

## Original Article

# Reduced cullin1 expression enhances the effects of fluorouracil-leucovorin-oxaliplatin in resectable gastric cancer

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**Abstract:** To investigate the significance of Cullin1 as a biomarker in adjuvant chemotherapy of gastric cancer. A total of 618 gastric cancer cases in tissue microarray were enrolled for analyzing the relationship of overall survival and Cul1 expression; the *in vitro* SGC7901 and BGC823 gastric cancer cell culture models were used for identifying the synergistic role and potential mechanisms of Cul1 expression and chemotherapeutic drugs fluorouracil and oxaliplatin (FO). The gastric cancer patients with low Cul1 expression and treated by FLO regimen after surgery had a significant longer survival time compared with the patients treated by surgery alone. The gastric cancer cells knock down Cul1 increased sensitivity to FO due to enhanced activations of caspase-8 and PARP and therefore induced apoptosis. Data in the present study suggested that the expression of Cul1 protein may be a predictive factor and benefit from FLO adjuvant chemotherapy in resectable human gastric cancer. Detection of Cul1 expression may be a potential biomarker for personalized chemotherapy for gastric cancer.

**Keywords:** Cullin1, gastric cancer, biomarker, prognosis, adjuvant chemotherapy

## Introduction

Gastric cancer (GC) is one of the most common cancers. Despite improvements in diagnostic and therapeutic methods, GC remains the second leading cause of cancer deaths worldwide [1]. And the incidence and mortality of it in China were especially high [2], with a 5-year survival rate less than 30% [3]. Although surgery is the main treatment of GC, even if radical resection is the mainstay of curative treatment of gastric cancer, disease recurrence is very common. Outcome of unresectable or metastatic gastric cancer is still extremely poor, although chemotherapy demonstrated to confer a benefit in terms of survival and quality of life [4]. There is no standard first-line chemotherapeutic regimen, although cisplatin and fluoropyrimidine combination is the backbone of treatment, with or without other agents such as epirubicin or docetaxel [5].

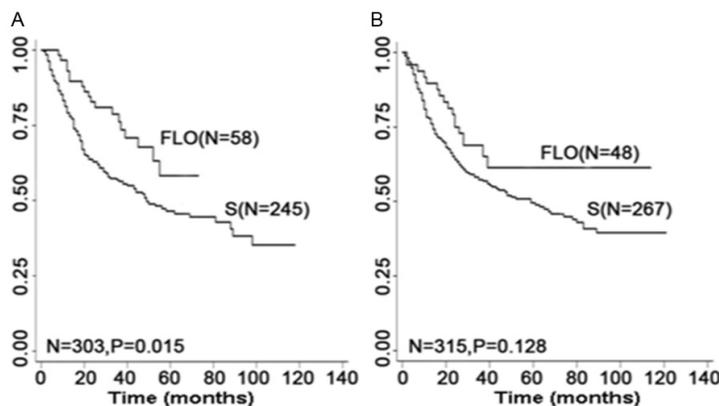
Cullin1 is the most representative member of Cullin protein family, which degrades many proteins through ubiquitin-proteasome pathway, especially the proteins involved in cell cycle. Thus, cullin1 is an essential regulator of cell cycle, and its abnormality can lead to tumorigenesis [6-8]. Our previous study found that Cul1 may be a novel candidate biomarker for human gastric cancer, and loss of Cullin1 inhibited cell growth by up-regulating p27 expression and reduced cell adhesion ability by suppressing Src and FAK expression [9]. However, whether cullin1 is a good marker for adjuvant platinum-based chemotherapy is still unknown. In this study, we investigated the relationship between Cul1 expression and FLO based adjuvant chemotherapy on gastric cancer patients; moreover, cell culture models were used for identify the potential molecular mechanisms of the synergistic role between cul1 and FO on gastric cancer cells.

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**Table 1.** Cul1 staining and clinicopathologic characteristics of 618 gastric cancer patients

