Increased Expression of DNA Methyltransferase-1 in Non-Neoplastic Epithelium Helps Predict Colorectal Neoplasia Risk in Ulcerative Colitis

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Abstract

\textbf{Aims:} To clarify the possible significance of increased expression of DNA methyltransferase (DNMT)-1 in the tumorigenesis of colorectal neoplasia in ulcerative colitis (UC) and to clarify whether analysis of DNMT1 expression in non-neoplastic epithelium can contribute to the prediction of increased risk for UC-associated neoplasia. \textbf{Methods:} Sixty-two patients with long-standing and extensive UC were included in this study: 31 with colorectal neoplasia (dysplasia in 11 and invasive cancer in 20) and 31 without. Immunohistochemical analysis and quantitative RT-PCR were performed to determine the expression of DNMT1 in rectal epithelium of UC patients without neoplasia, and in non-neoplastic rectal epithelium and colorectal neoplasia of UC patients with neoplasia. \textbf{Results:} The immunoreactive DNMT1 expression gradually increased from rectal epithelium of UC patients without neoplasia (0.13 ± 0.07) to non-neoplastic rectal epithelium of UC patients with neoplasia (0.32 ± 0.12, p < 0.001), and to colorectal neoplasia (0.54 ± 0.20, p < 0.001). Among 31 neoplasias, there was no difference in the immunoreactive DNMT1 expressions between dysplasia and invasive cancer (0.47 ± 0.52 vs. 0.58 ± 0.63). The expression level of DNMT1 mRNA tended to increase gradually from rectal epithelium of UC patients without neoplasia (0.53 ± 0.34) to non-neoplastic rectal epithelium of UC patients with neoplasia (0.88 ± 0.57, p = 0.06), and to colorectal neoplasia (1.38 ± 0.64, p = 0.07). \textbf{Conclusion:} Increased expression of DNMT1 in non-neoplastic epithelium may precede or be a relatively early event in UC-associated tumorigenesis, and may help predict the risk of colorectal neoplasia in UC.

Introduction

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]. The risk of UC-associated colorectal neoplasia increases with disease duration and extent [1, 3]. Therefore, surveillance colonoscopy with multiple-step biopsies has been widely recommended for patients with long-standing and extensive UC in...
order to diagnose UC-associated neoplasia at an early or precancerous stage [4, 5]. However, because UC-associated neoplasia is often difficult to detect endoscopically and to discriminate from inflammatory regenerative epithelium histologically, it remains a matter of contention whether conventional surveillance colonoscopy is effective for early detection [6–8]. 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.

Neoplastic progression in UC occurs in a histologically stepwise manner, from chronic epithelial inflammation to dysplasia, and to carcinoma, and the process of neoplastic progression involves accumulation of genetic and epigenetic alterations [9, 10]. Some of these alterations are known to occur in both the neoplastic and non-neoplastic epithelium of UC patients with neoplasia, and are considered to be widespread and to occur early in the process of neoplastic progression, suggesting that they could be applicable as markers for identifying patients who are likely to have, or to develop, neoplasia [11–21].

In several types of neoplasia, aberrant methylation of promoter-region CpG islands, as an epigenetic modification of DNA, is associated with transcriptional inactivation of tumor suppressor genes and plays a crucial role in the development and progression of neoplasia. DNA methylation results from a methyl transfer reaction performed by the three active DNA methyltransferases (DNMTs): DNMT1, DNMT3a and DNMT3b [22, 23]. Of these, DNMT1 is the most abundant DNMT targeted to replication foci and has a preference for hemimethylated DNA substrates [24]. It seems to be the main enzyme responsible for maintaining the methylation pattern after each round of DNA replication. Recent investigations have shown that DNMT1 is overexpressed in tumorigenic cells and several types of human tumors, and that increased expression of DNMT1 is dependent on cell proliferation [25–29]. However, to our knowledge, there are no reported data on DNMT1 expression in inflamed epithelium and neoplasia of UC.

In this study, to clarify the possible significance of increased expression of DNMT1 in the tumorigenesis of UC-associated neoplasia and to clarify whether analysis of DNMT1 expression in non-neoplastic epithelium can contribute to the prediction of increased risk for UC-associated neoplasia, we assessed the expression of DNMT1 in non-neoplastic rectal epithelium and colorectal neoplasia in patients with long-standing and extensive UC.

