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Original Article

Expression of CD44 and MDM2 in cholangiocarcinoma is correlated with poor clinicopathologic characteristics

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Abstract: The aim of the study was to investigate the expression of cluster of differentiation 44 (CD44) and mouse double minute 2 (MDM2) in cholangiocarcinoma, in addition to evaluating their association with clinicopathologic characteristics and overall survival time. Paraffin-embedded tumor tissues from 128 patients from 3 study centers in Thailand were evaluated using immunohistochemistry. The results demonstrated that positive expression of CD44 was associated with high histologic grade (*P*=0.013), large tumor size (*P*=0.027), lymph node metastasis (*P*=0.037), and distant metastasis (*P*=0.031). MDM2 expression was related to high histologic grade (*P*=0.013), lymph node metastasis (*P*=0.025), and distant metastasis (*P*=0.016). Furthermore, multivariate analyses revealed that combined expression of CD44 and MDM2 was significantly associated with worse overall survival time (OR=1.52; 95% CI=1.04-2.26; *P*=0.041) in patients with cholangiocarcinoma. CD44 and MDM2 significantly indicate poor clinicopathologic outcomes in patients with cholangiocarcinoma.

Keywords: CD44, MDM2, cholangiocarcinoma, clinicopathologic characteristics, prognosis

Introduction

Cholangiocarcinoma (CCA) originates from bile duct epithelial cells, and can be caused by chronic inflammation due to *Opisthochis viverini* infection. The highest prevalence of CCA is found in Southeast Asia, especially in Northeast Thailand [1, 2]. The mortality rate from CCA remains high because most patients are diagnosed at an advanced stage due to a delay in diagnosis; additionally, chemotherapeutic responses vary, even for the same disease stage. Many researchers have investigated prognostic markers for CCA. However, no specific or robust CCA biomarkers have been found.

The cluster of differentiation 44 (CD44) antigen is a cell surface glycoprotein involved in cell-cell interactions, cell adhesion, and migration that is important in many types of cancer [3]. Several studies have investigated the role of CD44 in CCA. High expression of CD44 in the biliary epithelium may indicate unfavorable patient outcome, and may serve as a useful, and practical adjunct to conventional prognostic indicators for CCA [4, 5]. The elimination of cancer stem cells (CSCs) by targeting CSC-specific CD44 isoforms may lead to more favorable treatment outcomes [6].

Mouse double minute 2 homologue (MDM2) is an important negative regulator of tumor protein TP53, which is involved in the carcinogenesis of most types of human cancer, including CCA [7]. TP53 is a tumor-suppressor that plays a key role in the control of various processes of cancer progression and apoptosis [8]. Mutation of the *TP53* gene has been linked with CD44 expression [9]. It was demonstrated that TP53 transcriptionally suppresses the expression of CD44 in both normal and tumor-derived mammary epithelial cells by directly binding to the *CD44* promoter. Moreover, the down-regulation of *CD44* expression was found to be a prerequisite for TP53-dependent growth regulation and the induction of apoptosis in mammary epithelium [10]. Horie *et al.* demonstrated that MDM2 overexpression is also correlated with TP53 overexpression and the Ki-67 labelling index, suggesting that MDM2 overexpression may play a role in late stage CCA [11]. The presence

of *TP53* mutations or the up-regulation of *MDM2* gene expression in CCA strongly suggests that the impairment of the TP53 pathway is an important and specific step in CCA pathogenesis [5]. As previous studies have reported, we hypothesized that the CD44 and MDM2 status might be associated with certain clinicopathologic characteristics that predict a poor prognosis for patients with CCA. In this study, we aimed to investigate CD44 and MDM2 expression and to evaluate the association with poor clinicopathologic outcomes for CCA. These findings may help to select patients who might benefit from use of this practical adjunct to conventional prognostic indicators for CCA.

Patients and methods

Patients and specimens

Samples were obtained from patients with CCA who underwent surgical resection at Suranaree University of Technology Hospital, Buriram Hospital, and Surin Hospital in Northeast Thailand between January 2011 and December 2016. Informed consent was obtained from all patients. Ethical approval to perform this study was granted by the Ethics Committee for Research Involving Human Subjects, Suranaree University of Technology (EC-16-2560). The methods were carried out in accordance with good clinical practice and the guidelines of the Declaration of Helsinki. The patients were clinically evaluated according to TNM staging for liver tumors developed by the American Joint Committee on Cancer (seventh edition, 2010) [12] for tumor stage, histologic grade, and metastasis. Fibrosis scores and overall survival rates were also examined. All tissues were fixed in 10% formalin, embedded in paraffin and cut into 4-μm sections.

