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Diagnostic Validity of DNMT-1 and 3b Immunoreactivity in Non-neoplastic Epithelium of UC Patients with and Without Neoplasia

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SUMMARY

It is important to improve the efficacy of surveillance in UC patients with neoplasia.

In the present study, we assessed the cut-off value of expression of DNMT-1 and DNMT-3b expression in the non-neoplastic rectal epithelium of patients with long-standing and extensive UC. Sixty patients with long-standing and extensive UC paticipated in this study (30 with colorectal neoplasia and 30 without). Immunohistochemical analysis was performed to determine the expression of DNMT-1 and 3b in non-neoplastic rectal epithelium of UC patients without neoplasia, and in non-neoplastic rectal epithelium of UC patients with neoplasia. The level of immunoreactive DNMT-1 and DNMT-3b expression was determined as the percentage of positive cells relative to the total number of cells counted under high power magnification. DNMT-1 and 3b expression in non-neoplastic rectal epithelium of UC patients with neoplasia (0.57, range 0.53-0.63) (0.32, range 0.18-0.67) was higher than in the non-neoplastic epithelium of UC patients without neoplasia (0.41, range 0.25-0.54, $P=.001$) (0.0, range 0.0-0.13, $P<.001$). ROC curve analysis confirmed 0.53 and 0.07 as the best DNMT-1 and DNMT-3b cut-off values for identifying individuals at increased risk of neoplasia (area under the curve = 0.798 and 0.842, respectively). The cut-off value for DNMT-1 and DNMT-3b expression in non-neoplastic rectal epithelium is therefore an efficient predictor for the increased risk of UC-associated neoplasia.

Key Words : DNA methyltransferase, surveillance, ulcerative colitis-associated neoplasia

INTRODUCTION

Patients with long-standing and extensive ulcerative colitis (UC) exhibit increased incidence of colorectal neoplasia[1,2]. The diagnosis of this condition at an early or precancerous stage is crucial[3,4]. The established method of surveillance is colonoscopy, but this is associated with difficulty in discriminating UC-associated neoplasia from inflammatory or regenerative epithelium[5]. In order to improve the efficacy of surveillance, there is an urgent need for sensitive and specific markers to identify individuals at increased risk of neoplasia among patients with long-standing and extensive UC. In order to help predict colorectal neoplasia risk, we previously used immunohistochemical

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Table 1 Clinicopathological characteristics of UC patients with and without neoplasia

	UC with neoplasia (n=30)	UC without neoplasia (n=30)
Mean age at study, years	48.6±10.4	46.9±7.5
Male/Female	16/14	20/10
Mean disease duration of UC, years	16.4±5.5	18.2±7.4
Disease extent (total/left)	26/4	24/6

methods to show that the expression of DNA methyltransferase-1 (DNMT-1) progressively increased from the rectal epithelium of UC patients without neoplasia to non-neoplastic rectal epithelium of UC patients with neoplasia[6]. However, it remained unclear whether the determination of immunoreactive DNMT-1 and 3b expression could be a useful predictor for colorectal neoplasia risk in UC patients. In the present study, we assessed the cut-off value of expression of DNMT-1 and DNMT-3b expression in the non-neoplastic rectal epithelium of patients with long-standing and extensive UC.

MATERIALS AND METHODS

Patients and samples

Sixty UC patients participated in the study : 30 (16 men, 14 women) with colorectal neoplasia and 30 (20 men, 10 women) without. We studied the non-neoplastic rectal epithelium of each patient. All patients were diagnosed at Dokkyo Medical University School of Medicine and its associated institutes between 2000 and 2009. The clinicopathological features of our UC patients are shown in Table 1. The mean (±SD) age of the UC patients with neoplasia was 48.6±10.4 years (range 26-74), and that of the UC patients without neoplasia was 46.9±7.5 years (range 33-59). Mean (±SD) duration of disease in the UC patients with neoplasia was 16.4±5.5 years (range 7-29), while that in the UC patients without neoplasia was 18.2±7.4 years (range 10-36). There were no significant differences in age, sex, disease duration, or disease extent, between UC patients with, and without neoplasia. Histologically, we confirmed that all of the 60 non-neoplastic samples from UC patients with and without neoplasia were negative for neoplasia in accordance with the Riddell classification of gastrointestinal epithelial neoplasia[7]. Histological evaluations were confirmed by two experienced gastrointestinal pathologists (TF, SF) and a gastrointestinal surgeon (HT). All UC patients without neoplasia were diagnosed as neoplasia-free via endoscopy and histological assessments throughout periodic surveillance colonoscopy on the basis of multiple-step biopsy samples. The Ethics Committee of Dokkyo Medical University School of Medicine approved all protocols, and informed consent for tissue procurement was obtained from all the patients.

