COLORECTAL CANCER

Methylation of the oestrogen receptor gene in non-neoplastic epithelium as a marker of colorectal neoplasia risk in longstanding and extensive ulcerative colitis

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Gut 2005;54:1287–1292. doi: 10.1136/gut.2004.062059

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Revised version received
31 March 2005
Accepted for publication
26 April 2005
Published online first
3 May 2005

Background: Surveillance colonoscopy is widely recommended in patients with longstanding and extensive ulcerative colitis (UC) in order to detect colorectal neoplasia at an early stage. However, it still remains questionable whether surveillance colonoscopy effectively enables early detection of UC associated neoplasia. There is a great need for sensitive markers to identify individuals at increased risk of neoplasia. The oestrogen receptor (OR) gene shows age related methylation in the colorectal epithelium and is methylated frequently in sporadic colorectal neoplasia, suggesting that OR methylation may predispose to colorectal neoplasia.

Aim: To clarify whether analysis of methylation of the OR gene in non-neoplastic epithelium can contribute to prediction of increased neoplasia risk in UC patients.

Materials and methods: A total of 165 non-neoplastic colorectal epithelia from 30 patients with longstanding and extensive UC, including 13 UC patients with neoplasia and 17 patients without, were evaluated. Methylation specific polymerase chain reaction was performed to determine the methylation status of the OR gene.

Results: Methylation of the OR gene was detected in 54 of 70 (77.1%) non-neoplastic colorectal epithelia in UC with neoplasia but in only 23 of 95 (24.2%) without neoplasia. Methylation of the OR gene was significantly more frequent in non-neoplastic epithelium from UC with neoplasia than in chronic colitic epithelium from UC without neoplasia. Furthermore, in UC with neoplasia, the OR gene was extensively methylated in non-neoplastic epithelia throughout the colorectum compared with those in UC without neoplasia.

Conclusion: These results suggest that analysis of OR gene methylation may have potential as a useful marker for identifying individuals at increased risk of neoplasia among those with longstanding and extensive UC.

Patients with ulcerative colitis (UC) show an increased incidence of colorectal neoplasia, and UC associated colorectal neoplasia represents a major cause of increased mortality in such patients.^{1–2} In order to improve the prognosis of patients with UC associated neoplasia, diagnosis at an early or precancerous stage is crucial. Predisposition to colorectal neoplasia in UC is generally considered to depend on two risk factors—namely, the presence of longstanding disease and extensive colitis.^{3–5} Therefore, surveillance colonoscopy with multiple step biopsy has been widely recommended for patients with longstanding and extensive UC.^{6–8} However, studies examining the efficacy of surveillance in UC have produced conflicting results, and have suggested that surveillance achieves detection of early stage neoplasia in only a minority of patients and cannot guarantee cancer detection at a curable stage.^{9–14} In order to improve the efficacy of surveillance, there is a great need for sensitive and specific markers to identify individuals at increased risk of neoplasia among those with longstanding and extensive UC.

In several neoplasias, aberrant methylation of promoter region CpG islands, as an epigenetic modification of DNA, is associated with transcriptional inactivation of tumour suppressor genes and plays a crucial role in the development and progression of neoplasia. In normal colorectal epithelium,

some genes are methylated with aging, and this alteration is known as age related methylation.¹⁵ Issa and colleagues¹⁶ reported that methylation of the oestrogen receptor (OR) CpG island increased with age in non-neoplastic colorectal epithelium, and that the same methylation occurred in most sporadic colorectal neoplasias. They concluded that methylation of the OR gene in aging colorectal epithelium could represent one of the earliest events predisposing to sporadic colorectal tumorigenesis.

In this study, to clarify whether analysis of OR gene methylation in non-neoplastic epithelium can contribute to the prediction of increased risk for UC associated neoplasia, we investigated the incidence of OR methylation in non-neoplastic epithelium from longstanding and extensive UC patients with and without colorectal neoplasia.