Immunohistochemistry

The avidin-biotin complex method (ABC; Thermo Fisher, Illinois, USA.) was used for immunohistochemical detection of CD44 and MDM2 expression. Consecutive sections were deparaffinized using xylene and were rehydrated in a graded series of alcohol solutions (100, 95, 80 and 70%) to water. The sections were unmasked by heating in a microwave oven at 500 W for 5 min in a 10 mM citrate buffer (pH 6.0) and were incubated with 5% normal serum for 1 h at room temperature to block nonspecific background staining. The slides were then incubated with monoclonal mouse homing cell adhesion molecule (HCAM) (1:100, clone DF1485; Santa Cruz Biotechnology, Inc. Dallas, TX, USA) or MDM2 antibody (1:100, clone SMP 14; Santa Cruz Biotechnology, Inc.) overnight at 4°C in a moist chamber. Subsequently, the sections were washed three times in phosphate-buffered saline and were incubated with the biotinylated goat anti-mouse secondary antibody (1 μg/ml) for 30 min, followed by incubation with ABC-conjugated avidin-biotin-complex (Thermo Fisher, Rockford, IL, USA). The colour was developed using an aminoethyl carbazole substrate solution (Life Technologies Corp, Carlsbad, CA, USA), followed by counterstaining with Mayer's haematoxylin.

Immunohistochemistry evaluation

The assessment of CD44 and MDM2 staining was based on the percentage of positively stained cells, 500 cells per field in at least five different fields were counted under high magnification (×400) and were scored as follows: lack of staining was scored as 0; 1-10% staining was classified as 1+; >10 and ≤50% staining as 2+; and >50% as 3+. Cases classified as 0 were defined as negative, whereas 1+, 2+ and 3+ were considered positive cases. The results were reviewed independently by three pathologists who were blinded to the clinicopathologic details of the patients. The discrepancy between the pathologists' analyses was minimal when present and was resolved by consensus.

Statistical analysis All statistical analyses were conducted using SPSS version 20 software (IBM, Armonk, NY, USA). Differences in age, gender, underlying condition, and family history of biliary tract cancer between CD44 or MDM2 positive and negative patients were assessed using chi-square test or Fisher's exact test. Associations between CD44 or MDM2 protein expression and clinicopathologic characteristics of CCA were evaluated by univariate and multivariate Cox proportional hazard regression analysis. To assess the prognostic index, a univariate Cox regression model analysis was first used. *P*-Values of less than 0.05 from the univariate analysis were then assessed in the final analysis by using multivariate Cox proportional hazards regression modelling with a step-wise forward selection methodology. A survival analysis was performed using the

Table 1. Clinicopathologic characteristics of patients with cholangiocarcinoma according to cluster of differentiation 44 (CD44) and mouse double minute 2 (MDM2) expression

	CD44			MDM2		
	Positive	Negative	*p*-value	Positive	Negative	*p*-value
Age (year ± SD)	62 ± 9.74	59 ± 8.46	0.427	64 ± 5.63	58 ± 6.35	0.146
Male, n (%)	78.1	73.4	0.382	70.3	65.6	0.094
Underlying condition, n (%)						
HT, n (%)	12 (9.37)	8 (6.25)	0.069	6 (4.68)	14 (10.93)	0.074
DM, n (%)	6 (4.68)	4 (3.12)	0.514	2 (1.56)	8 (6.25)	**0.048**
Hyperlipidemia, n (%)	4 (3.12)	2 (1.56)	0.094	4 (3.12)	2 (1.56)	0.094
Smoking, n (%)	18 (14.06)	14 (10.93)	0.771	12 (9.37)	20 (15.62)	0.083
Alcohol, n (%)	22 (17.18)	16 (12.50)	0.337	18 (14.06)	20 (9.37)	0.941
Family history of biliary tract cancer, n (%)	4 (3.12)	2 (1.52)	0.094	4 (3.12)	2 (1.52)	0.094

HT: Hypertension; DM: diabetes mellitus. Statistically significant *P*-values are shown in bold.

