

Original Article

Downregulation of ring-finger protein 43 in glioma associates with poor prognosis

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Received October 25, 2014; Accepted December 22, 2014; Epub January 1, 2015; Published January 15, 2015

Abstract: Ring-finger protein 43 (RNF43) has been identified as a RING-type E3 ubiquitin ligase. The overexpression of RNF43 results in a significant enhancement of cell growth, and knockdown of the expression of RNF43 by siRNAs exerts a growth-suppressing effect. RNF43 has been proposed as a tumor suppressor in colorectal cancers. However, its expression significance in glioma has not been well studied. In the present study, we found that the decreased expression of RNF43 was associated with poor prognosis by the Kaplan-Meier survival analysis ($P < 0.001$). Importantly, multivariate analysis suggested RNF43 as an independent predictor of overall survival ([hazard ratio (HR) 0.547, 95% confidence interval (95% CI) 0.382-0.784, $P = 0.001$]). Collectively, our study demonstrated that RNF43 could be served as a potential prognostic marker for patients with this deadly disease.

Keywords: Ring-finger protein 43 (RNF43), glioma, survival, prognostic marker

Introduction

Glioma is the most common human primary central nervous tumor, and astrocytic glioma composes the largest subgroup of gliomas [1-3]. Radiation exposure and certain genetic syndromes are well-known and well-defined risk factors for glioma [4]. The World Health Organization (WHO) grade is an estimate of the malignancy of a glioma [5]. This grading has been systematically evaluated and successfully applied to a spectrum of diffusely infiltrative astrocytic tumors that range from grade I to IV.

The survival time of glioma patients is known to be variable, and this variability might be related to the WHO grade, tumor location (i.e., supratentorial or subtentorial), surgical treatment, postoperative course, radiotherapy, chemotherapy, patient age, glioblastoma multiforme (GM) recurrence, imaging characteristics, serum, molecular markers and other factors. However, few factors are likely to be good predictors of long-term survival. More than two decades of research has identified several

important molecular events, such as the over-activation of the epidermal growth factor receptor (EGFR) and phosphatidylinositol 3 kinase (PI3K)/Akt pathways and inactivation of the p53/Rb pathways, as important contributors to glioma development. These alterations facilitate cell survival, proliferation, and migration and are thus crucial for tumorigenesis [6]. Another important factor that has recently emerged as a significant determinant of glioma initiation and progression is the canonical Wnt/ β -catenin signaling pathway, and this contribution is independent of the direct survival advantage conferred by Wnt signaling to the tumor cells [7].

Ring-finger protein 43 (RNF43) has been identified as a RING-type E3 ubiquitin ligase and has been reported to be highly expressed in colorectal cancers. The overexpression of RNF43 results in a significant enhancement of cell growth, and knockdown of the expression of RNF43 by siRNAs exerts a growth-suppressing effect. These findings suggest that RNF43 participates in the process of colorectal

Table 1. Relationship between the expression of RNF43 and clinicopathologic characteristics

Variable	RNF43			P value
	No.	Low expression No. (%)	High expression No. (%)	
Age				0.055
< 50 y	172	60 (34.9)	112 (65.1)	
≥ 50 y	57	28 (49.1)	29 (50.9%)	
Sex				0.279
Male	125	52 (41.6)	73 (58.4)	
Female	104	36 (34.6)	68 (65.4)	
Tumor location				0.309
Supratentorial	199	79 (39.7)	120 (60.3)	
Subtentorial	30	9 (30)	21 (70)	
WHO grade				0.631
Low grade (grade I to II)	93	34 (36.6)	59 (63.4)	
High grade (grade III to IV)	136	54 (39.7)	82 (60.3)	
Status				0.002
Live	105	29 (27.6)	76 (72.4)	
Death	124	59 (47.6)	65 (52.4)	
Relapse				0.778
Yes	141	41 (46.6)	47 (53.4)	
No	88	63 (44.7)	78 (55.3)	

carcinogenesis [8]. Moreover, RNF43 has also been found to be an important tumor suppressor gene in mucinous ovarian tumours [9]. However, the role and mechanism of RNF43 in the progression of carcinogenesis have not been fully elucidated. The present study is the first study of the role of RNF43 in glioma.