MATERIALS AND METHODS

Patients and samples

We studied non-neoplastic colorectal epithelia from 30 patients with longstanding (>7 years) and extensive (proximal to the splenic flexure) UC. Thirteen UC patients had

Abbreviations: UC, ulcerative colitis; OR, oestrogen receptor; MSP, methylation specific polymerase chain reaction; PCR, polymerase chain reaction

neoplasia and 17 patients had no neoplasia. In all patients, whenever possible, multiple samples were taken from six regions (rectum, sigmoid colon, descending colon, transverse colon, ascending colon, and caecum) of the colorectum. Non-neoplastic samples of UC with neoplasia were retrieved from colectomy specimens, which were resected at Dokkyo University School of Medicine Hospital and our collaborative institutions between 1999 and 2003. Samples of UC without neoplasia were obtained from biopsy specimens in surveillance colonoscopy between 2002 and 2003. The ethics committee of Dokkyo University School of Medicine approved the study, and all patients gave informed consent for inclusion.

Histological evaluation

Histologically, we classified the inflammatory activity of each specimen into the following categories: no inflammation, mild to moderate inflammation, or severe inflammation. We confirmed that all of the samples were negative for neoplasia in accordance with the Vienna classification of gastrointestinal epithelial neoplasia.¹⁷ This new classification is practical, and has been recommended for resolving the large discrepancies between Western and Japanese pathologists in the diagnosis of gastrointestinal epithelial neoplasias and to better aid understanding of research data in the field of gastroenterology. We defined both dysplasia and carcinoma as neoplasia in accordance with this classification.

DNA extraction

Formalin fixed paraffin embedded samples were cut serially at a thickness of 5 or 10 µm. Based on histological findings, the tissue of each region was microdissected and DNA was

then extracted using a DNA isolator PS kit (Wako Pure Chemical, Osaka, Japan) according to the supplied protocol.

Methylation analysis

DNA methylation status of the OR gene was determined by methylation specific polymerase chain reaction (MSP).¹⁸ Briefly, 500 ng of genomic DNA were modified by sodium bisulfite (Nacalai Tesque, Inc., Kyoto, Japan) using low melting agarose (SeaPlaque agarose; BioWhittaker Molecular Applications, Rockland, Maine, USA), as described previously.¹⁹ DNA from the unmethylated breast cancer cell line MCF-7 and the methylated colon cancer cell line HT29 were used as negative and positive controls, respectively.¹⁶ Agarose beads were incubated for 18 hours at 50°C to ensure complete sodium bisulfite modification. A quarter of the beads containing modified DNA were amplified in a total volume of 25 µl of polymerase chain reaction (PCR) mixture. The reaction mixture contained 2.5 µl of 10×PCR Gold Buffer, 2 µl of 25 mM MgCl₂, 2.5 µl of dNTP mixture, 0.75 units of AmpliTaq Gold (Applied Biosystems, Tokyo, Japan), 10 pmol of forward primer, and 10 pmol of reverse primer. The primer sequences of OR for the methylated reaction were 5'- GTG TAT TTG GAT AGT AGT AAG TTC GTC -3' (forward primer) and 5'- CGT AAA AAA AAC CGA TCT AAC CG -3' (reverse primer), which amplify a 118 bp product, and the primer sequences for the unmethylated reaction were 5'- GGT GTA TTT GGA TAG TAG TAA GTT TGT -3' (forward primer) and 5'- CCA TAA AAA AAA CCA ATC TAA CCA -3' (reverse primer), which amplify a 120 bp product. MSP was carried out using the following amplification profile: five minutes at 94°C once; 30 seconds at 94°C, 30 seconds at 55°C, 30 seconds at 72°C for 40 cycles; then four minutes at 72°C. PCR products

Table 1 Clinical and pathological features of patients with and without ulcerative colitis (UC) neoplasia