Table 2. The distribution of clinicopathologic characteristics of cholangiocarcinoma according to expression of cluster of differentiation 44 (CD44) protein

Characteristic	Subgroup	Univariate analysis			Multivariate analysis	
		Expression of CD44 protein, n (%)			Expression of CD44 protein, n (%)	
		Negative	Positive	*P*-value	OR (95% CI)	*P*-value
Histologic grade	1, 2	42 (58.33)	30 (41.67)			
	3, 4	12 (21.42)	44 (78.57)	**0.024**	2.24 (2.06-2.87)	**0.013**
Primary tumor	T1, 2	26 (41.93)	36 (58.06)			
	T3, 4	18 (27.27)	48 (72.72)	**0.018**	1.82 (1.29-1.94)	**0.027**
Regional lymph nodes	N0	26 (46.42)	30 (53.57)			
	N1	14 (19.44)	58 (80.55)	**0.029**	1.79 (1.18-2.04)	**0.037**
Distant metastasis	M0	56 (54.90)	46 (45.10)			
	M1	6 (23.07)	20 (76.92)	**0.016**	1.63 (0.92-1.36)	**0.031**
Location of tumor	Intrahepatic	20 (43.47)	26 (56.52)			
	Extrahepatic	26 (31.70)	56 (68.28)	**0.038**	1.02 (0.97-1.25)	0.069
Fibrosis score	None to moderate	42 (53.84)	36 (46.15)			
	Severe	22 (44.00)	28 (56.00)	0.337		

OR: Odds ratio; CI: confidence interval. Statistically significant *P*-values are shown in bold.

Kaplan-Meier method, and survival differences were analyzed using the log-rank test. *P*-Values of less than 0.05 were considered significant.

Results

Patient characteristics

The characteristics of the patients enrolled in the study are summarized in **Table 1**. The study included 100 males and 28 females, with ages ranging from 40-83 (median=62.89) years. There were no significant differences between the samples that were positive or negative for CD44 and MDM2 expression according to age, gender, or underlying conditions except that in patients with diabetes, the frequency of MDM2 positivity was significantly lower than MDM2 negativity (**Table 1**).

Association between CD44 expression and clinicopathologic characteristics

A total of 128 CCA cases were examined, of which 72 patients had well- or moderately differentiated adenocarcinoma (grade 1-2), while 56 patients had poorly differentiated adenocarcinoma (grade 3-4), according to TNM staging. Tumor location was analysed, and 82 patients were classified as having an extrahepatic CCA tumor (**Table 2**). There were 62 cases of T1-T2 stage disease, and 66 cases were T3-T4 stage.

