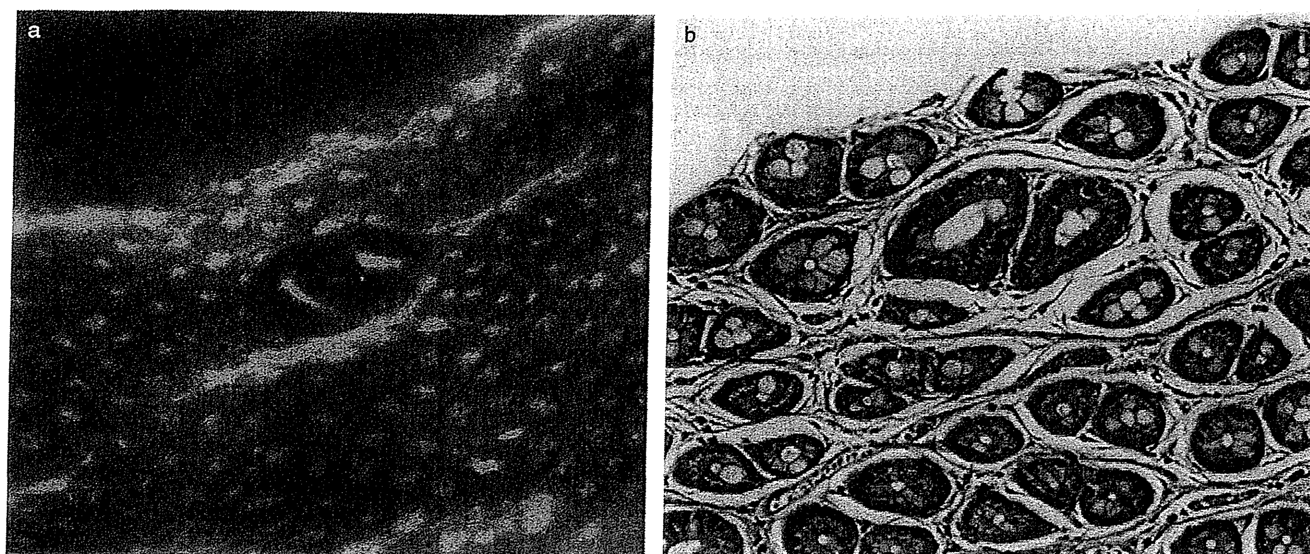

Figure 2 Aberrant crypt foci (ACF) induced by azoxymethane. (a) ACF observed under a light microscope with methylene blue staining, consisting of two large crypts; (b) histological findings of the corresponding ACF.

the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) technique. In brief, formalin-fixed paraffin-embedded sections of rat colorectal mucosa were placed on silanized slides (Dako). After deparaffinization, they were immersed in a protein-digestion enzyme solution at room temperature for 5 min and incubated for 10 min at 37°C in a terminal deoxynucleotidyl transferase substrate solution. Subsequently, the sections were immersed in 3% hydrogen peroxide to block endogenous peroxidase activity and then incubated with peroxidase-conjugated antibody at 37°C for 10 min. The DAB was added, and counterstaining was performed with methyl green.

Statistics. The number of ACF was compared between the manumycin A and vehicle groups by Student's *t*-test. Positivity rates for p-ERK and Ki-67 immunostaining and TUNEL staining were also compared between the manumycin A and vehicle groups by Student's *t*-test.

Results

General observation. Rats administered manumycin A appeared to be healthy throughout the experiment, and no apparent signs of toxicity were observed. There were no appreciable changes in bodyweight gain or diet intake between the manumycin A and vehicle groups (data not shown). There were no significant differences in the weights of liver, kidney, lung, or spleen between the two groups.

Manumycin A reduced the number of ACF in AOM-injected rats. In the first setting, manumycin A was administered for 8 weeks from the first day of AOM administration, and the rats were sacrificed at 8 weeks for observation of ACF (Fig. 1a,b). ACF in methylene blue-stained colorectum were identified and counted under a stereoscopic microscope. Figure 2a shows a typical ACF consisting of two large crypts densely stained with methylene blue. Figure 2b shows the histological findings of the corresponding ACF, which consisted of crypts twofold to threefold larger than the surrounding normal crypts. Nuclear disorientation, irregular arrangement, and chromatin condensation were observed in the epithelial cells in the aberrant crypts.

The mean number of ACF in the manumycin A group was 72.9 ± 20.1, which was significantly smaller than in the vehicle group (155.6 ± 56.7) ($P = 0.003$). When the number of ACF was assessed according to the size of the ACF (1, 2, 3, 4 crypts or 5 crypts≤), a significant difference was observed between the manumycin A and vehicle groups for all sizes of ACF. However, the inhibitory effect was more pronounced for larger ACF (Table 1). In the second setting, manumycin A was administered for 4 weeks, from week 5 to week 8, and the suppressive effect on ACF formation was evaluated. The mean number of ACF in the manumycin A group was 92.2 ± 13.0, which was significantly lower than in the vehicle group (222.3 ± 83.3) ($P = 0.004$). When the number of ACF was assessed in terms of the size of ACF, the suppressive effect on ACF formation was more evident for larger sized ACF.