Variables	Cul1 staining			P <sup>a</sup>
	Low	High	Total	
Age (y)				
≤ 65	168 (50.0%)	168 (50.0%)	336	0.628
> 65	135 (47.9%)	147 (52.1%)	282	
Gender				
Male	228 (48.2%)	245 (51.8%)	473	0.506
Female	75 (51.7%)	70 (48.3%)	145	
Tumor diameter (cm)				
≥ 5	191 (51.8%)	178 (48.2%)	369	0.102
< 5	112 (45.0%)	137 (55.0%)	249	
Histological type				
Intestinal	117 (43.7%)	151 (56.3%)	268	0.015
Diffuse	185 (53.6%)	160 (46.4%)	345	
Depth of invasion				
T1/T2	127 (57.0%)	96 (43.0%)	223	0.004
T3/T4	174 (44.6%)	216 (55.4%)	390	
Lymph node metastasis				
N0	141 (54.2%)	119 (45.8%)	260	0.028
N1/N2/N3	161 (45.2%)	195 (54.8%)	356	
Distant metastasis				
M0	295 (50.2%)	293 (49.8%)	588	0.014
M1	8 (26.7%)	22 (73.3%)	30	
TNM stage				
I	85 (51.2%)	81 (48.8%)	166	0.809
II	71 (50.7%)	69 (49.3%)	140	
III	139 (47.3%)	155 (52.7%)	294	
IV	8 (44.4%)	10 (55.6%)	18	

<sup>a</sup>Two-sided Fisher's exact tests.



**Figure 1.** Kaplan-Meier curves depicting overall survival according to CUL1 expression and adjuvant chemotherapy. A. Patients with low CUL1 expression had a longer survival time when received FLO based chemotherapy ( $P = 0.015$ ). B. Patients with high CUL1 expression can not receive benefit from FLO based chemotherapy ( $P > 0.05$ ).

## Materials and methods

### Patient and specimens

Totally 618 GC patients who underwent curative surgery and adjuvant chemotherapy during 1st January 1999 to 31st December 2006, were recruited from Yixing People's Hospital, Yixing city, Jiangsu Province, China [10]. The clinicopathologic features of the 618 primary gastric cancer biopsies were summarized in **Table 1**. (For more details, see the supplementary data, which is available online).

### Tissue microarray (TMA) construction and immunohistochemistry

The gastric cancer TMAs were constructed as previously described [9, 10]. A standard protocol was used for immunostaining of the TMAs, which was also described in supplementary data.

### Evaluation of immunohistochemistry

Assessment of the immunohistochemistry was the same as the former study. Positive Cul1 immunostaining is defined as cytoplasmic with or without nuclear staining. The percentage of Cul1-positive stained cells was scored into 4 categories: 1 (0-25%), 2 (26%-50%), 3 (51%-75%), and 4 (76%-100%). In the cases with a discrepancy between duplicated cores, the higher score from the 2 tissue cores was taken as the final score. The level of Cul1 staining was evaluated by immunoreactive score (IRS) [11], which is calculated by multiplying the scores of staining intensity and the percentage of positive cells. Based on the IRS, Cul1 staining pattern was defined as negative (IRS: 0), weak (IRS: 1-2), moderate (IRS: 3-6), and strong (IRS: 8-12).

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**Table 2.** Multivariate Cox model analyses the effect of FLO therapy on overall survival of gastric cancer patients with low/high Cullin1 expression

	Cullin1 low expression			Cullin1 high expression		
	HR (95% CI)	beta	P <sup>a</sup>	HR (95% CI)	beta	P <sup>a</sup>
Age (≤ 65 vs. > 65)	0.91 (0.65-1.27)	-0.094	0.565	0.95 (0.71-1.27)	-0.051	0.730
Gender (male vs. female)	0.96 (0.66-1.39)	-0.041	0.824	0.85 (0.59-1.20)	-0.163	0.351
Histological type (intestinal vs. diffused)	1.70 (1.20-2.41)	0.531	0.003	1.84 (1.37-2.47)	0.610	0.000
Tumor diameter (≤ 5 cm vs. > 5 cm)	1.24 (0.86-1.80)	0.215	0.250	1.34 (1.00-1.82)	0.293	0.053
TNM stage (I-II vs. III/IV)	5.27 (3.43-8.08)	1.662	0.000	4.14 (2.93-5.84)	1.421	0.000
Adjuvant therapy (surgery alone vs. FLO)	0.40 (0.24-0.67)	-0.916	0.000	0.65 (0.42-1.02)	-0.431	0.062

<sup>a</sup>Multivariate Cox regression analysis including age, gender, histological type, TNM stage, tumor diameter, adjuvant therapy (FLO vs. surgery alone).

### Cell culture

SGC7901 and BGC-823 gastric cancer cell lines were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). These cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and 100 U/ml penicillin/100 µg/ml streptomycin at 37°C in 5% CO<sub>2</sub> incubator.

### Transfection of *cul1* siRNA in BGC-823 and SGC-7901 cells

SGC-7901 and BGC-823 Cul1-knockdown cells were transfected with small interfering RNA (siRNA) and corresponding control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using Lipofectamine 2000 (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions. All the experiments were conducted 72 h after transfection.