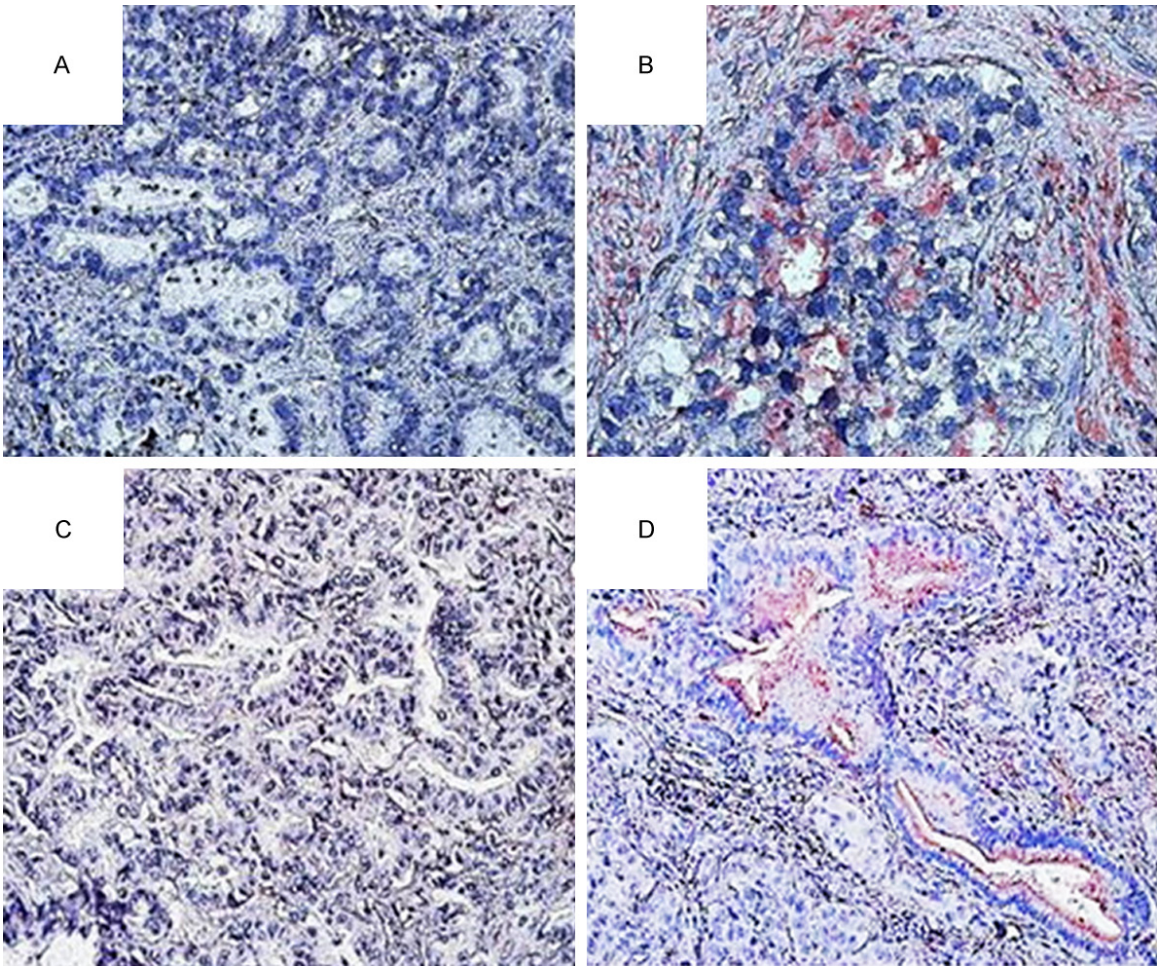

Figure 1. Representative photomicrographs of immunohistochemistry for cluster of differentiation 44 (CD44) and mouse double minute 2 (MDM2) expressions in cholangiocarcinoma tissues. Negative and positive staining for (A, B) CD44, and (C, D) MDM2. The red staining indicates the presence of CD44 and MDM2 protein in cholangiocarcinoma tissues (magnification, ×200).

Lymph node metastasis was observed in 72 cases and 26 patients had distant metastasis. Moreover, severe fibrosis was found in 50 patients (**Table 2**).

CD44 staining was detected in 102/128 of the samples (79.7%), which was mainly localized to the membrane and cytoplasm of the cancer cells (**Figure 1**). According to univariate analysis, CD44-positive staining was associated with high histologic grade (P=0.024), large tumor size (P=0.018), lymph node metastasis (P=0.029), distant metastasis (P=0.016), and extrahepatic tumor location (P=0.038). In contrast, no significant association was observed between CD44-positive staining and fibrosis score (P=0.337) (**Table 2**). The variables that were significantly associated with CD44 expression in the univariate analysis were further analysed by multivariate analysis using a Cox regression model. In the multivariate analysis, extrahepatic CCA was not associated with CD44-positive staining (P=0.069) (**Table 2**). However, high histologic grade (P=0.013), large tumor size (P=0.027), lymph node metastasis (P=0.037) and distant metastasis (P=0.031) were associated with CD44-positive staining (**Table 2**).