Materials and Methods

Patient Samples and Histological Evaluation

Sixty-two UC patients were included in this study: 31 (16 men, 15 women) with colorectal neoplasia and 31 (21 men, 10 women) without. The clinicopathological features of the UC patients are shown in Table 1. All patients had long-standing (≥7 years) and extensive (proximal to the splenic flexure) UC. Of the 31 UC patients with neoplasia, 20 had the highest histological grade of invasive cancer, 10 had high-grade dysplasia, and 1 had low-grade dysplasia. All UC patients without neoplasia were diagnosed as neoplasia-free by endoscopic and histological assessments throughout periodic surveillance colonoscopy on the basis of multiple-step biopsy samples. The mean (± SD) age of the UC patients with neoplasia was 49.9 ± 10.8 years (range 39–79), and that of UC patients without neoplasia was 46.9 ± 7.5 years (range 33–66). On the other hand, the mean (± SD) duration of disease in the UC patients with neoplasia was 17.2 ± 6.3 years (range 7–33), and that in the UC patients without neoplasia was 18.1 ± 7.3 years (range 10–36). There were no significant differences in age, sex, disease duration, disease extent, medication for UC between UC patients with, and those without, neoplasia.

Thirty-one paired samples of colorectal neoplastic tissue and corresponding non-neoplastic rectal epithelium from the 31 UC patients with neoplasia were retrieved from total colectomy specimens. The rectal epithelium samples from the 31 UC patients without neoplasia were obtained from step biopsy specimens during surveillance colonoscopy. Histologically, we confirmed that all of the 62 non-neoplastic samples from UC patients with and without neoplasia were negative for neoplasia in accordance with the Riddell classification of gastrointestinal epithelial neoplasia [9]. We also classified the inflammatory activity of each specimen into three categories: mild, moderate and severe inflammation. Histological evaluations were confirmed by two experienced gastrointestinal pathologists. The Ethics Committee of Dokkyo University School of Medicine approved all protocols, and informed consent for tissue procurement was obtained from all patients.

Immunohistochemical Analysis of DNMT1 Protein

As described previously, immunohistochemical analysis was carried out with a goat anti-human polyclonal antibody for DNMT1 (N16, dilution 1:50; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) in formalin-fixed paraffin-embedded tissue sections using an LSAB2 kit (Dako, Carpinteria, Calif., USA) [27, 30]. Finally, the immunoreactivity was visualized with 3,3′-diaminobenzidine tetrahydrochloride with 0.05% H2O2, followed by counterstaining with Carazzi’s hematoxylin. In each specimen, lymphocytes and macrophages on the same slide were used as an internal positive control for DNMT1 immunoreactivity.

The immunoreactivity DNMT1 was assessed in areas showing the highest density of cells with positively staining nuclei. A minimum of 500 nuclei in selected areas was counted under ×300 magnification. The level of immunoreactive DNMT1 expression was expressed as the percentage of positive cells relative to the total number of cells counted. Assessment of immunoreactive DNMT1 expression was done without knowledge of patient source.
Laser Capture Microdissection and RNA Extraction

Thirty-seven samples of fresh-frozen tissue were embedded in optimal cutting temperature (OCT) compound (Sakura Fine Technical, Tokyo, Japan) and stored at −80°C until study. These included 8 samples of neoplastic tissue, 15 samples of non-neoplastic epithelium from UC patients with neoplasia, and 14 samples of rectal epithelium from UC patients without neoplasia. Frozen sections 20 μm thick were cut in a cryostat, and then mounted on a clear polyethylene membrane attached to an aluminum frame slide (Molecular Machines & Industries AG, Zurich, Switzerland). To obtain only epithelial crypts for RNA extraction, laser capture microdissection was performed using a UV laser microdissection system (Molecular Machines & Industries AG). The caps were placed in a microcentrifuge tube, and the RNA was extracted using an RNeasy Micro kit (Qiagen, Tokyo, Japan) according to the manufacturer’s protocol.