Manumycin A inhibited ERK phosphorylation and Ki-67 positivity in ACF. Manumycin A, a RAS farnesyltransferase inhibitor, is thought to act by inhibiting signal transduction via the RAS/RAF/MEK/ERK pathway and to suppress cell proliferation. To clarify the mechanism of action, AOM-injected rats received manumycin A at dose of 30 mg/kg fourtimes beginning at 5 weeks. They were then sacrificed at 6 weeks, and the ACF tissues were immunohistochemically examined for p-ERK and Ki-67 expression. Figure 3 shows representative results of immunostaining for p-ERK. The apparent expression of p-ERK in the cytoplasm and nucleus of ACF cells was detected in

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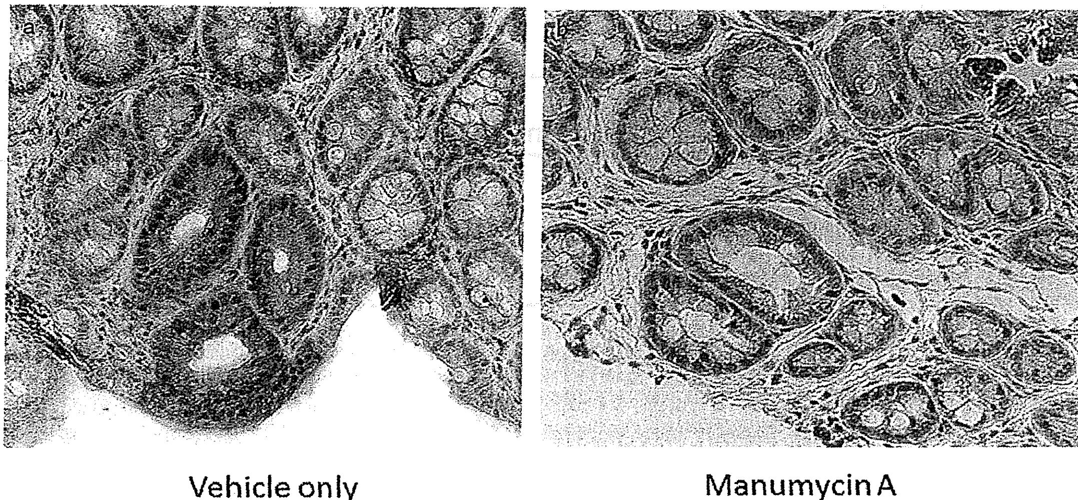
Table 1 Effect of manumycin A on number of aberrant crypt foci (ACF) in rats induced by azoxymethane

	Total No. of ACF	No. of ACF				
		1 crypt	2 crypts	3 crypts	4 crypts	5 crypts ≤
8-week treatment						
Vehicle	155.6 ± 56.7	35.4 ± 16.2	51.8 ± 23.4	36.1 ± 16.9	19.8 ± 12.8	12.6 ± 7.1
Manumycin A	72.9 ± 20.1	35.4 ± 16.2	25.1 ± 11.2	15.0 ± 9.8	7.6 ± 2.7	4.1 ± 2.0
P value	0.003	0.018	0.015	0.015	0.006	0.006
4-week treatment						
Vehicle	222.3 ± 83.3	44.0 ± 19.8	82.7 ± 38.0	40.8 ± 29.8	23.8 ± 11.1	31.0 ± 23.3
Manumycin A	92.2 ± 13.0	25.2 ± 7.6	39.0 ± 4.9	20.0 ± 4.2	7.0 ± 3.4	1.0 ± 1.1
P value	0.004	0.045	0.016	0.010	0.006	0.003



	No. of rats	No. of ACF examined	Positive rate for p-ERK (/100 cell)	
Vehicle	3	30	50.2 ± 19.8	$P < 0.01$
Manumycin A	3	30	13.5 ± 19.2	

Figure 3 Representative immunohistochemical staining for p-ERK in aberrant crypt foci (ACF) from azoxymethane-injected rats. ACF from rats in the vehicle group (a) and in the manumycin A group (b). Strong staining for p-ERK was observed in ACF cells from rats in the vehicle group. However, no staining was observed in ACF cells from rats in the manumycin A group. The positive rate for p-ERK in the manumycin A group was significantly lower than in vehicle group.

the vehicle group. In contrast, no p-ERK expression was observed in the manumycin A group. A total of 30 ACF tissues were evaluated in the manumycin A and vehicle groups (30 per group). The mean p-ERK positive rate in the manumycin A group was 13.5 ± 19.2%, which was significantly lower than in the vehicle group (50.2 ± 19.8%, $P < 0.01$).