Association between MDM2 expression and clinicopathologic characteristics

MDM2 staining was detected in 100/128 of the samples (78.13%), which stained mainly in the membrane and cytoplasm of the cancer cells (**Figure 1**). Based on univariate analysis, MDM2 positivity was significantly associated with clinicopathologic features including high

Table 3. The distribution of clinicopathologic characteristics of cholangiocarcinoma according to expression of mouse double minute 2 (MDM2) oncoprotein

Characteristic	Subgroup	Univariate analysis			Multivariate analysis	
		Expression of MDM2 protein, n (%)			Expression of MDM2 protein, n (%)	
		Negative	Positive	*P*-Value	OR (95% CI)	*P*-Value
Histologic grade	1, 2	42 (58.33)	30 (41.67)			
	3, 4	18 (32.15)	38 (67.85)	**0.024**	1.82 (1.46-2.57)	**0.013**
Primary tumor	T1, 2	34 (54.83)	28 (45.17)			
	T3, 4	30 (45.45)	36 (54.55)	0.237		
Regional lymph nodes	N0	32 (57.14)	24 (42.86)			
	N1	22 (30.56)	50 (69.44)	**0.036**	1.74 (1.84-3.21)	**0.025**
Distant metastasis	M0	56 (54.90)	46 (45.10)			
	M1	4 (15.38)	22 (84.62)	**0.015**	1.98 (1.62-2.06)	**0.016**
Location of tumor	Intrahepatic	18 (39.13)	28 (60.87)			
	Extrahepatic	34 (41.46)	48 (58.54)	0.086		
Fibrosis score	None to moderate	48 (61.54)	30 (38.46)			
	Severe	12 (24.00)	38 (76.00)	0.489		

OR: Odds ratio; CI: confidence interval. Statistically significant *P*-values are shown in bold.

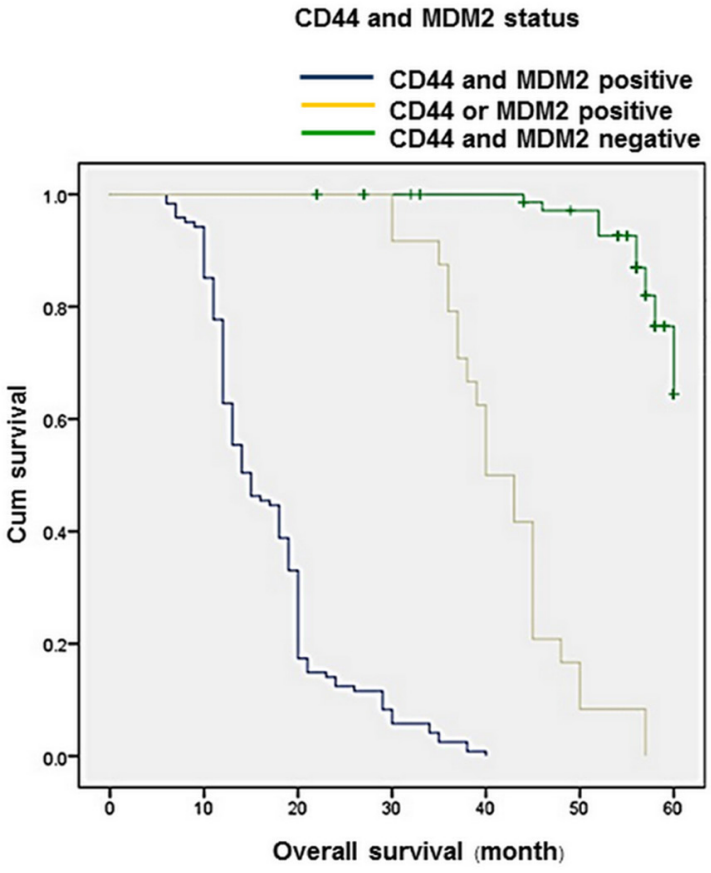

Figure 2. Association between combined expression of CD44 and MDM2 in patients with cholangiocarcinoma and overall survival. Combined expression of CD44 and MDM2 (blue line) showed significantly worse overall survival time than those with negative expression (green line; *P*=0.016).

histologic grade (*P*=0.024), lymph node metastasis (*P*=0.036), and distant metastasis (*P*=0.015). No significant association was observed between staining group and tumor size, tumor location or severe fibrosis (*P*>0.05) (**Table 3**). Similarly, the multivariate analysis demonstrated that high histologic grade (*P*=0.013), lymph node metastasis (*P*=0.025), and distant metastasis (*P*=0.016) were directly associated with MDM2 positivity (**Table 3**).