Quantitative RT-PCR of DNMT1 mRNA

Total RNA was reverse transcribed using an oligo-dT primer (Applied Biosystems, Foster City, Calif., USA), and quantitative real-time PCR of DNMT1 and an internal reference gene (β-actin) was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Each amplification was done in 25 μl of a reaction mixture containing forward and reverse primer (5 μmol/l each), cDNA (2.5 μl) and 2 × SYBR Premix Ex TaqII (Takara, Shiga, Japan). Forward primers 5'-GGCCTTGCGCCCTCATA-3' and 5'-GGCCTTGCGCCCTCATA-3' and reverse primers 5'-GGCCTTGCGCCCTCATA-3' and 5'-GGCCTTGCGCCCTCATA-3' were used for DNMT1 and β-actin, respectively. To avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons or in a different exon. The PCR cycling conditions were 95°C for 10 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. The Human Universal Reference total RNA (Clontech, Palo Alto, Calif., USA) was used to construct a standard curve. Five serial 10-fold dilutions of the reference RNA were amplified to create standard curves for DNMT1 and β-actin. The relative amount of the mRNA was determined using the ratio of the target gene concentration relative to that of β-actin. The real-time PCR reactions were performed in triplicate for each sample–primer set, and the average value of triplicate determinations was used as the relative mRNA expression level in each sample.

Statistical Analysis

Age, disease duration, and expression levels of DNMT1 were expressed as mean ± SD, and the differences were analyzed using the t test. The correlation analyses were assessed using Pearson’s correlation coefficient. For all tests, differences of p < 0.05 were considered statistically significant, and all p values were two-sided.

Results

Immunoreactivity of DNMT1

The immunoreactivities of DNMT1 in samples of rectal epithelium from UC patients without neoplasia, non-neoplastic rectal epithelium from UC patients with neoplasia, and colorectal neoplastic tissues are shown in figure 1a. Examples of immunohistochemical staining for DNMT1 are shown in figure 2. The mean (± SD) levels of immunoreactive DNMT1 expression were 0.13 ± 0.07 in rectal epithelium from UC patients without neoplasia, 0.32 ± 0.12 in non-neoplastic rectal epithelium from UC patients with neoplasia, and 0.54 ± 0.20 in colorectal neoplastic tissues. The immunoreactive DNMT1 expressions increased gradually from rectal epithelium of UC patients without neoplasia to non-neoplastic rectal epithelium of UC patients with neoplasia (p < 0.001 compared with rectal epithelium from UC patients without neoplasia), and to colorectal neoplastic tissues (p < 0.001 compared with rectal epithelium from UC patients without neoplasia, p < 0.001 compared with non-neoplastic rectal epithelium from UC patients with neoplasia).

Table 1. Clinicopathological features of the patients

<table>
<thead>
<tr>
<th>Feature</th>
<th>UC with neoplasia (n = 31)</th>
<th>UC without neoplasia (n = 31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age at study, years</td>
<td>49.9 ± 10.8</td>
<td>46.9 ± 7.5</td>
</tr>
<tr>
<td>Male/female</td>
<td>16/15</td>
<td>21/10</td>
</tr>
<tr>
<td>Mean disease duration in UC, years</td>
<td>17.2 ± 6.3</td>
<td>18.1 ± 7.3</td>
</tr>
<tr>
<td>Disease extent (total/left)</td>
<td>27/4</td>
<td>24/7</td>
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<tr>
<td>Medication for UC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SASP/5-ASA</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Immunosuppressive drug</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Location of neoplasia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Left colon</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Right colon</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Histology of neoplasia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LGD</td>
<td>1</td>
<td></td>
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<tr>
<td>HGD</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Carcinoma*</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Grade 2</td>
<td>3</td>
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<td>Grade 3</td>
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<tr>
<td>UICC stage</td>
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<tr>
<td>UICC I</td>
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<tr>
<td>UICC II</td>
<td>1</td>
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<tr>
<td>UICC III</td>
<td>9</td>
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<tr>
<td>UICC IV</td>
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</table>

Total = Total colitis; left = left-sided colitis. SASP = salicylazosulapyridine; 5-ASA = 5-aminosalicylic acid. LGD = low-grade dysplasia; HGD = high-grade dysplasia.

*Histological grading and staging of carcinoma were done according to UICC (Union International Against Cancer) and WHO criteria.

DNMT1 Expression in Ulcerative Colitis
Among the 31 samples of non-neoplastic rectal epithelium from UC patients with neoplasia, there was no significant difference in the immunoreactive DNMT1 expression between non-neoplastic rectal epithelium from UC patients with dysplasia and those with invasive cancer (0.33 ± 0.38 vs. 0.32 ± 0.34, p = 0.90). Among the 31 samples of neoplasia including 11 dysplasias and 20 invasive cancers, the mean (±SD) levels of immunoreactive DNMT1 expression were 0.47 ± 0.52 and 0.58 ± 0.63, respectively. There was no statistically significant difference between them (p = 0.63).