In order to examine cell proliferative activity in ACF, immunostaining for Ki-67 was performed using the same specimens. Figure 4 shows representative results of immunostaining for Ki-67 in the manumycin A and vehicle groups. Strong Ki-67 expression in the nucleus of ACF cells was observed in the vehicle group. However, very weak expression of Ki-67 in ACF cells was observed in the manumycin A group. A total of 30 ACF tissues were evaluated in the manumycin A and vehicle groups (30 per group). The positive rate of Ki-67 staining was 2.2 ± 3.4% in the manumycin A group, which was significantly lower than in the vehicle group (14.7 ± 8.2%, $P < 0.01$). These results strongly suggested that manumycin A inhibited signal transduction via the RAS/RAF/MEK/ERK pathway, thereby leading to suppression of cell proliferative activity.

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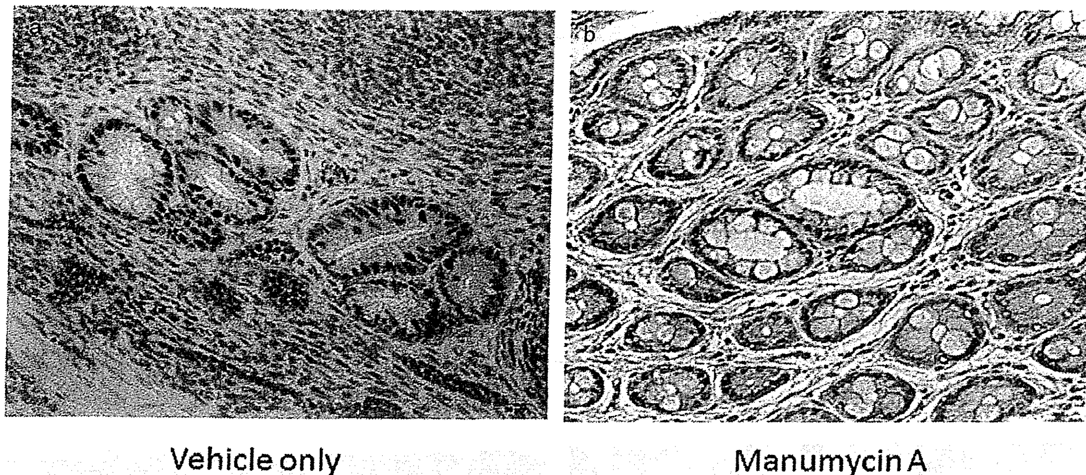


	No. of rats	No. of ACF examined	Positive rate for Ki67 (/100 cell)	
Vehicle	3	30	14.7 ± 8.2	$P < 0.01$
Manumycin A	3	30	2.2 ± 3.4	

Figure 4 Representative immunohistochemical staining for Ki-67 in aberrant crypt foci (ACF) from azoxymethane-injected rats. ACF from rats in the vehicle group (a) and the manumycin A group (b). Strong staining for Ki-67 was evident in ACF cells from rats in the vehicle group; however, staining was very weak in ACF cells from rats in the manumycin A group. The positive rate for Ki-67 in the manumycin A group was significantly lower than in the vehicle group

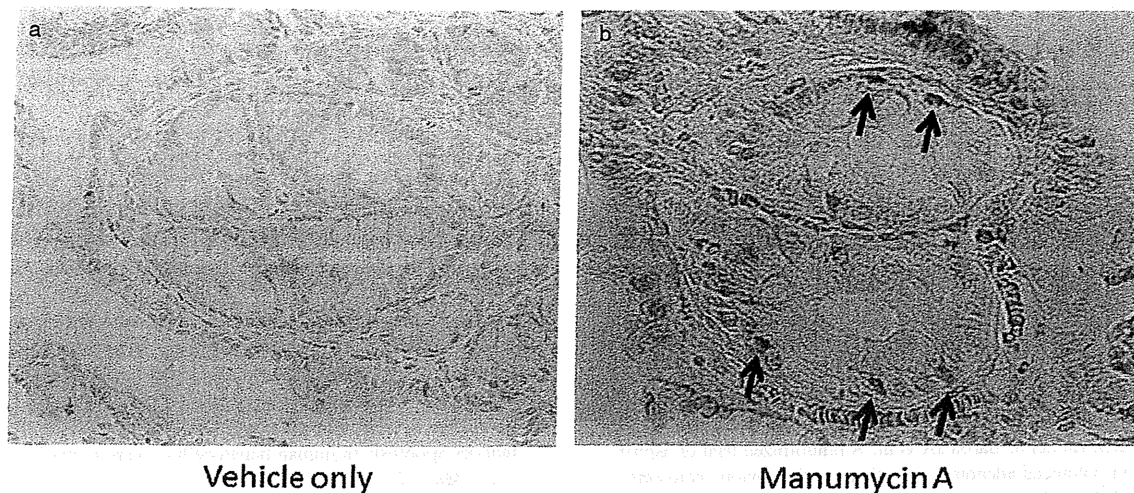
Manumycin A enhances TUNEL positivity in ACF. Because it has been reported that manumycin A induces apoptosis in some cancer cell lines *in vitro*, apoptotic cells in ACF from colorectal tissues obtained, as described earlier, were examined by TUNEL staining. Representative TUNEL staining results are shown in Figure 5. Numerous TUNEL-positive cells with brown nuclei were detected in the manumycin A group, whereas fewer TUNEL-positive cells was observed in the vehicle group. A total of 30 ACF tissues were evaluated in the manumycin A and vehicle groups (30 per group). The mean number of TUNEL-positive cells in the manumycin A group was 8.6 ± 9.7%, which was significantly greater than in the vehicle group (2.9 ± 2.0%). These results indicate that manumycin A not only inhibited cell proliferation signal but also induced apoptosis in ACF tissues.