Methods

Patients and tissue specimens

In the present study, paraffin-embedded pathologic specimens from 229 patients with glioma were obtained from the archives of the Department of Pathology, Sun Yat-sen University Cancer Center, China, between November 2000 and December 2008. The cases were selected based on the distinctive pathologic diagnoses of gliomas of patients who had undergone primary and curative resections for tumors without preoperative chemotherapy or radiotherapy, the availability of resection tissue, and the follow-up data. The glioma cases included 125 men and 104 women, and the mean age of the patients was 58.8 years. The average follow-up time was 52.46 months (median, 45.25 months; range, 4-116 months). Patients whose cause of death was unknown were excluded from the study. The clinicopathologic characteristics of these patients, including age, sex, tumor location,

WHO grade and relapse, are detailed in **Table 1**. WHO grade was defined according to the 4th edition of WHO Classification of Tumors of the Central Nervous System [10]. The institute research medical ethics committee of Sun Yat-sen University Cancer Center granted approval for this study. All patients provided signed consent forms indicating that they agreed to donate the tissue for the medical study when they were hospitalized. Therefore, our ethics committee waived the need for consent.

Tissue microarray (TMA) construction

Tissue microarray was constructed for the study. Briefly, formalin-fixed, paraffin-embedded tissue blocks and the corresponding Hematoxylin and Eosin -Stained slides were overlaid for TMA sampling. A senior pathologist (J. Z) reviewed the slides to determine and mark out representative tumor areas. Duplicates of 1 mm diameter cylinders were punched from representative tumor areas of individual donor tissue block and re-embedded into a recipient paraffin block at defined position, using a tissue arraying instrument. (Beecher Instruments, Silver Spring, MD).

Immunohistochemical (IHC) staining

The TMA slides were dried overnight at 37°C, deparaffinized in xylene, rehydrated through