Immunohistochemical analysis of DNMT-1, and DNMT-3b protein

Immunohistochemical staining for DNMT-1 and 3b was performed universal immunoperoxidase polymer method as described previously[6]. In brief, sections (4μm thick) placed on silane-coated slides were deparaffinized, rehydrated, and then pretreated with 3% H_2O_2 in methanol for 5 min at room temperature to quench endogenous peroxidase activity. The sections were doused in 0.01 M citrate buffer (pH 7.0) and heated to 95℃ in a microwave oven (MI-77, Azumaya, Tokyo, Japan ; 400 W) for 40 min to facilitate antigen retrieval. The sections were incubated with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 30 min, and then with a goat anti-human polyclonal DNMT-1 antibody (N16, dilution 1 : 50 ; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) or anti-mouse DNMT3b antibody (IMG-184A ; IMGENEX, San Diego, CA, USA ; dilution 1 : 20) for 30 min. Thereafter, the sections were incubated with Envison$^+$/HRP System (Dako, Carpinteria, CA, USA) for 60 min, and washed with PBS. Finally, they were incubated in 3,3'-diaminobenzidine tetrahydrochloride with 0.05% H_2O_2 for 3 min and then counterstained with Carazzi's hematoxylin. The immunoreactivity of DNMT-1 and DNMT-3b was assessed in areas showing the highest density of cells with positively staining nuclei. The lev-

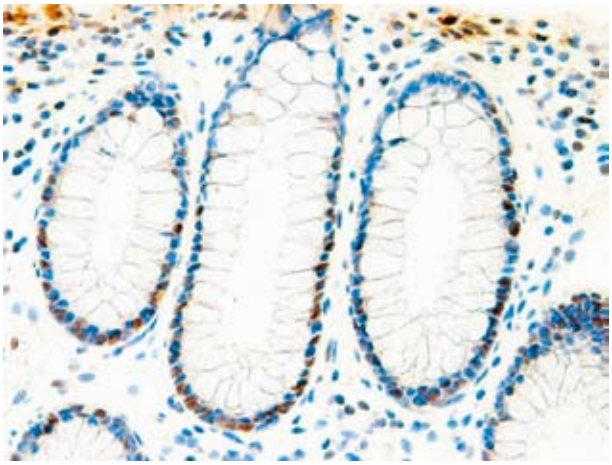

Fig. 1-a Non-neoplastic epithelium of UC patients without neoplasia (DNMT-1)

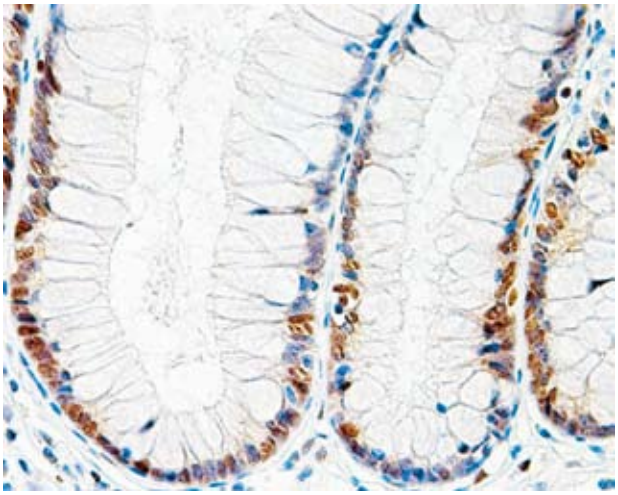

Fig. 1-b Non-neoplastic epithelium of UC patients with neoplasia (DNMT-1)