Patient No	Age (y)	Sex (M/F)	Duration of disease (y)	Extent of disease†	Location of neoplasia	Histology of neoplasia‡	Dukes' stage
UC with neoplasia							
1	58	F	22	Total	Rectum	Muc	C
2	41	F	15	Left side	Sigmoid	Poor	C
3	60	F	19	Total	Sigmoid	Dysplasia	
4	57	F	12	Total	Rectum	Well	A
5	51	M	17	Total	Rectum	Well	C
6	79	F	23	Total	Rectum	Moderate	C
7	44	M	18	Total	Rectum	Dysplasia	
8	48	F	6	Total	Rectum	Well	A
9	74	F	14	Total	Transverse	Well	A
10	44	M	19	Total	Sigmoid	Well	C
11	45	M	14	Total	Sigmoid	Moderate	A
12	50	M	14	Total	Rectum	Moderate	B
13	45	M	22	Total	Rectum	Well	A
UC without neoplasia							
14	43	M	12	Total			
15	44	M	25	Total			
16	44	M	10	Total			
17	41	M	12	Total			
18	50	F	24	Left side			
19	49	M	13	Total			
20	57	M	9	Total			
21	51	M	15	Total			
22	66	F	14	Total			
23	42	M	16	Total			
24	53	M	36	Total			
25	59	F	31	Total			
26	51	M	33	Total			
27	42	F	17	Total			
28	42	M	19	Total			
29	42	M	19	Total			
30	33	F	10	Total			

†Total, total colitis; Left side, left sided colitis; Well, well differentiated adenocarcinoma; moderate, moderately differentiated adenocarcinoma; poor, poorly differentiated adenocarcinoma; muc, mucinous carcinoma.

Figure 1 Methylation analysis of the oestrogen receptor (OR) gene in each region of the colorectum. Methylation specific polymerase chain reaction products in lanes marked U show the presence of unmethylated templates whereas products in lanes marked M show the presence of methylated templates. R, rectum; S, sigmoid colon; D, descending colon; T, transverse colon; A, ascending colon; C, caecum. (A) Representative samples of non-neoplastic epithelia from a patient with neoplasia. The OR gene was methylated in all regions of the non-neoplastic colorectal epithelium. (B) Representative samples of non-neoplastic epithelia from a patient without neoplasia. The OR gene was methylated in only rectal epithelium whereas no methylation of the OR gene was detected in other regions.

were electrophoresed on 2% agarose gels and visualised under UV illumination using ethidium bromide staining.

Statistical analysis

Age and disease duration of UC patients with and without neoplasia are expressed as mean (SD), and differences between groups were analysed using Welch's *t* test; differences at $p < 0.05$ were considered significant. The χ^2 test or Fisher's exact test was used to compare the inflammatory activity of colitis and the incidence of methylation in UC

patients with and without neoplasia, and to determine the correlation between the inflammatory activity of colitis and methylation status; differences at $p < 0.05$ were considered significant.

RESULTS

Clinical and pathological features

The clinical and pathological features of the patients with and without UC neoplasia are summarised in table 1. In a total of 13 patients with UC neoplasia, six patients were men and seven were women, and mean age was 54.5 (SD 11.8) years (range 41–79). All patients except one (case 8) had longstanding UC; known duration of the disease was mean 16.5 (SD 4.7) years (range 6–23). Twelve patients had pancolitis and the remaining patient had left sided colitis. Eleven of the patients underwent resections because of complications related to cancer, and two of the patients underwent resection because of dysplasia progression. There were 17 patients without neoplasia: 12 were men and five were women, and mean age was 47.6 (SD 8.0) years (range 41–66). The known duration of the disease was mean 18.5 (SD 8.4) years (range 9–36) and 16 patients had pancolitis and the remaining patient left sided colitis. There were no significant differences in mean age or known disease duration between patients with neoplasia and those without.

Incidences of OR gene methylation

A total of 165 non-neoplastic epithelia obtained from 30 patients were available for the MSP study. At some regions, because of lack of an available tissue specimen, no methylation analysis was performed. OR methylation was present in 54 of 70 (77.1%) non-neoplastic epithelia from patients with neoplasia (fig 1A) compared with 23 of 95 (24.2%) non-neoplastic epithelia from patients without neoplasia (fig 1B). The incidence of OR methylation in non-neoplastic epithelium was higher in patients with neoplasia than in those without ($p < 0.001$).

OR methylation status in each region of the colorectum is shown schematically in fig 2. OR methylation in UC patients with neoplasia occurred extensively in the non-neoplastic epithelium throughout the colorectum, and OR gene methylated in both non-neoplastic epithelia from UC patients with carcinoma and dysplasia. Eleven of 13 patients with

Figure 2 Oestrogen receptor (OR) methylation status in each region of the colorectum is shown schematically. Methylated and unmethylated regions are indicated (blank regions, not done). R, rectum; S, sigmoid colon; D, descending colon; T, transverse colon; A, ascending colon; C, caecum.