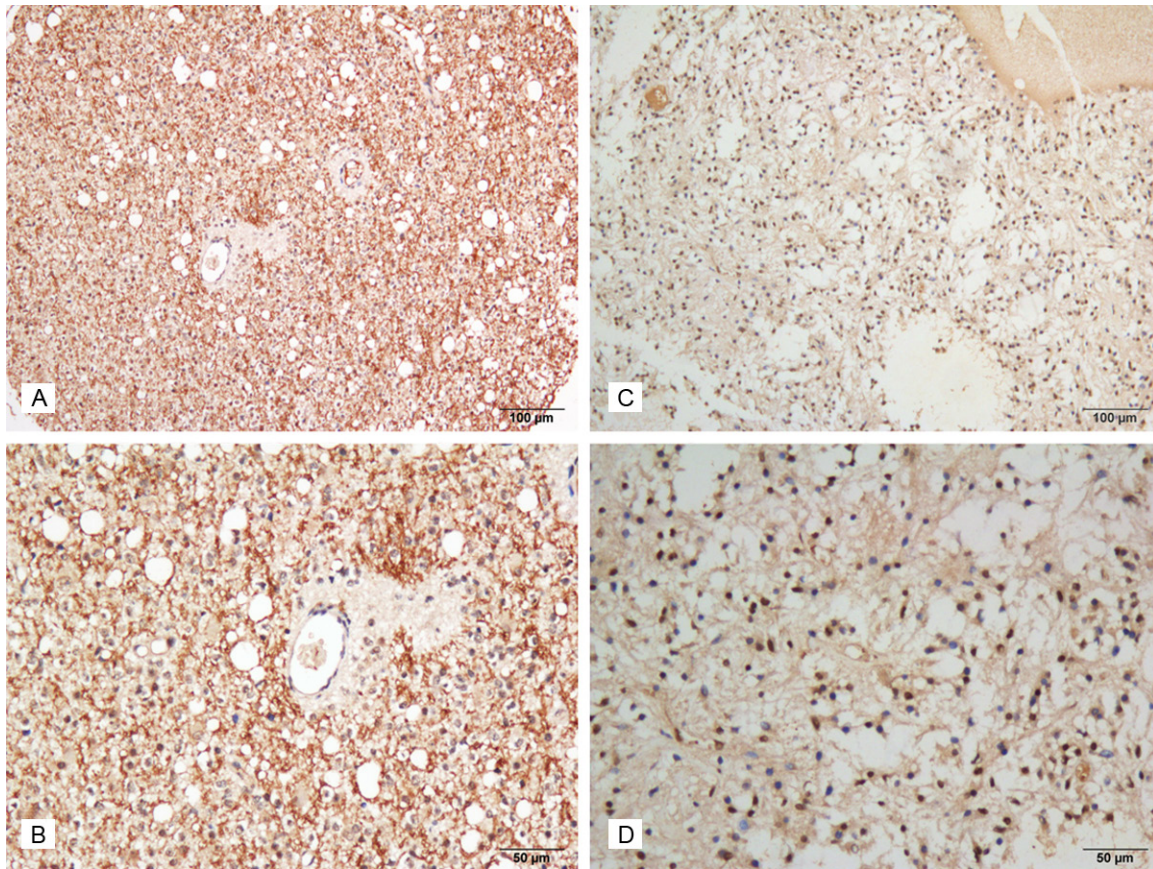


Figure 1. The expression patterns of RNF43 immunostaining in Glioma tissue. A. The high expression of RNF43 in a glioma case, showing the immunoreactivity as a block mass (upper panel, $\times 100$; lower panel, $\times 200$). B. The low expression of RNF43 in a glioma case, showing the immunoreactivity as a block mass (upper panel, $\times 100$; lower panel, $\times 200$).

graded alcohol, immersed in 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity, and antigen retrieved by pressure cooking for 3 minutes in EDTA buffer (pH 8.0). The slides then were preincubated with 10% normal goat serum at room temperature for 30 minutes to reduce nonspecific reaction. Subsequently, the slides were incubated with mouse polyclonal anti-RNF43 (1:50 dilution, ab31218; Abcam, Cambridge, UK) at a concentration of 3 ng/mL for overnight at 4°C. The slides were incubated with a secondary antibody (Envision; Dako, Glostrup, Denmark) for 1 hour at room temperature and stained with DAB (3,3-diaminobenzidine). Finally, the sections were counterstained with Mayer hematoxylin, dehydrated, and mounted. A negative control was obtained by replacing the primary antibody with a normal murine immunoglobulin G. Known immunostaining positive slides were used as positive controls.

Evaluation of IHC staining

The IHC stained samples were assessed as follows: each TMA spot was assigned an intensity score from 0-3 (I0, I1-3), and the proportion of tumor cells for that intensity over the total number of tumor cells was recorded in 5% increments over the range of 0-100 (P0, P1-3); a final H score (range 0-300) was calculated by adding the sum of the scores obtained for each intensity and the proportion of area stained ($H\ score = I1 \times P1 + I2 \times P2 + I3 \times P3$); and the expression of RNF43 was scored by two independent pathologists (S.X. and J.Z.) who were blinded to the clinicopathologic data.

Statistical analysis

Statistical analysis was performed by using the SPSS statistical software package (standard version 16.0; SPSS, Chicago, IL). The correlation between the expression patterns of RNF43

Table 2. Univariate and multivariate analysis of different prognostic factors in 229 patients with glioma

Variable	All cases	Univariate analysis*		Multivariate analysis†	
		Mean survival (months)	P value	HR (95% CI)	P value
Age (y)			< 0.001		< 0.001
≤ 50	172	76.7		1.0	
> 50	57	21.1		2.371 (1.592-3.529)	
Sex			0.536		
Male	104	62.1			
Female	125	64.8			
Tumor location			0.020		0.251
Supratentorial	199	58.0			
Subtentorial	30	96.3			
WHO grade			< 0.001		< 0.001
Low grade (grade I to II)	93	90.4		1.0	
High grade (grade III to IV)	136	43.8		3.431 (2.199-5.355)	
Relapse			< 0.001		< 0.001
Yes	104	28.8		3.526 (2.362-5.262)	
No	125	84.6		1.0	
RNF43 expression			0.001		0.001
Low expression	88	46.5		1.0	
High expression	141	73.1		0.547 (0.382-0.784)	

*: Kaplan-Meier survival analysis. †: Multivariate Cox regression analysis.

and clinicopathologic features of glioma patient was evaluated by χ^2 -test. Univariate and multivariate survival analyses were performed using the Cox proportional hazards regression model. Survival curves were obtained with the Kaplan-Meier method. Differences were considered significant if the *P*-value from a two-tailed test was < 0.05.