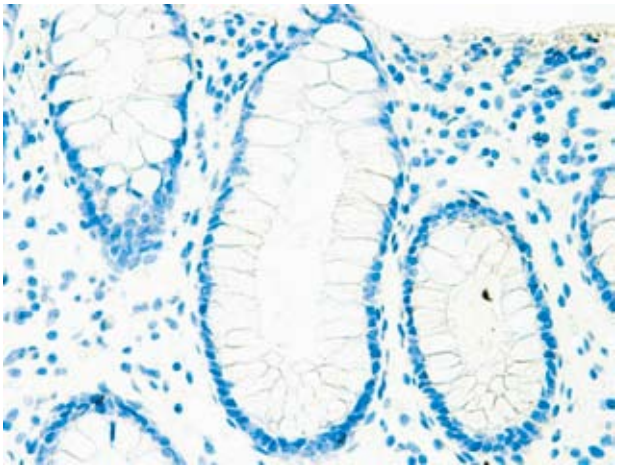

Fig. 1-c Non-neoplastic epithelium of UC patients without neoplasia (DNMT-3b).

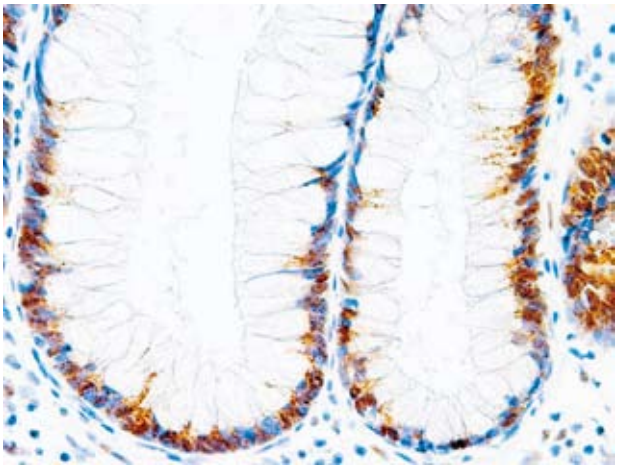

Fig. 1-d Non-neoplastic epithelium of UC patients with neoplasia (DNMT-3b).

el of immunoreactive DNMT-1 and DNMT-3b expression is given as the percentage of positive cells relative to the total number of cells counted under high power magnification[6].

Statistical analysis

JMP 8 statistical software (SAS Institute Inc, Cary, NC) was used for all analyses. Continuous variables were expressed as mean ± standard deviation (SD), or, where indicated, median and interquartile range (IRQ). For continuous variables, 2-group comparisons were performed with the parametric 2-sample t test or with the nonparametric Mann-Whitney U test. For all tests, differences of $P<0.05$ were considered statistically significant ; all P values were two-sided. A receiver operating characteristics (ROC) curve was generated and the area under the curve calculated to determine the best discriminating DNMT-1 and DNMT-3b expression of non-neoplastic epithelium and neoplastic epithelium. Sensitivity, specificity, positive and negative predictive values and likelihood ratios, and accuracy were calculated with standard formulas.

RESULT

Immunohistochemical staining was carried out upon non-neoplastic rectal epithelium samples from 30 UC patients with neoplasia, and on non-neoplastic rectal epithelium samples from 30 UC patients without neoplasia. We were able to evaluate 28 non-neoplastic rectal epithelia from UC patients with neoplasia and 15 non-neoplastic rectal epithelia from 30 UC patients without. DNMT-1 and DNMT-3b expression levels in