Figure 3 Incidence of oestrogen receptor (OR) gene methylation in non-neoplastic epithelium from ulcerative colitis (UC) patients with and without neoplasia in each region of the colorectum. In the rectum, sigmoid colon, descending colon, and caecum, the incidence of OR methylation in UC with neoplasia was significant higher than in those without neoplasia. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. R, rectum; S, sigmoid colon; D, descending colon; T, transverse colon; A, ascending colon; C, caecum.

neoplasia had at least three regions with methylation and three of 13 had all analysed regions with methylation, whereas 15 of 17 patients without neoplasia had less than two regions with methylation and five of 17 had no region with methylation. Figure 3 demonstrates the incidence of OR gene methylation in non-neoplastic epithelium from UC patients with and without neoplasia in each region of the colorectum. In the rectum, sigmoid colon, descending colon, and caecum, the incidences of OR methylation in UC with neoplasia were significant higher than in those without neoplasia (rectum 92.3% *v* 31.2%, $p < 0.01$; sigmoid colon 100% *v* 31.2%, $p < 0.001$; descending colon 76.9% *v* 25.0%, $p < 0.05$; transverse colon 63.6% *v* 31.3%, $p = 0.20$; ascending colon 54.5% *v* 13.3%, $p = 0.069$; caecum 66.7% *v* 12.5%, $p < 0.05$).

Relationship between OR gene methylation and inflammatory activity of colitis

With regard to inflammatory activity in the 70 samples from UC patients with neoplasia, 22 (31.4%) had no inflammation, 26 (37.1%) had mild inflammation, 15 (21.4%) had moderate inflammation, and seven (10.0%) had severe inflammation. Among 95 samples from UC patients without neoplasia, 20 (21.1%) had no inflammation, 51 (53.7%) had mild inflammation, 16 (16.8%) had moderate inflammation, and eight (8.4%) had severe inflammation. There were no significant differences in inflammatory activity between patients with and without neoplasia.

The relationship between OR gene methylation status and the inflammatory activity of colitis is shown in table 2. The incidence of OR gene methylation was 52.4% in non-neoplastic epithelium without inflammation, 40.3% in that with mild inflammation, 48.4% in that with moderate inflammation, and 60.0% in that with severe inflammation. There was no relationship between OR gene methylation status and the inflammatory activity of colitis.

DISCUSSION

In this study, we have shown that the incidence of OR gene methylation in non-neoplastic epithelium was higher in patients with neoplasia than in those without. In UC with neoplasia, OR gene methylation was detected not only in regions in which neoplasia was located but also in other regions widely throughout the colorectum. Furthermore, these epigenetic alterations occurred in both non-neoplastic epithelia from UC patients with carcinoma and dysplasia. These results suggest that an increase in the incidence of age related methylation—in other words, progression of aging in the colorectal epithelium—is preceding or is a relatively early event in UC associated tumorigenesis. Analysis of OR gene methylation in non-neoplastic colorectal epithelium may be a useful adjunct for identifying individuals at increased risk of neoplasia among those with longstanding and extensive UC, and contribute to more effective cancer surveillance.

In order to detect neoplasia at a surgically curative and preferably preinvasive stage, periodic colonoscopy combined with extensive biopsy sampling throughout the colorectum is recommended. According to several studies that have discussed the efficacy of surveillance, however, an appreciable number of cancers were detected at an advanced stage despite surveillance colonoscopy, and many of these cases had less than ideal outcomes. Thus it still remains questionable whether surveillance colonoscopy with multistep biopsy effectively enables early detection of UC associated neoplasia.