### Western blotting

Western blotting was conducted as standard protocol previously described. The cells were lysed in RIPA buffer (50 mM Tris [pH 7.4]; 150 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS) supplemented with protease inhibitor. Antibodies used for immunoblotting were included Cul1 (Epitomics Inc, 863 Mitten Road, Burlingame, CA, USA); caspase-3, caspase-8, caspase-9, PARP-1 (Cell Signaling Technology Inc., Beverly, MA, USA), β-actin (Boster Biotechnology, Wuhan, China). Immunoreactive bands were detected with the Phototope-HRP Western blot detection kit (Cell Signaling Technology Inc., Beverly, MA, USA).

### Cell growth inhibition assay

The *in vitro* growth of SGC-7901, BGC-823 cells were determined by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye absorbance at 570 nm of living cells [12]. Briefly, cells (5×10<sup>3</sup> cells/well) were first seeded into 96-well microtiter plates (Coster). After 12 h, the culture medium was replaced with new one which contains 5-Fu (36.25 µg/ml) and L-OHP (20 µg/ml). The culture medium was carefully removed at indicate time, then 20 µl of MTT (5 µg/ml of PBS) was added to the plates. After 2 to 4 h incubation at 37°C, 150 µl DMSO was added to each well and plates were agitated for 5 minutes. Absorbance was measured at 570 nm. All experiments were performed at least three times.

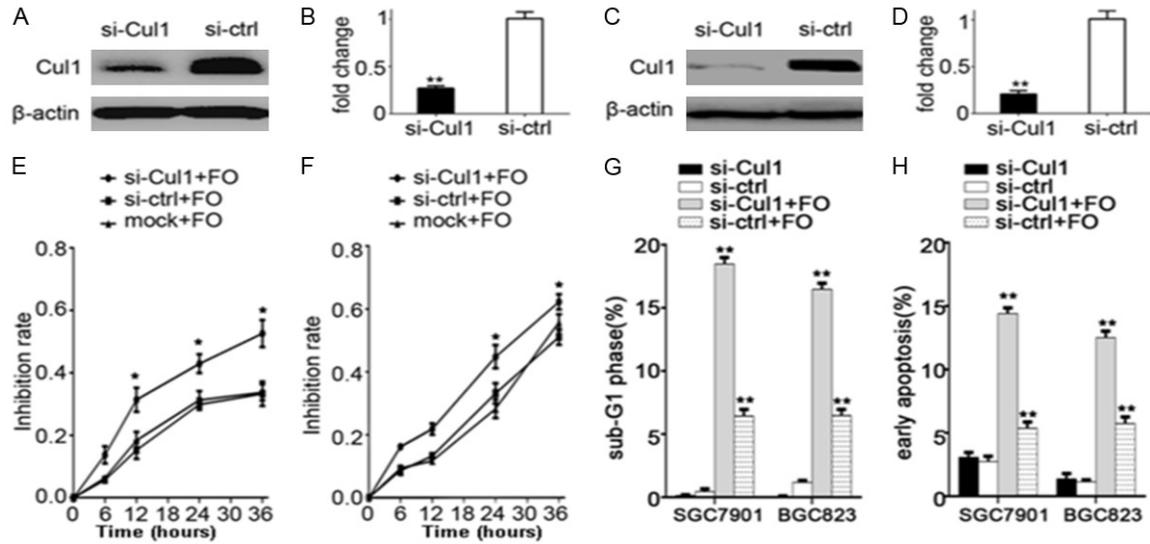
### Cell cycle analysis

SGC-7901, BGC-823 cells were treated with 5-Fu (36.25 µg/ml) and L-OHP (20 µg/ml) for 24 h. Cell cycle phase was detected using PI single staining according to the published protocol [13]. Data were analyzed with the ModFit soft (Verity Software House, United States). At least three biological experiments were performed to verify observations.

### Early apoptotic cells detection

SGC-7901, BGC-823 cells were treated with 5-Fu (36.25 µg/ml) and L-OHP (20 µg/ml) for 24 h. The cells were harvested and stained according to the published protocol [14]. Direct fluorescence staining of apoptotic cells for flow cytometric analysis was performed with the Annexin V-FITC apoptosis detection kit I (BD Pharmingen, San Jose, CA, USA). All experi-

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**Figure 2.** Gastric cancer cells with low CUL1 expression were more sensitive to FLO. CUL1 protein levels were reduced in SGC7901 (A) and BGC823 (C) by siRNA treatment, and the Western blot was quantitatively normalized by β-actin in SGC7901 (B) and BGC823 (D). MTT assay showed that the inhibitory rate of SGC-7901 (E) and BGC-823 (F) cells treated with FLO. (G) PI single staining displayed these cells treated with FLO showed a decrease in sub-G1 levels by flow cytometry analysis. (H) The amount of early apoptotic cells were determined by flow cytometry using FITC-Annexin V/PI staining.

ments were performed at least three times to verify the results.