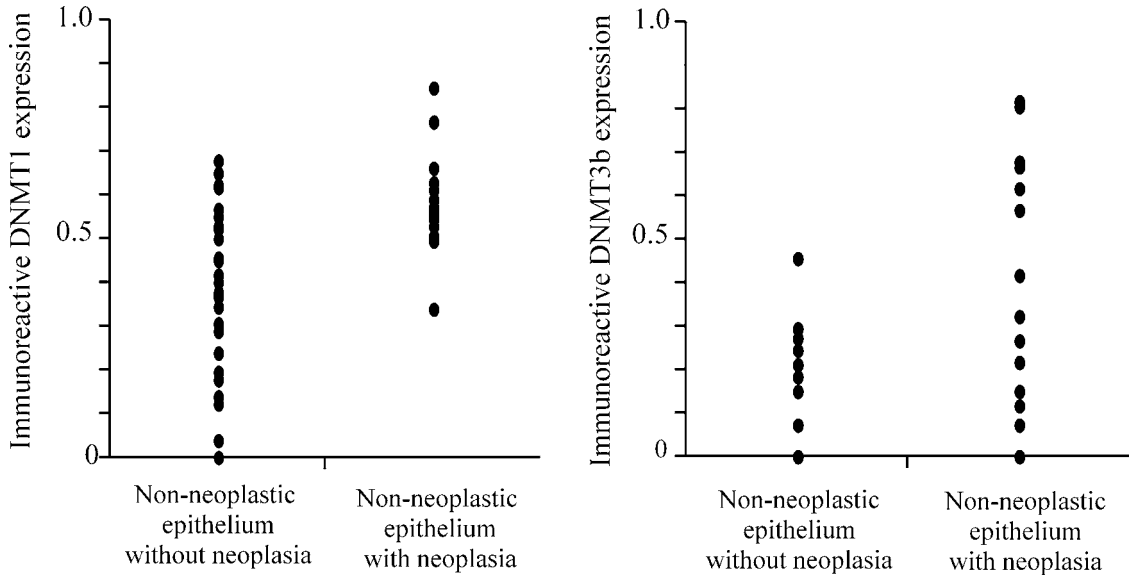

Fig. 2 Level of immunoreactive DNMT-1 and 3b expression in non-neoplastic rectal epithelium.

non-neoplastic rectal epithelium from UC patients with and without are shown in Figure 1. The median and interquartile (IQR) of immunoreactive DNMT-1 expression were 0.41 (0.25-0.54) and 0.57 (0.53-0.63) in non-neoplastic rectal epithelium of UC patients without and with neoplasia, respectively. Non-neoplastic rectal epithelium of UC patients without and with neoplasia differed significantly in the level of immunostaining for DNMT1 expression ($P=0.001$) (Figure 2). The median and interquartile (IQR) of immunoreactive DNMT-3b expression were 0.00 (0.00-0.13) and 0.32 (0.18-0.67) in non-neoplastic rectal epithelium of UC patients without and with neoplasia. Non-neoplastic rectal epithelium of UC patients without and with neoplasia differed significantly in the level of immunostaining for DNMT3b expression ($P<0.001$) (Figure 2). ROC curve analysis confirmed 0.53 and 0.07 as the best DNMT-1 and DNMT-3b cut-off value for identifying individuals at increased risk of neoplasia (area under the curve = 0.798 and 0.842 respectively). In the diagnostic validity of cut-off value of immunoreactive DNMT-1 expression, sensitivity of the diagnostic test was 80.0%, and the specificity was 75.0%, the positive predictive value 63.2% and the negative predictive value 87.5%. The positive likelihood ratio was 3.20 and the negative likelihood ratio 0.31. The accuracy was 76.7% (Table 2). In the diagnostic validity of cut-off value of immunoreactive DNMT-3b expression, sensitivity of the diagnostic test was 86.7%, specificity 75.0%, the positive predictive value 65.0% and the negative predictive value 91.3%. The positive likelihood ratio was 3.44 and the negative likelihood ratio 0.28. The accuracy was 79.1% (Table 3).

DISCUSSION

In the present study, we have shown that the expression of DNA methyltransferase-1 and 3b in non-neoplastic rectal epithelium was higher in patients with neoplasia than in those without. We also determined the cut-off value for DNA methyltransferase-1 and 3b expression, and examined whether the cut-off value may be useful in identifying individuals at increased risk of neoplasia. Immunohistochemistry proved to be a sufficient method with which to determine DNMT-1 and 3b expression and tissues differed significantly in terms of DNMT-1 and 3b immunoreactivity. In terms of diagnostic validity of the best DNMT-1 and DNMT-3b cut-off values, we determined that the positive likelihood ratio was 3.20, 3.44, its ratio is equivalent. Since Warren was the first to suggest that UC-associated dysplasia was a precursor of colitic cancer in UC patients[8]. Sinece then UC-associated dysplasia has been observed in UC patients. In western countries, recommendations are to employ periodic surveillance using colonoscopy on the basis of multiple-step biopsy[9,10]. Issa et al. reported that first reported that age-related methylated genes were highly methylated in non-neoplastic epithelium from

Table 2 Diagnostic validity of DNMT-1 immunoreactivity in non-neoplastic epithelium of UC patients with and without neoplasia.