The unsatisfactory efficacy of current surveillance colonoscopy for early detection of UC associated neoplasia can be attributed to difficulties in endoscopic and histological diagnoses of UC associated neoplasia at an early stage. Endoscopically, early stage UC associated neoplasias show various macroscopic changes. These changes are not clear, and are sometimes missed in areas of chronically inflamed epithelium.^{20–22} Therefore, detection of UC associated neoplasias at the precancerous and early stages is difficult by endoscopy, and physicians have no choice but to depend on histological evaluation of multiple step biopsies obtained by surveillance colonoscopy. Histologically, UC associated neoplasia often involves one part of the crypt, a few crypts in the epithelium, or is found throughout the inflamed epithelium.⁶ Therefore, it is not unusual for pathologists to experience difficulty in discriminating between neoplastic lesions and inflammatory regenerative epithelium using biopsy specimens stained with haematoxylin and eosin. Furthermore, different pathologists may use different diagnostic criteria for determining neoplasia.^{6, 23}

Table 2 Relationship between oestrogen receptor (OR) gene methylation status and the inflammatory activity of colitis

	Inflammatory activity of colitis			
	None (n = 42)	Mild (n = 77)	Moderate (n = 31)	Severe (n = 15)
OR methylation status				
Methylated	22 (52.4%)	31 (40.3%)	15 (48.4%)	9 (60.0%)
Unmethylated	20 (47.6%)	46 (59.7%)	16 (51.6%)	6 (40.0%)

In order to overcome such difficulties, adjunctive diagnostic modalities such as chromoendoscopy for identifying neoplasia in non-neoplastic inflamed epithelium and analysis of p53 alteration for distinguishing neoplastic lesion from regenerative epithelium have been reported.^{24–28} However, it would be impractical to perform these adjunctive modalities for surveillance of all UC patients with longstanding and extensive colitis because of their labour intensive nature and expense. If refinement of high and low risk subgroups of UC patients with longstanding and extensive colitis were possible, it would enable physicians to conduct more intensive surveillance with these modalities (chromoendoscopy and analysis of p53 alteration) for patients at higher risk of developing colorectal neoplasia.

Recently, numerous studies have revealed higher frequencies of molecular alterations to non-neoplastic epithelium in UC patients with neoplasia than in non-neoplastic epithelium in UC patients without neoplasia, suggesting that these molecular alterations may be used as new markers for identifying individuals with UC at increased risk of neoplasia.^{27–33} In the present study, we observed age related methylation of the OR gene in non-neoplastic epithelium throughout the entire colorectum of patients with longstanding and extensive UC. Age related methylation has been demonstrated for several genes, including OR, insulin-like growth factor II, MYOD1, N33, and E-cadherin in the colorectal epithelium, and patients with a high level of methylation in their colorectal epithelium may be at higher risk of developing sporadic colorectal neoplasia.^{16 34 35} In this study, we selected and analysed the OR gene because OR gene methylation is predominantly a phenomenon of the epithelium. Ahuja and colleagues³⁴ examined differences in the methylation status of the OR, MYOD, and N33 genes between epithelial and stromal components of colorectal mucosa, and clarified that the OR gene showed four times higher methylation in the epithelial components than in the stromal components. In contrast, the N33 and MYOD genes showed equivalent levels of methylation in both components. Therefore, we considered that OR gene methylation status would not be susceptible to the influence of contamination by stromal cells.

In UC associated neoplasia, Issa and colleagues³⁶ first reported that the OR, MYOD, and CSPG2 genes, and p16 gene exon 1, which behave as age related methylation in colorectal epithelium, were intensively methylated in neoplastic epithelium from high grade dysplasia/cancer patients with UC. Furthermore, they showed that these genes were highly methylated in non-neoplastic epithelium from UC patients with high grade dysplasia/cancer compared with non-neoplastic epithelium from UC patients without dysplasia. They suggested that age related methylation could be used as a molecular marker for identifying individuals with ulcerative colitis at increased risk of developing neoplasia but they did not mention methylation status by region in the colorectum. In our present study, we analysed OR gene methylation in multiple samples taken from six regions (rectum, sigmoid colon, descending colon, transverse colon, ascending colon, and caecum) throughout the colorectum, and showed that OR methylation in UC patients with neoplasia occurred more frequently and extensively in the non-neoplastic epithelium throughout the colorectum than in those without neoplasia. From these findings it is expected that analysis of a single biopsy sample (for example, a rectal biopsy) may have the possibility of helping identify UC patients at particularly high risk of developing neoplasia in contrast with the large number of biopsy samples needed for surveillance colonoscopy at present.