Association between cancer patients' survival and the combination of CD44 and MDM2 expression

The Kaplan-Meier survival curve showed that CD44 or MDM2-positive patients exhibited shorter survival than CD44 and MDM2-negative patients (median 42.12 months; mean, 58.72 months) (*P*=0.027), particularly for patients who had both CD44 and MDM2 expression (median, 16.88 months) (*P*=0.016) (**Figure 2**). Then, we used Cox multivariate regression model to test the independent value of each variable for predict-

ing overall survival (OS). The result showed that combined expression of CD44 and MDM2 was associated with OS time of gastric cancer patients (OR=1.52; 95% CI, 1.04-2.26; *P*= 0.041). On the other hand, no significant differences were observed in patients with CD44 or MDM2-positive expression (*P*>0.05). These results indicated that combined expression of CD44 and MDM2 could be used as independent prognostic factors of poor prognosis.

Discussion

As far as we are aware, this study provides the first report on the association between CD44 expression, MDM2 expression, and the clinicopathologic characteristics of CCA. Our results revealed that positive expression of CD44 in tumor that were classified as histologic grade G3-G4, large tumor size, and as having lymph node or distant metastasis were significantly associated with a poor prognosis in patients with CCA. MDM2 expression was related to high histologic grade, lymph node metastasis, and distant metastasis. Moreover, combined expression of CD44 and MDM2 was found to be a statistically significant risk factor for worse OS. In recent years, the incidence and mortality of CCA have been increasing worldwide. Despite the substantial advances in surgery and chemotherapy for CCA over the past few decades, therapeutic failure and disease progression are still quite frequent. Moreover, there are no effective prognostic markers for CCA. In this study, we identified a potentially valuable marker that is associated with CCA and might be used to predict prognosis and to optimize therapy.

As the CSC hypothesis has become one of the predominant theories explaining tumor capacity and the heterogeneity of tumor cells, various stem cell markers have been correlated with clinicopathologic characteristics and prognosis has been examined [13, 14]. CD44 was first described as a CSC marker in breast cancer, and it can be used as a CSC marker and prognostic factor in various types of human cancer, including CCA [4, 15, 16]. Primary tumor growth is not the main cause of cancer-related death; rather the main cause of cancer-related death is the formation of metastases in distant organs. Evidence has indicated CD44 plays an important role in this complicated process and is linked to the development and spread of cancer [17, 18]. An increasing number of studies have noted that CD44 is overexpressed in multiple human cancer types including CCA, and contributes to tumor progression and metastasis, resulting in poor survival rates [4, 5, 19]. Our study also found an association between CD44 and clinicopathologic characteristics related to poor prognosis in CCA. Positive CD44 expression in tumors was associated with high histologic grades, large tumor size, lymph node metastasis, and distant metastasis. These findings support the role of CD44 in patients with CCA and demonstrate that a CD44-positive tumor suggests a poor prognosis. Thus, CD44 positivity might be a candidate CSC marker in CCA, as well as a marker for prognosis.

The growth of cancer tissue depends on both cell proliferation and the rate of cell death [20]. Therefore, it is conceivable that neoplastic growth may be caused or promoted by factors that inhibit cell death. MDM2 is an oncoprotein that inhibits the TP53 tumor-suppressor protein, resulting in cancer progression. The overexpression of this protein has been observed in various human malignancies, and these abnormalities have a role in tumorigenesis [21]. Horie *et al.* reported the overexpression of MDM2 by immunohistochemistry and the relationship between its expression and histologic grade, clinicopathologic features, TP53 overexpression, and the Ki-67 labelling index in intrahepatic CCA [11]. We also observed that MDM2 expression was associated with clinicopathologic characteristics such as high histologic grade, lymph node metastasis, and distant metastasis. Interestingly, the combined expression of CD44 and MDM2 was more strongly associated with OS than either expression alone. Our observations indicate that immunohistochemical staining of CD44 and MDM2 expression may be a candidate marker that might be used to predict poor prognosis in CCA. The limitations of this study were an insufficient number of samples tested and the limited OS survival data. This may have been the reason why the OS survival and CD44 or MDM2 expression were not significantly correlated.

In conclusion, our study demonstrated that in patients with CCA positive CD44 and MDM2 expression was associated with unfavorable clinicopathologic characteristics and poor survival rates. These results show that the dual expression of CD44 and MDM2 may be a biomarker predicting poor prognosis and may be useful in selecting individual treatment modalities for CCA.

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Disclosure of conflict of interest

None.

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