Discussion

In this study, the RAS inhibitor manumycin A clearly suppressed ACF formation in the AOM-induced colorectal carcinogenesis model, strongly suggesting that the drug has a chemopreventive effect on colorectal cancer. There has been only one previously published investigation of the chemopreventive effect of RAS inhibitors on cancers, that is, a farnesyltranferase inhibitor, FTI276, which lowered the occurrence of lung cancer in a carcinogen-induced mouse model,[27] but there have been no published reports on the preventive effect of RAS inhibitors on other types of cancer, including colorectal cancer. Our data also suggest that RAS inhibitors would potentially be effective as chemopreventive agents not only for colorectal cancer but also for other cancers with RAS mutation including pancreatic cancer, hematological malignancies, and biliary duct cancer.

ACF formation was sufficiently suppressed by not only 8-week administration of manumycin A but also when 4-week administration in the latter half of the study was performed. Because ACF are reported to develop at about 2–4 weeks after AOM administration,[28] our results suggest that manumycin A could potentially eradicate pre-existing ACF. Patients with colorectal adenoma resected endoscopically are considered to be a high-risk group for colorectal cancer and are plausible candidates for cancer chemoprevention. We and other researchers have so far found that a majority of patients with adenoma have numerous ACF.[12,26,30] Moreover, we recently found that short-term administration of sulindac eradicated ACF in polypectomized patients; the occurrence of polyps (adenoma) 1 year later was also significantly suppressed.[31] Therefore, if RAS inhibitors can eradicate pre-existing ACF, it is expected that they could also reduce the occurrence of colorectal adenoma in humans. In this context, it was important to show that manumycin A (a RAS inhibitor) could eradicate pre-existing ACF in this study.

Aside from K-RAS mutation, activation of the PI3kinase/AKT pathway and β-catenin pathway in ACF has been reported in studies using the AOM rodent model.[32–34] Therefore, it is plausible that the ACF that developed in the manumycin A group were negative for K-RAS mutation and associated with activation of



	No. of rats	No. of ACF examined	TUNEL positive rate (/100 cell)	
Vehicle	3	30	2.9 ± 2.0	$P < 0.05$
Manumycin A	3	30	8.6 ± 9.8	

Figure 5 Representative terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining in aberrant crypt foci (ACF) from azoxymethane-injected rats. ACF from rats in the vehicle group (a) and in the manumycin A group (b). Several TUNEL-positive cells with brown nuclei were observed in ACF cells from rats in the vehicle group. However, no TUNEL-positive cells were detected in ACF cells from rats in the manumycin A group. The TUNEL positive rate in the manumycin A group was significantly higher than in the vehicle group.

PI3kinase or β-catenin pathways. Moreover, it has been reported that approximately 30% of ACF show histologically dysplastic features (i.e. high-grade dysplasia) in the AOM rat model.[35] Because dysplastic ACF consist mostly of large-sized ACF, there is possibility that dysplastic ACF were predominantly suppressed by manumycin A, although we did not analyze the precise number of dysplastic ACF in this study. In addition, there was some discrepancy in the number of ACF between the 8-week and 4-week vehicle groups (Table 1), although the difference between the two groups was not statistically significant. It may be explained by systematic (theoretical) experimental errors. Otherwise, it is possible that the vehicle including DMSO might have affected the metabolism of AOM concomitantly administered in the 8-week vehicle group.

As for the underlying mechanism of ACF suppression by manumycin A, the significant decrease of p-ERK and Ki-67 positivity in ACF cells that was observed in manumycin A group suggests that suppression of cell proliferation occurred through inhibition of the RAS/RAF/MEK/ERK pathway. The involvement of this mechanism was supported by the finding that larger sized ACF were more efficiently suppressed. In addition, manumycin A led to a significant increase in the number of TUNEL-positive cells, indicating that it induces apoptosis. This is consistent with our experimental results indicating that pre-existing ACF could be eradicated by administration of manumycin A for 4 weeks during the latter half of the study.

Regarding the mechanism of apoptosis by manumycin A, Pan and associates reported that manumycin A induced the production of reactive oxygen species (ROS), release of cytochrome c, and activation of caspases leading to apoptosis in anaplastic thyroid cancer cells.[36] Similarly, Sears and colleagues reported that manumycin A induced apoptosis via ROS production and subsequent activation of caspases by disruption of MEK and AKT activation in myeloma cell lines.[37] Therefore, it is plausible that in the present study manumycin A induced apoptosis in ACF tissues by stimulating ROS production and subsequent caspase activation.