	DNMT-1 immunoreactivity		Total
	Positive	Negative	
Non-neoplastic epithelium with neoplasia	12	3	15
Non-neoplastic epithelium without neoplasia	7	21	28

Sensitivity 80.0%, specificity 75.0%, the positive predictive value 63.2% and the negative predictive value 87.5%, the positive likelihood ratio 3.20, the negative likelihood ratio 0.31. the accuracy 76.7%

Table 3 Diagnostic validity of DNMT-3b immunoreactivity in non-neoplastic epithelium of UC patients with and without neoplasia.

	DNMT-3b immunoreactivity		Total
	Positive	Negative	
Non-neoplastic epithelium with neoplasia	13	2	15
Non-neoplastic epithelium without neoplasia	7	21	28

Sensitivity 86.7%, specificity 75.0%, the positive predictive value 65.0% and the negative predictive value 91.3%, the positive likelihood ratio 3.44 and the negative likelihood ratio 0.28, the accuracy 79.1%.

UC patients with high-grade dysplasia/cancer, compared with compared with non-neoplastic epithelium from UC patients without neoplasia[11,12]. In our own previous studies, we investigated the methylation status of the ESR-1 gene using the combined bisulfite restriction analysis method in surveilance. ESR-1 gene was highly methylated in non-neoplastic epithelium from UC patients with high-grade dysplasia/cancer compared with non-neoplastic epithelium from UC patients without neoplasia[13,14]. Other reports have revealed that RUNX-3, MINT-1, ESR-1, N-33 were associated with high methylation levels[15,16]. However the combined bisulfite restriction analysis method for investigating gene methylation status of gene is very time consuming, and it is therefore very difficult to envisage how this might be applied clinically. Therefore, in the present study we investigated whether immunohistochemistry could be a more viable technique for clinical application. Using this method, we found that DNA methyltransferase-1 was highly methylated in non-neoplastic epithelia from UC patients with high-grade dysplasia/cancer compared with non-neoplastic epithelium from UC patients without neoplasia[6]. We therefore consider that this methodology could be applied clinically but highlight the need to determine a cut-off value for surveillance. To this end, we also attempted to determine a cut-off value for DNMT-1 and 3b immunoreactivity. The assessment of DNMT-1 and 3b immunoreactivity therefore represents a cut-off value for predicting the risk of colorectal neoplasia in UC patients. If we assess DNMT-1 and 3b immnreactivity at a timepoint close to the definitive diagnosis, we will be able to increase the cut-off value in order to increase specificity. ROC curve was generated in order to determine the best sensitivity and specificity for predicting colorectal neoplasia risk in UC patients. Consequently, the best cut-off values for DNMT-1 and DNMT-3b immunoreactivity were 0.53 and 0.07 respectively. Previously, DNMT positive cells in more than 20% of the cells exhibiting nuclear immunostaining. Methods for assessing DNMT-3b immunoreactivity have included determination of the specific strength

of staining[17~19]. Several study have assessed the neoplastic epithelium using such methodology and have examined the relationship between DNMT expression and recurrence, and between DNMT expression and prognosis. The specific aim of this study was to evaluate the assessment DNMT-1 and 3b immunoreactivity as a means of predicting the risk of colorectal neoplasia in UC patients. However, earlier methods have reported difficulty in distinguishing UC patients with neoplasia from UC patients without neoplasia. Therefore, our ROC analysis was important in distinguishing UC patients with neoplasia from UC patients without neoplasia. DNMT-1 and 3b immunoreactivity proved to be very useful in involved remains to be fully elucidated. Futher studies will therefore be required to clarify the mechanisms involed. Aberrant gene methylation patterns arising in a wide range of colorectal epithelial cells in UC patients implies that abnormal methylation may be one underlying aspect of colitis-associated tumorigenesis. Accordingly, it is important for future studies to elucidate the precise relationship between aberrant gene methylation and colitis-associated tumorigenesis. In summary, the cut-off value of DNMT-1 and 3b immunoreactivity in non-neoplastic rectal epithelia was considerd as a useful factor for differential diagnosis in the prediction of colorectal neoplasia risk in UC patients. We also concluded that it is reasonable for a new simple surveillance protocol to assess the cut-off value of expression of DNMT-1 and DNMT-3b immunoreactivity in non-neoplastic rectal epithelium in patients with long-standing and extensive UC.

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