Results

Immunochemical expression of RNF43 in glioma

To investigate the expression pattern of RNF43 in glioma tissues, we performed IHC staining on 229 cases of glioma tissues. Positive staining of RNF43 protein was observed in most of glioma cases. The positive for the immunochemical staining of RNF43 was cytoplasmic staining. As the median score of the expression was 100.5, we classified the expression score that less than and equal to 100 was low expression, otherwise was high expression (As shown in **Figure 1**).

Relationship between the expression of RNF43 and clinicopathologic characteristics

The relationship between the expression of RNF43 and the clinicopathological characteristics

such as age, sex, tumor location, WHO grade, status or relapse were analyzed by the correlation analysis. We found that the expression of RNF43 was significantly related to the status of patients (As shown in **Table 2**).

Relationship of RNF43 expression and glioma patients' survival: univariate survival analysis

Kaplan-Meier survival analysis was used to demonstrate the prognostic factors of glioma patients' survival. Such as age (< 50 y or ≥ 50 y), WHO grade [low grade (grade I and II) or high grade (grade III and IV)], tumor location (supratentorial or subtentorial) and the high or low expression of RNF43. Patients with a high RNF43 expression were likely to have a significantly longer survival (*P* = 0.001). As our result showed, age, WHO grade, tumor location and relapse were also significant prognostic factors in glioma by the analysis of Kaplan-Meier survival analysis (As shown in **Table 2** and **Figure 2**). Stratified survival analysis was further performed with regard to the RNF43 expression in subsets of glioma patients with different WHO grade and age. As shown in the **Figures 3** and **4**, in high grade glioma, high expression of RNF43 indicated the better survival (*P* < 0.001). In the younger patients, high expression of RNF43 indicated the better survival (*P* = 0.009).