No clinical study of manumycin A has been reported to date, although it was shown to have insignificant toxicity.[38] RAS inhibitors such as manumycin A may prove to be useful for chemoprevention in future. A clinical trial evaluating a newly developed RAS farnesyltransferase inhibitor, salirasib, for the treatment of pancreatic and lung cancer is currently underway. However, the therapeutic response to date has not been sufficient.[39] Since ACF are minute (< 1 mm in diameter) precancerous lesions and have a very simple genetic mutation (K-RAS mutation but not adenomatous polyposis coli or p53 mutation), it is expected that RAS inhibition will be very effective for eradication. Thus, RAS inhibitors appear most promising as chemopreventive agents rather than cancer treatment agents. However, vemurafenib and dabrafeinib, inhibitors of RAF downstream from RAS, are now in clinical use for cancer therapy, and a clinical trial on the MEK inhibitor selumetinib as a cancer therapeutic agent is currently underway.[40]

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Because all of these inhibitors eventually suppress p-ERK expression and subsequently cell proliferation, as does manumycin A, these drugs could also be potentially effective for the chemoprevention of colorectal cancer.

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ORIGINAL PAPER

Polymorphisms in DNA repair genes *XRCC1*, *XRCC3* and *XPD*, and colorectal cancer risk: a case–control study in an Indian population

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Abstract
Purpose Genetic polymorphisms in DNA repair genes may influence variations in individual DNA repair capacity, which could be associated with the development of cancer. We detected the distributions of three single-nucleotide polymorphisms (*XRCC1* Arg399Gln, *XRCC3* Thr241Met and *XPD* Lys751Gln) in DNA repair genes, and assessed the associations of these genetic polymorphisms with colon and rectal cancer susceptibility as well as evaluated the interactions of gene–gene and gene–environment in a case–control study of an Indian population.
Methods This case–control study was conducted with 302 cases (including 59 colon and 243 rectal cancer patients) and 291 cancer-free healthy controls. Genotypes were determined by PCR–RLFP assays. The effects [odds ratios (ORs) and 95% confidence intervals (95% CIs)] of genetic polymorphisms on colorectal cancer were estimated using unconditional logistic regression.
Results The *XRCC1* 399Gln allele was found to be associated with a significantly increased rectal cancer risk among men (OR = 1.65, 95% CI 1.04–2.64). Whereas the *XRCC3* 241Met allele showed a protective tendency against rectal cancer (OR = 0.68, 95% CI 0.46–1.02) for both men and women. Furthermore, a combination of the *XRCC1* 399Gln allele with *XRCC3* Thr/Thr genotype and the *XPD* 751Gln allele demonstrated the highest rectal cancer risk (OR = 3.52, 95% CI 1.43–9.44).
Conclusions The combined effects of putative risk alleles/genotypes for different DNA repair pathways may strengthen the susceptibility to rectal cancer.

Keywords Colorectal cancer · Susceptibility · Single nucleotide polymorphism · DNA repair genes

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Introduction

Colorectal cancer is a complex disease resulting from both environmental and genetic factors. Although the development of colorectal cancer has mainly been attributed to environmental factors, such as diet, lifestyle and environmental pollution (Doll and Peto 1981; Thomas 1993), inter-individual differences in susceptibility to colorectal cancer

may be due to genetic alterations, including those involved in DNA repair (Potter 1999; de Jong et al. 2002). Four major DNA repair pathways have been identified in mammalian cells, i.e., base excision repair (BER), nucleotide excision repair (NER), double-strand break repair and mismatch repair (Christmann et al. 2003). Humans are routinely exposed to mutagenic and carcinogenic chemicals originating from cigarette smoking, well-cooked food, combustion of fossil fuels and other sources (Vineis 1994), all of which can form DNA adducts and lead to DNA damage (Vineis et al. 1996). Most damaged DNA can be removed and recovered by DNA repair enzymes (Lunn et al. 1999; Matullo et al. 2001a; Hou et al. 2002). Polymorphisms in DNA repair genes that lead to amino acid substitution may influence the individual capacity to repair DNA damage, and insufficient DNA repair capacity (DRC) may result in genetic instability and carcinogenesis (Miller et al. 2001; de Boer 2002).

Among known genetic polymorphisms in DNA repair genes, X-ray repair cross-complementing groups 1, 3 (*XRCC1* and *XRCC3*) and the xeroderma pigmentosum group D (*XPD*, also known as *ERCC2*) have been frequently investigated as cancer susceptibility genes (Goode et al. 2002). The DNA repair gene *XRCC1* codes for a scaffolding protein physically associated with DNA polymerase beta, DNA ligase III, human AP endonuclease, polynucleotide kinase, and poly(ADP-ribose) polymerase (Caldecott et al. 1994; Gryk et al. 2002; Whitehouse et al. 2001; Vidal et al. 2001), which functions in a complex to facilitate BER and single-strand break-repair processes. The BER pathway mainly removes non-bulky base adducts produced by methylation, oxidation or reduction by ionizing radiation or oxidative damage (Beckman and Ames 1997; Ladiges et al. 2003). Three polymorphisms occurring at conserved sequences in *XRCC1* gene have been reported, and amino acid substitutions were detected at codons 194 (Arg-Trp), 280 (Arg-His) and 399 (Arg-Gln) (Shen et al. 1998). The 399Gln allele that was identified as associated with reduced DRC, was found to be significantly associated with the increase in both aflatoxin B1-DNA adducts and glycophorin A variants (Lunn et al. 1999).