### Statistical analysis

Student's t-test or one-way ANOVA was applied for results *in vitro*. Probability of differences in OS as a function of time was ascertained by use of the Kaplan-Meier method, with a log-rank test probe for significance. Univariate and multivariate Cox proportional hazards regression analysis were performed to estimate the crude HR (hazard ratios), adjusted HR and 95% CI (confidence interval). The difference of the clinicopathological parameters among adjuvant FLO regime has been showed before [10]. All the statistical analyses were performed by Statistical Analysis System software (version 9.1.3; SAS Institute, Cary, NC), STATA statistical software (version 10.1; StataCorp, College Station, TX). A *P*-value of < 0.05 was deemed statistically significant, and all tests were 2-sided.

### Results

#### Patients with low *cul1* expression showed more sensitive to adjuvant chemotherapy

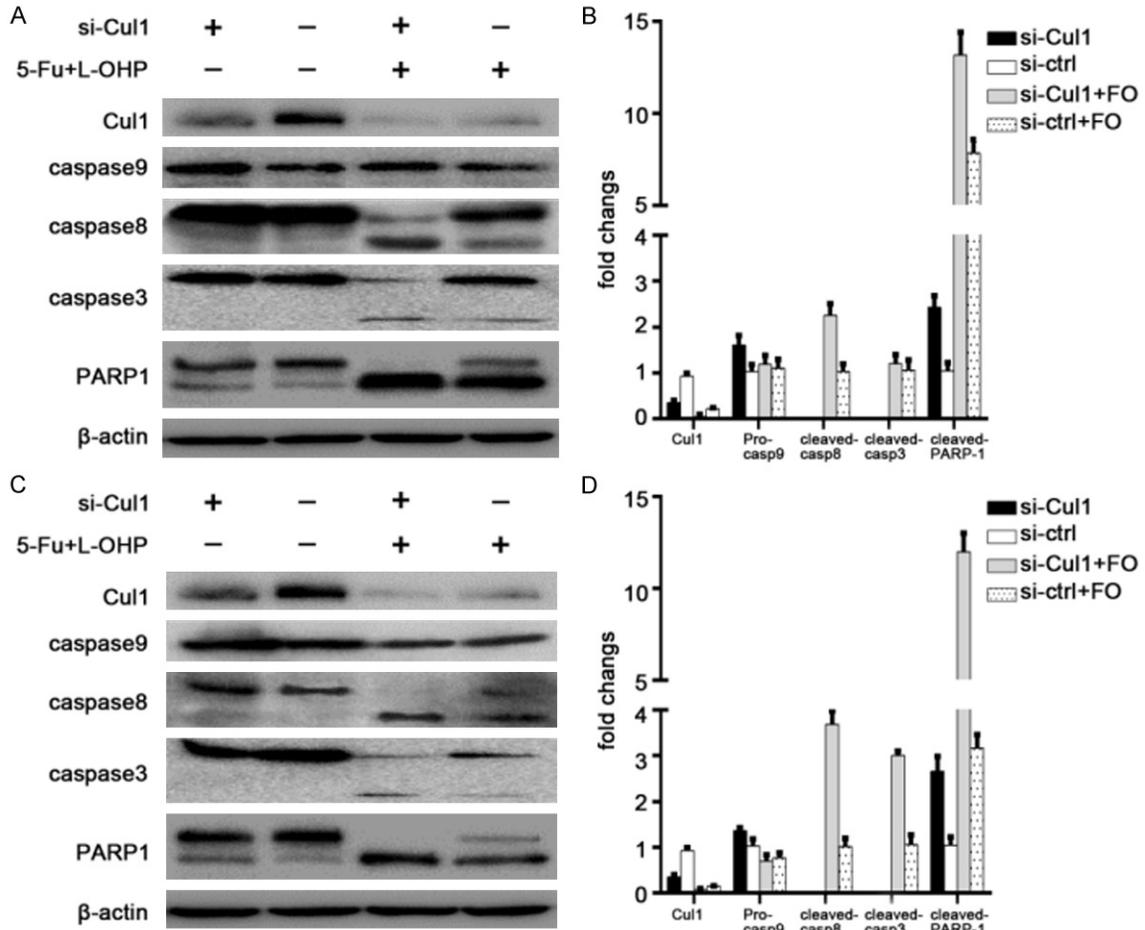
Kaplan-Meier curve revealed that the patients who received FLO treatment and with low Cull