Although the mechanism of age related methylation is not well known, there may be several contributory factors, such

as exogenous carcinogens, reactive oxygen species, and host genetic differences.^{15 37–39} Chronic inflammation in UC would be associated with an elevated level of exogenous carcinogens and reactive oxygen species, and UC colonocytes would be subjected to a high level of epigenetic damage. In the present study, the inflammatory activity of colitis did not have any relationship with the incidence of OR gene methylation, suggesting that OR gene methylation does not simply reflect transient inflammation and that cumulative and repetitive inflammation may lead to the occurrence of methylation.

In our study, although there were no significant differences in age and disease duration between UC patients with and without neoplasia, suggesting that all UC patients would theoretically have been exposed to an equal degree of damage by exogenous carcinogens and reactive oxygen species, why was there a wide difference in the incidence of OR gene methylation between non-neoplastic epithelium from UC patients with and without neoplasia? Possible reasons include differences in cumulative inflammation among patients in the two groups despite the similarity of disease duration, differences between actual disease duration and those listed in the hospital records, and individual differences in susceptibility to gene methylation after exposure to exogenous carcinogens and reactive oxygen stress. Clarification of the reasons for this difference awaits further study.

Our results show that OR methylation is worthy of study to explore its potential as a biomarker in the surveillance of UC patients. We demonstrated the occurrence of gene methylation; quantitative information on the status of methylation was lacking. Therefore, we are currently conducting a quantitative analysis of age related methylation using a laser capture microdissection technique and the combined bisulfite restriction analysis (COBRA) method. Further longitudinal studies are needed to address the predictive power and clinical utility of the methylation analysis. It is anticipated that analysis of OR gene methylation using rectal biopsy specimens may make it possible to identify UC patients at high risk of future neoplastic progression, and that in future it could be realistic, on the basis of OR methylation status, to adjust a patient's surveillance interval and to select UC patients who should undergo intensive surveillance that includes adjunctive modalities such as chromoendoscopy and analysis of p53 alteration.

ACKNOWLEDGEMENTS

This study was supported by a grant in aid (No 16790390) for Scientific Research from the Japan Society for the Promotion of Science (JSPS). The authors greatly thank Dr S Kameoka and Dr M Itabashi (Department of Surgery II, Tokyo Women's Medical college), Dr B Iizuka (The Institute of Gastroenterology, Tokyo Women's Medical college), Dr H Mitooka (Division of Gastroenterology, Kobe Kaisei Hospital), Dr T Tanaka (Division of Gastroenterology, Shizuoka City Shizuoka Hospital) and Dr N Kitajima (Division of Gastroenterology, Kasai City Hospital) for kindly supplying the tissue materials. The authors also thank Dr H Kawamata (Department of Oral and Maxillofacial Surgery, Dokkyo University School of Medicine) for critical reading and valuable advice on this manuscript, and Ms C Sato-Matsuyama, A Shimizu, T Ohtsuki, M Katayama, and S Miyahara (Department of Surgical and Molecular Pathology, Dokkyo University School of Medicine) for technical assistance.

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Conflict of interest: None declared.

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EDITOR'S QUIZ: GI SNAPSHOTS.....

Answer

From question on page 1282

The diagnosis was pseudoachalasia due to neurofibromatosis of the oesophagus. Computerised tomography showed circumferentially thickened and dilated oesophagus. Neurofibromas were visible infiltrating the muscularis propria at endoscopic ultrasonography.

A diagnostic laparoscopy was performed and showed multiple nodules around the gastro-oesophageal junction (GOJ) and also throughout the small bowel mesentery. Histology of the nodules confirmed neurofibromatosis.

Subsequently, the patient underwent pneumatic dilatations of up to 40 mm but his dysphagia recurred rapidly. Because of refractory symptoms and after discussion with the patient, oesophagectomy through a left thoracoabdominal incision and a left cervical incision was performed. Reconstruction was via a gastric tube anastomosed to the cervical oesophagus. The patient has made an uneventful recovery with no significant complications over the past 12 months of follow up.

The specimen consisted of a dilated thick walled oesophagus with an obvious narrowing just above the GOJ where multiple neurofibromas were present. Microscopic examination confirmed plexiform neurofibromatosis.

doi: 10.1136/gut.2004.063297