The XRCC3 protein, involved in the homologous recombinational repair (HRR) of DNA double-strand break repair and cross-links, is a member of an emerging family of Rad-51-related proteins that likely participate in HRR to maintain genomic stability and repair DNA damage (Brenneman et al. 2000). XRCC3 has been shown to interact directly with HsRad51 (Pierce et al. 1999), and *XRCC3*-deficient cells were found to be unable to form Rad51 foci after radiation damage as well as demonstrating genetic instability and increased sensitivity to DNA-damaging agents (Griffin 2002). The *XRCC3* gene has a sequence variation in exon 7 (C–T), resulting in an amino acid substitution at codon 241 (Thr-Met) that may affect the enzyme's function (Matullo et al. 2001b).

The *XPD* gene that encodes a helicase, a subunit of transcription factor IIH (TFIIH), is responsible for opening DNA around the damaged site, a crucial step in initiating the NER process (Egly 2001), which repairs bulky adducts and UV-induced DNA damage (Weeda and Hoeijmakers 1993). Several *XPD* polymorphisms in the coding regions have been identified (Shen et al. 1998), including two single nucleotide polymorphisms, Asp312Asn in exon 10 and Lys751Gln in exon 23. The variant *XPD* Asp312Asn and Lys751Gln genotypes were reported to be consistently associated with a lower proficiency in repairing the damage induced by UV and chemical carcinogens (Spitz et al. 2001; Qiao et al. 2002). However, it has also been found that the 751Gln allele conferred higher proficiency in repairing the damage induced by ionizing radiation (Moller et al. 1998), and the 312Asn allele had no effect on DRC (Lunn et al. 2000).

As described in our previous study, although the incidence of colorectal cancer is low, there is a 20-fold difference between areas of the highest and lowest incidence (North America and Australia vs. India), and rectal cancer remains more common in India, where a significant increase in colorectal cancer has been reported for both men and women over the last two decades (Wang et al. 2006). We had already identified the associations between common environmental factors, such as diet, lifestyle, and single-nucleotide polymorphisms in *MTHFR* (C677T; A1298C), *PPAR*-gamma (C161T; Pro12Ala), *Cyclin D1* (A870G) and the susceptibility to colorectal cancer in an Indian population (Wang et al. 2006; Jiang et al. 2005, 2006). However, there are few studies linking DNA repair genes with colorectal cancer risk in Indian populations. We conducted this case–control study in an Indian population to detect the distribution of DNA repair genes *XRCC1*, *XRCC3* and *XPD* genotypes and to assess the potential role of these genetic polymorphisms on the risk of colorectal cancer, as well as to evaluate the interactions of gene–gene and gene–environment with susceptibility to colorectal cancer.

Subjects and methods

Subjects

This case–control study was conducted with 302 cases (including 59 colon and 243 rectal cancer patients) and 291 controls. As described elsewhere (Wang et al. 2006), all subjects were recruited at the Cancer Institute, Chennai in South-Eastern India. Cases were first diagnosed as primary colorectal carcinoma between 1999 and 2001. Colon cancer

cases aged from 22 to 72 years old (mean ± SD: 48.5 ± 12.0) included 67.8% men, and rectal cancer cases aged from 17 to 75 years old (mean ± SD: 49.1 ± 14.1) included 64.6% men. Controls were cancer-free healthy individuals, frequency matched to cases for sex and age (within 5 years), aged from 20 to 75 years old (mean ± SD: 47.3 ± 12.6) included 62.5% men, and selected from relatives/visitors to patients other than those with cancers in the gastrointestinal tract during the same period as the case collection. The data collection on smoking status and alcohol consumption has also been previously described. Informed consent was obtained from all study subjects.

Genotyping

XRCC1 Arg399Gln, *XRCC3* Thr241Met and *XPD* Lys751Gln genotypes were determined by PCR–RLFP assays using genomic DNA isolated from peripheral blood lymphocytes. *XRCC1* Arg399Gln PCR products were amplified with the primers 5′-TTGTGCTTTCTCTGTG TCCA-3′ and 5′-TCCTCCAGCCTTTTCTGATA-3′, and digested with *Msp*I (Lunn et al. 1999). Arg allele revealed 374 and 221 bp fragments, while Gln allele was not digested. The PCR primers for the *XRCC3* Thr241Met polymorphism were 5′-GGTCGAGTGACAGTCCAAAC-3′ and 5′-TGCAACGGCTGAGGGTCTT-3′, while PCR products were digested by the restriction enzyme *Nla*III (Smith et al. 2003). The wild type (*Thr/Thr*) produced two bands (316 and 140 bp), the homozygous variant genotype (*Met/Met*) resulted in three bands (211, 140 and 105 bp), and heterozygote (*Thr/Met*) displayed all four bands (316, 211, 140 and 105 bp). The *XPD* Lys751Gln genotypes were analyzed using primers 5′-GCCCGCTCTGGATTATACG-3′ and 5′-CTATCATCTCCTGGCCCCC-3′, and restriction enzyme *Pst*I (Xing et al. 2002). *Pst*I digestion resulted in two fragments of 290 and 146 bp for the wild type (*Lys/Lys*); three fragments of 227, 146 and 63 bp for the variant homozygotes (*Gln/Gln*), and four fragments at 290, 227, 146 and 63 bp for the heterozygotes (*Lys/Gln*).