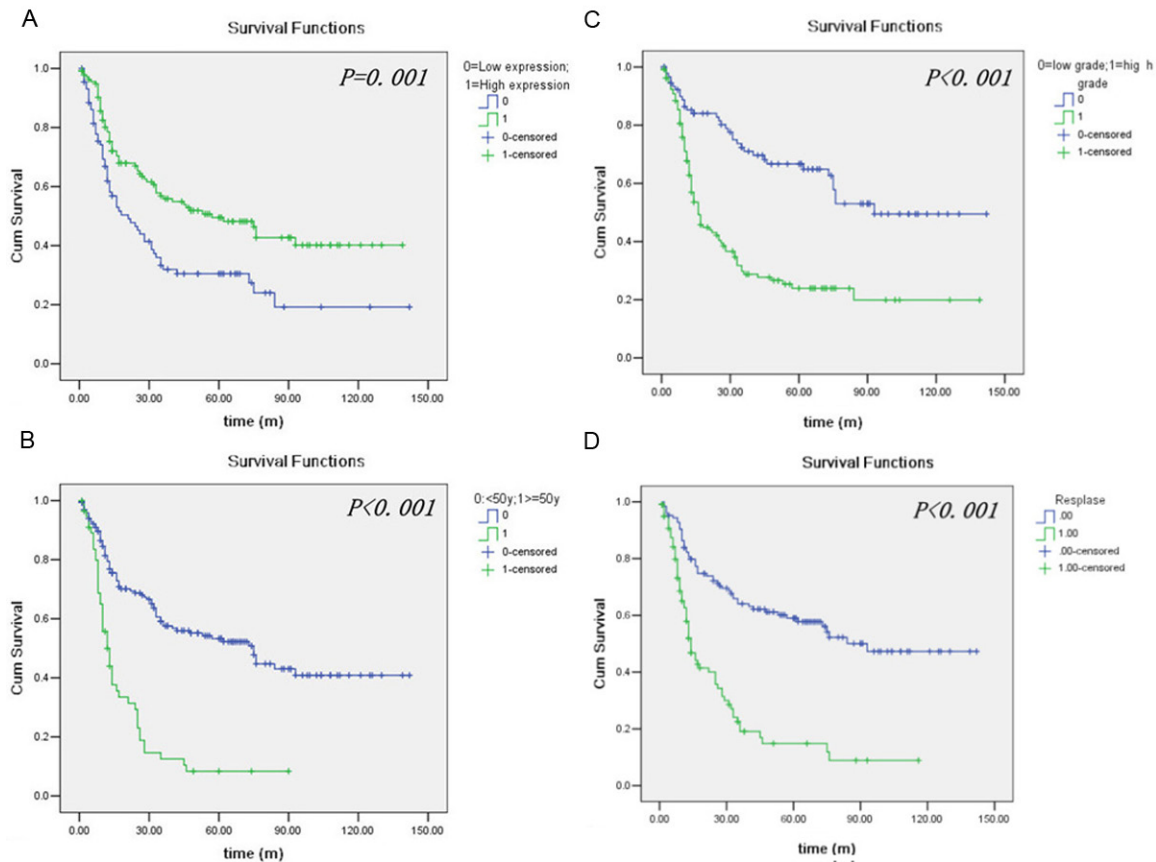


Figure 2. Kaplan-Meier survival analysis of RNF43 expression and the clinicopathological features in patients with glioma (log-rank test). A. The survival analysis of the expression of RNF43 in glioma patients: low expression, $n = 88$; high expression, $n = 141$. B. The survival analysis of age in glioma patients: not elder than 50 years, $n = 172$; elder than 50 years, $n = 57$. C. The survival analysis of the WHO grade in glioma patients: low grade, $n = 93$; high grade, $n = 136$. D. The survival analysis of relapse in glioma patients: not relapse, $n = 125$; relapse, $n = 104$.

Independent prognostic factors of glioma: multivariate Cox regression analysis

Since variables observed to have prognostic significance by univariate analysis might covariate, the expression of RNF43 as well as other clinicopathological features (WHO grade, age, tumor location and relapse) that were significant in univariate analysis was examined in multivariate analysis (Table 2). Our results showed that high expression of RNF43 was an independent prognostic factor for patient survival [hazard ratio (HR) 0.547, 95% confidence interval (95% CI) 0.382-0.784, $P = 0.001$] (Table 2). Of the clinicopathological features, WHO grade ($P < 0.001$), age ($P < 0.001$) and relapse ($P < 0.001$) were also evaluated as good independent prognostic factors.

Discussion

Glioma was the most common tumor in the central nervous system. RING finger proteins

act by mediating diverse protein-protein interactions. The main biological function of RING finger proteins is the catalysis of ubiquitylation as an ubiquitin-protein isopeptide ligase [11, 12]. The relevance of ubiquitination to cancer might arise from oncogenic mutations that disrupt ubiquitination of the proteins that control cell growth and death [13]. RNF43 has frequently been found to be up-regulated in human colorectal cancer. In this study, to elucidate the clinical role of RNF43 in glioma, we applied TMA and IHC to examine its expression in a cohort of Chinese patients. As our knowledge, this is the first study to analyze RNF43 expression in glioma using TMA-based IHC method.

In results of Kaplan-Meier survival analysis, we found that patients with high RNF43 expression had longer survival. This might be explained by the findings that high RNF43 expression positively correlated with a higher proliferation