Correlation of DNMT1 Immunoreactivity Level in Rectal Epithelium with Patient Age, Disease Duration and Inflammatory Activity of Colitis

In both UC with and without neoplasia, there were no significant correlations between the immunoreactive DNMT1 expression in rectal epithelium and patient age (r = 0.315, p = 0.102; r = −0.027, p = 0.885, respectively). Similarly, there were no significant correlations between the immunoreactive DNMT1 expression in rectal epithelium and disease duration (r = 0.169, p = 0.390; r = 0.062, p = 0.741, respectively).

The correlation between the immunoreactive DNMT1 expression and inflammatory activity of colitis was eval-
uated in the 62 samples of rectal epithelium from patients with and without neoplasia (38 mild, 16 moderate, 8 severe inflammation). The immunoreactive DNMT1 expression in rectal epithelium with mild, moderate, severe inflammation were 0.23 ± 0.13, 0.20 ± 0.14 and 0.26 ± 0.17, respectively. There were no significant differences in immunoreactive DNMT1 expressions among the different severities of inflammation.

**DNMT1 mRNA Expression**

The expression levels of DNMT1 mRNA in samples of rectal epithelium from UC patients without neoplasia, non-neoplastic rectal epithelium from UC patients with neoplasia, and colorectal neoplastic tissues are shown in figure 1b. The mean (±SD) expression levels of DNMT1 mRNA were 0.53 ± 0.34 in rectal epithelium from UC patients without neoplasia, 0.88 ± 0.57 in non-neoplastic rectal epithelium from UC patients with neoplasia, and 1.38 ± 0.64 in colorectal neoplastic tissues. The expression level of DNMT1 mRNA in samples of colorectal neoplasia was significantly higher than that in rectal epithelium from UC patients without neoplasia (p < 0.001). Although the difference was not statistically significant, the expression level of DNMT1 mRNA tended to increase gradually from rectal epithelium of UC patients without neoplasia to non-neoplastic rectal epithelium of UC patients with neoplasia (p = 0.06 compared with rectal epithelium from UC patients without neoplasia), and to colorectal neoplasia (p = 0.07 compared with non-neoplastic rectal epithelium from UC patients with neoplasia).

**Correlation of Immunoreactivity with mRNA Expression of DNMT1**

As shown in figure 3, when the immunoreactive and mRNA expressions were compared on an individual basis, immunoreactive DNMT1 expression correlated well with mRNA expression from the same tissue (r = 0.576, p < 0.001).

**Discussion**

In this study, we have shown that expression of DNMT1 increased with progression from rectal epithelium of UC patients without neoplasia to non-neoplastic rectal epithelium of UC patients with neoplasia, and then to UC-associated colorectal neoplasia. Furthermore, the immunoreactive expression of DNMT1 in dysplasia was not significantly different from that in invasive cancer. These results suggest that an increase in the expression of DNMT1 may precede, or be a relatively early event in UC-associated tumorigenesis, and that analysis of DNMT1 expression in non-neoplastic rectal epithelium may be of potential use for identifying individuals at increased risk of neoplasia among patients with long-standing and extensive UC.

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 [4, 5]. However, according to several studies that have assessed the efficacy of surveillance, an appreciable number of neoplasia were detected at an advanced stage despite surveillance colonoscopy, and many of these cases had less-than-ideal outcomes [6–8]. Thus, it still remains questionable whether surveillance colonoscopy with multiple-step 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 endoscopical and histological diagnosis 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 [31]. 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, similarly to sporadic colorectal neoplasia, diagnosis of UC-associated neoplasia is based on a combination of architectural and cytological alterations. However, because these alterations are often unremarkable and are limited to the lower half of the crypt or a few crypts in the inflamed epithelium, it is not unusual for pathologists to experience difficulty in discriminating between a neoplastic lesion and inflammatory regenerative epithelium using biopsy specimens stained with hematoxylin and eosin [9]. Furthermore, different pathologists may use different diagnostic criteria for determining UC-associated neoplasia [32].

In order to overcome such difficulties, adjunctive diagnostic modalities such as chromoendoscopy and narrow-band imaging endoscopy for identifying neoplasia in chronically inflamed epithelium, and analysis of p53 alteration for distinguishing neoplastic lesions from regenerative epithelium have been reported [30, 33–37]. However, it would be impractical to perform these adjunctive modalities for surveillance of all UC patients with longstanding and extensive colitis. If refinement of high- and low-neoplasia risk subgroups of UC patients with longstanding and extensive colitis were possible, it would enable physicians to conduct more intensive surveillance using the adjunctive modalities, chromoendoscopy and narrow-band imaging endoscopy and analysis of p53 alteration, for patients at higher risk of developing colorectal neoplasia, and to improve the effectiveness of surveillance colonoscopy.