Statistical analysis

Differences in the distribution of genotypes between cases and controls were assessed using χ^2 test. Within the controls, we also compared the observed genotype frequencies to those expected under the Hardy–Weinberg law using the χ^2 test. The effects [odds ratios (ORs) and 95% confidence intervals (95% CIs)] of genetic polymorphisms on colorectal cancer were estimated using unconditional logistic regression adjusted for potential confounding factors, such as age, sex, household income, education, religion, mother tongue, tobacco, alcohol, chewing habit and vegetarianism. The combined effects of *XRCC1* Arg399Gln, *XRCC3* Thr241Met and *XPD* Lys751Gln polymorphisms, and the interactions of gene–smoking and gene–alcohol were also tested, using low-risk genotypes or non-smokers (non-drinkers) with low-risk genotypes as the referent group, respectively. The computer software package SAS (version 8.2) was used for the statistical calculations. A likelihood ratio test was used to examine the associations of variables with respect to the risk of colorectal cancer. All statistical tests were two sided, and statistical significance was determined as $P < 0.05$.

Results

The general characteristics of the study participants were previously presented in detail (Wang et al. 2006), they were omitted here. Frequencies of the *XRCC1* 399Gln, *XRCC3* 241Met, and *XPD* 751Gln alleles were, respectively, 0.33, 0.18 and 0.33 among controls, and the genotype distributions were all consistent with the Hardy–Weinberg equilibrium (Table 1). Frequencies of the *XRCC1* 399Gln and *XPD* 751Gln alleles were similar to those reported in North and South Indian populations (Vettriselvi et al. 2007; Sobti et al. 2007; Gangwar et al. 2009; Sreeja et al. 2008). The *XRCC1* 399Gln allele was found no significant association with either colon cancer (OR = 1.45, 95% CI 0.81–2.66) or rectal cancer (OR = 1.32, 95% CI 0.92–1.90). However, the *XRCC3* 241Thr/Met genotype showed no significant association with colon cancer (OR = 1.39, 95% CI 0.74–2.60) and a significantly decreased risk with rectal cancer (OR = 0.64, 95% CI 0.42–0.97); the same tendency was found for *XRCC3* 241Met allele carriers with colon cancer (OR = 1.31, 95% CI 0.70–2.42) and rectal cancer (OR = 0.68, 95% CI 0.46–1.02). The *XPD* Lys751Gln genetic polymorphism was also found to show no significant association with either colon or rectal cancer risk. When the associations of these polymorphisms with rectal cancer were taken into account by gender (Table 2), a statistically significant association of the *XRCC1* 399Gln allele with rectal cancer was found among men (OR = 1.65, 95% CI 1.04–2.64), but not among women (OR = 0.90, 95% CI 0.50–1.62). An inverse association of the *XRCC3* 241Met allele with rectal cancer was also found among both men (OR = 0.78, 95% CI 0.46–1.31) and women (OR = 0.60, 95% CI 0.31–1.12), although none reached statistical significance. We also examined any possible difference in age stratification, but nothing significant was found (data not shown).

The combined effects of *XRCC1* Arg399Gln genotypes with the *XRCC3* Thr241Met or *XPD* Lys751Gln polymorphism to pose a risk of rectal or colorectal cancer were analyzed (Table 3). Using the combined low-risk genotypes (*XRCC1* 399Arg/Arg genotype and *XRCC3* 241Met allele)

Table 1 Genotype frequencies and adjusted OR for colon, rectal and colorectal cancers with polymorphisms of DNA repair genes