Ring-finger protein 43 in glioma

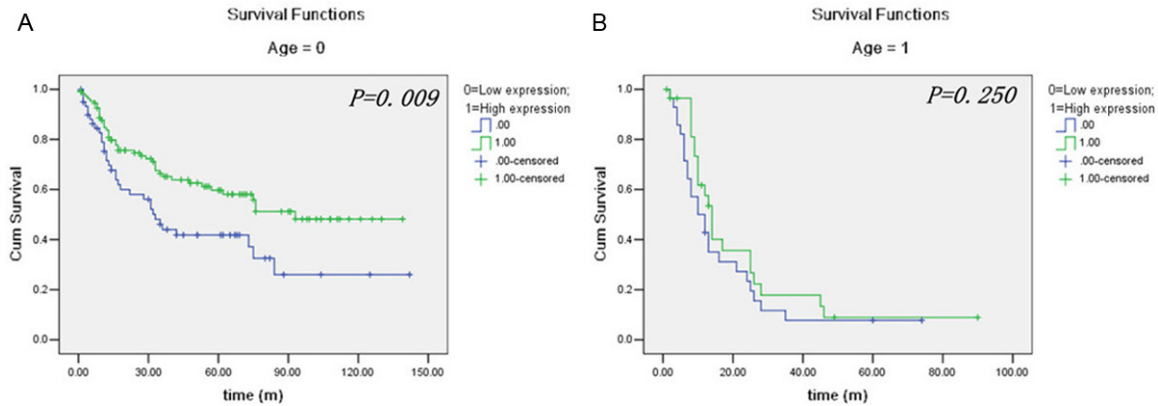


Figure 3. Kaplan-Meier survival analysis of RNF43 expressed in a subset of Glioma patients with different age (log-rank test). A. The survival of the different RNF43 expression in glioma patients at not more than 50 years old: low expression, n = 60; high expression, n = 112. B. The survival of the different RNF43 expression in glioma patients at more than 50 years old: low expression, n = 28; high expression, n = 29.

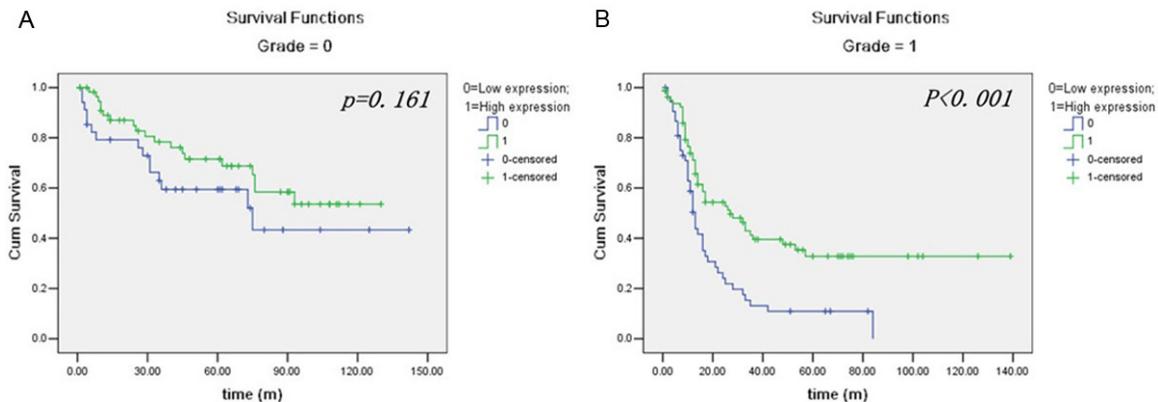


Figure 4. Kaplan-Meier survival analysis of RNF43 expressed in a subset of Glioma patients with different WHO grade (log-rank test). A. The survival of the different RNF43 expression in glioma patients at low grade: low expression, n = 34; high expression, n = 59. B. The survival of the different RNF43 expression in glioma patients at high grade: low expression, n = 54; high expression, n = 82.

index [14]. The expression of RNF43 has been shown to be regulated by the Wnt-signaling pathway [15-17]. RNF43 is also a direct target gene of Tcf4/b-catenin complex, it functions as a negative feedback regulator that is modulated in the Wnt-signaling pathway by the Tcf4/b-catenin complex, which acted as a significant determinant of glioma initiation and progression [7]. Furthermore, Cox regression analysis suggested RNF43 as an independent prognostic factor, indicating that RNF43 may be a pivotal modulator involved in cancer development. This may be explained by the role of RNF43 in Wnt-signaling pathway, and the reactivation of Wnt/ β -catenin signaling could cause an increase in the proliferation of glioma cells [18].

Stratified survival analysis of glioma, according to age, tumor location and WHO grade, evalu-

ated RNF43 expression to be closely correlated with survival of glioma patients with younger patients and grade III-IV. This suggested that downregulation of RNF43 in glioma could be of clinical use for distinguishing a set of patients with poor prognosis. Age and WHO grade are the most commonly agreed survival indices for affecting the prognosis of glioma patients [19, 20]. In this study, our results indicated RNF43 expression evaluated by IHC could be used as an additional tool in identifying those patients at risk of glioma progression.

Taken together, our study provides compelling clinical evidence that RNF43 can be served as an independent prognostic marker for survival in glioma. Although the current results which are based on a cohort of Chinese patients should be further confirmed in other glioma

cohorts, our findings suggest RNF43 as a new and promising prognostic biomarker for glioma progression and prognosis.

Acknowledgements

This study was supported by Science and Technology Planning Project of Guangdong Province, China (No. 2011B031800159).

Disclosure of conflict of interest

None.

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