Numerous studies have revealed molecular alterations (e.g. p53 mutation, chromosomal instability, p16 hypermethylation, p14 hypermethylation, microsatellite instability, age-related methylation, telomere length shortening, mitochondrial DNA mutation, gene expression using DNA microarray) of non-neoplastic epithelium in UC patients with neoplasia [11–19]. Several of these studies have indicated higher frequencies of molecular alterations in non-neoplastic epithelium of UC patients with neoplasia than in that of UC patients without neoplasia, suggesting that these molecular alterations may be applicable as new markers for identifying individuals with UC at increased risk of neoplasia.

Recently, we evaluated the methylation status of the estrogen receptor (ER), which is affected by age-related methylation in the colorectal epithelium, in samples of non-neoplastic colorectal epithelium from UC patients with and without neoplasia, and we demonstrated that the ER gene was highly methylated in non-neoplastic epithelium from UC with neoplasia compared to that without neoplasia [20, 21]. In addition, methylation of the ER gene was detected not only in regions of neoplasia, but also in other regions widely scattered throughout the colon-rectum. These findings implied that analysis of ER gene methylation in non-neoplastic colorectal epithelium of UC patients may be a useful molecular marker for helping predict the risk of colorectal neoplasia. However, analysis of methylation involves a great deal of time and expense, and would be difficult to put to practical use as a molecular marker. Therefore, a new molecular marker that is readily available in clinical laboratories should be established.

Although conflicting results between the DNA methylation status of specific genes and DNMT1 expression have been described in the literature, increased expression of DNMT1 potentially results in hypermethylation of CpG islands [25–28]. In the present study, therefore, we investigated whether analysis of DNMT1 expression in non-neoplastic epithelium is a candidate molecular marker for predicting increased risk of UC-associated neoplasia, similarly to methylation of the ER gene. The most noteworthy result of this study was that the immunoreactive DNMT1 expression in non-neoplastic rectal epithelium from UC patients with neoplasia was significantly higher than that in rectal epithelium of UC patients without neoplasia, even though there was no remarkable difference histologically between the two types of epithelium. Similarly, although the difference determined by quantitative RT-PCR assay did not reach statistical significance, the expression level of DNMT1 mRNA tended to be higher in non-neoplastic rectal epithelium from UC patients with neoplasia than in rectal epithelium from UC patients without neoplasia. These results imply that the increased expression of DNMT1 in non-neoplastic rectal epithelium may be indicative of concurrent or future neoplasia somewhere in colorectum.

Although the mechanism responsible for increased expression of DNMT1 in UC is not well known, the previous reports that DNMT1 protein overexpression was significantly associated with the PCNA-labeling index in bladder cancer suggests that the expressions of DNMT1 immunoreactivity and mRNA we observed in UC may be attributable to increased cell proliferation [38]. In addi-
tion, previous studies have demonstrated increased DNMT1 expression in non-neoplastic liver tissue with chronic hepatitis or liver cirrhosis and non-neoplastic pancreatic tissue with chronic pancreatitis, suggesting that the increase in expression may be related to chronic inflammation [27, 29]. In the present study, the inflammatory activity of colitis was unrelated to the immunoreactive DNMT1 expression, suggesting that such expression does not simply reflect transient inflammation, and that cumulative and repetitive inflammation may lead to an increase of DNMT1 expression.

Because immunostaining using formalin-fixed, paraffin-embedded sections from the biopsy specimens used for routine histopathological diagnosis is simple, reliable, and reproducible, immunohistochemical analysis of DNMT1 may be sufficiently acceptable as a routine procedure. Therefore, DNMT1 expression would become a readily available molecular marker for identifying UC patients at high risk of neoplasia elsewhere in the colorectum or future neoplastic progression. In this preliminary study, due to the small sample size, it was not possible to determine with accuracy the precise expression level of DNMT1 that would identify UC patients at high risk for neoplasia. Although further longitudinal studies with large numbers of subjects are needed to clarify the role of DNMT1 in the pathogenesis of UC-associated neoplasia, and to validate the predictive power and clinical utility of analysis of DNMT1 expression, these preliminary results could allow, on the basis of DNMT1 expression status, for the adjustment of a patient’s surveillance interval and to select UC patients who should undergo intensive surveillance.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of the article.

References