Genotype	Controls (*n* = 291) *n* (%)	Colon cancer (*n* = 59) *n* (%)	ORs (95% CI)	Rectal cancer (*n* = 243) n (%)	ORs (95% CI)	Colorectal cancer (*n* = 302) *n* (%)	ORs (95% CI)
XRCC1 Arg399Gln							
Arg/Arg (GG)	139 (47.8)	24 (40.7)	1.00 (Ref)	100 (41.1)	1.00 (Ref)	124 (41.1)	1.00 (Ref)
Arg/Gln (GA)	113 (38.8)	25 (42.4)	1.44 (0.76–2.75)	113 (46.5)	1.40 (0.96–2.06)	138 (45.7)	1.41 (0.99–2.03)
Gln/Gln (AA)	39 (13.4)	10 (16.9)	1.48 (0.60–3.47)	30 (12.4)	1.08 (0.61–1.90)	40 (13.2)	1.20 (0.71–2.03)
With Gln (A)	152 (52.2)	35 (39.3)	1.45 (0.81–2.66)	143 (58.9)	1.32 (0.92–1.90)	178 (58.9)	1.36 (0.97–1.91)
XRCC3 Thr241Met							
Thr/Thr (CC)	197 (67.7)	36 (61.0)	1.00 (Ref)	177 (72.8)	1.00 (Ref)	213 (70.5)	1.00 (Ref)
Thr/Met (CT)	85 (29.2)	22 (37.3)	1.39 (0.74–2.60)	57 (23.5)	0.64 (0.42–0.97)	79 (26.2)	0.78 (0.53–1.15)
Met/Met (TT)	9 (3.1)	1 (1.7)	0.57 (0.03–3.42)	9 (3.7)	1.09 (0.40–2.97)	10 (3.3)	0.97 (0.37–2.58)
With Met (T)	94 (32.3)	23 (39.0)	1.31 (0.70–2.42)	66 (27.2)	0.68 (0.46–1.02)	89 (29.5)	0.80 (0.55–1.16)
XPD Lys751Gln							
Lys/Lys (AA)	137 (47.1)	28 (47.5)	1.00 (Ref)	110 (45.3)	1.00 (Ref)	138 (45.7)	1.00 (Ref)
Lys/Gln (AC)	117 (40.2)	22 (37.3)	0.94 (0.49–1.76)	108 (44.4)	1.18 (0.80–1.72)	130 (43.0)	1.12 (0.78–1.60)
Gln/Gln (CC)	37 (12.7)	9 (15.2)	1.14 (0.45–2.65)	25 (10.3)	0.92 (0.50–1.66)	34 (11.3)	0.95 (0.55–1.63)
With Gln (C)	154 (52.9)	31 (52.5)	0.99 (0.55–1.77)	133 (54.7)	1.12 (0.78–1.60)	164 (54.3)	1.08 (0.77–1.51)

Adjusted for gender, age, household income, education, religion, mother tongue, smoking, drinking, chewing and vegetarianism

Table 2 Distributions of XRCC1, XRCC3 and XPD genotypes and risk for rectal cancer by gender

Genotype	Males		Females	
	Cases/controls	ORs (95% CI)[a]	Cases/controls	ORs (95% CI)[a]
XRCC1 Arg399Gln				
Arg/Arg (GG)	58/89	1.00 (Ref)	42/50	1.00 (Ref)
Arg/Gln (GA)	79/69	1.81 (1.11–2.98)	34/44	0.95 (0.51-1.77)
Gln/Gln (AA)	20/24	1.20 (0.56–2.52)	10/15	0.77 (0.29-1.93)
With Gln (A)	99/93	1.65 (1.04–2.64)	44/59	0.90 (0.50-1.62)
XRCC3 Thr241Met				
Thr/Thr (CC)	116/128	1.00 (Ref)	61/69	1.00 (Ref)
Thr/Met (CT)	33/49	0.64 (0.36–1.12)	24/36	0.63 (0.32-1.20)
Met/Met (TT)	8/5	2.52 (0.73–9.41)	1/4	0.25 (0.01-1.94))
With Met (T)	41/54	0.78 (0.46–1.31)	25/40	0.60 (0.31-1.12)
XPD Lys751Gln				
Lys/Lys (AA)	75/89	1.00 (Ref)	35/48	1.00 (Ref)
Lys/Gln (AC)	68/70	1.22 (0.75–1.98)	40/47	1.12 (0.61-2.09)
Gln/Gln (CC)	14/23	0.79 (0.35–1.74)	11/14	1.07 (0.42-2.68)
With Gln (C)	82/93	1.12 (0.71–1.77)	51/61	1.11 (0.62-1.99)

[a] Adjusted for age, household income, education, religion, mother tongue, smoking, drinking, chewing and vegetarianism

as the referent group, the combination of the *XRCC1* 399Arg/Gln and *XRCC3* 241Thr/Thr genotypes showed a significantly positive association with rectal cancer (OR = 2.10, 95% CI 1.08–3.26). Gene–gene interactions of the *XRCC1* Arg399Gln, *XRCC3* Thr241Met and *XPD* Lys751Gln polymorphisms were also estimated (Table 4). A combination of the *XRCC1* 399Gln allele, *XRCC3* Thr/Thr genotype and *XPD* 751Gln allele demonstrated the highest rectal cancer risk (OR = 3.52, 95% CI 1.43–9.44).

The interaction of gene–smoking and gene–alcohol for rectal or colorectal cancer were evaluated (Table 5). These genetic polymorphisms were not found to significantly modify the effect of tobacco consumption (interaction $P > 0.05$, respectively). With respect to alcohol intake, we found a positive association of the *XRCC1* 399Gln allele with rectal (OR = 1.56, 95% CI 1.05–2.33) or colorectal (OR = 1.61, 95% CI 1.11–2.34) cancer among non-drinkers, and weak evidence that *XRCC1* Arg/Arg genotype