expression in tumor tissues had a significant higher survival time than the patients who received surgery alone (log-rank test, *P* = 0.015, **Figure 1A**). However, patients with high Cull expression could not receive benefit from FLO treatment (log-rank test, *P* = 0.128, **Figure 1B**). Multivariate Cox proportional hazard regression analysis including 6 variables (age, gender, TNM stage, histological types, tumor diameter, and chemotherapy treatment) was used to indicate the benefit of chemotherapy on OS. Interestingly, we found that FLO treatment increased OS as compared with surgery alone in low Cull expression patients (HR = 0.40; 95% CI = 0.24-0.67, *P* < 0.001, **Table 2**). However, this effect was not observed in the patients with high Cull expression (HR = 0.65, 95% CI = 0.42-1.02, *P* = 0.062, **Table 2**).

#### Loss of *cul1* enhances effects of chemotherapy drugs in human gastric cancer cells

Specific si-RNA was used to knockdown CUL1 expression in gastric cancer cells. Western blotting revealed that compared to siRNA control cells, CUL1 expression was significantly down regulated to 25% and 20% in siRNA treated SGC7901 (**Figure 2A, 2B**) and BGC823 (**Figure 2C, 2D**) cells, respectively. MTT assays

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**Figure 3.** Knock down Cul1 expression enhances activities of caspase-8 and caspase-3 and spliced variant of PARP1. Apoptosis related protein was measured by Western blot in SGC-7901 (A) and BGC-823 (C) cells. Western blot measured CUL1 expression was normalized by  $\beta$ -actin in SGC7901 (B) and BGC823 (D).

indicated that the effects of chemotherapy drugs (5-Fu 36.25  $\mu$ g/ml, L-OHP 20  $\mu$ g/ml) on siRNA-cul1 treated cells were more sensitive than in siRNA control cells ( $P < 0.01$ ; **Figure 2E, 2F**). We further performed flow cytometry analysis to determine whether CUL1 low expression cells produced more apoptosis when treated with FO. As a result, PI single staining showed that siRNA-CUL1 group with FO-treatment had more proportion of cells in sub-G1 phase, indicating that those cells in late apoptosis. There were statistically significant among the four groups ( $P < 0.01$ ; **Figure 2G**). We also determined early apoptosis cells by FITC-Annexin V/PI double staining. The data showed that the siRNA-cul1 cells treated with chemotherapy drugs had earlier apoptotic cells than the other groups (**Figure 2H**,  $P < 0.01$ ).

### *Knock down cul1 via caspase-8 and PARP1 pathway increases apoptosis in human SGC-7901 and BGC-823 cells*

To understand the mechanisms involved in knock down cul1 increased apoptosis in human GC cells, we examined the expression of caspase-8, caspase-9, caspase-3 and PARP1 by Western blotting. As indicated in **Figure 3A-D**, the siRNA-cul1 group cells enhanced activation of caspase-8 and caspase-3, but not in caspase-9; and cleaved caspase-8 and caspase-3 increased significantly compared with si-ctrl group. PARP1, an effector molecule of caspase-3, also appeared more spliced in siRNA-cul1 group than in si-ctrl group, but not observed in caspase-9.

## Discussion

Though clinical outcomes of gastric cancer patients have improved by combination of effective chemotherapy, the side effects of toxicities of the drugs remain inevitable. In recent years, some molecules were reported as potential prognostic and therapeutic markers in gastric cancer [15-19]. However, very little is known about the molecular mechanisms of response to chemotherapy efficacy and clinical toxicity. Up to now, only the trastuzumab could be use in GC with the subset of patients with HER2-positive [20].

Cul1 is the core components of the ubiquitin ligase complex, and has a wide range of biological effects, especially in tumorigenesis [21]. As the biggest family of E3 ubiquitin ligases, the Skp1/Cullin/Rbx1/F-box (SCF) protein complexes ubiquitinate a broad range of proteins involved in cell-cycle progression, signal transduction, and transcription [22]. The SCFSkp2 is an oncogene, and Foxo3a is found to be a transcriptional repressor of Skp2 gene expression by directly binding to the Skp2 promoter, thereby inhibiting SCFSkp2 protein expression [23]. The p38 mitogen-activated protein kinase (p38MAPK)-mediated phosphorylation of ER $\alpha$  at Ser-294 specifies its turnover by the SCF (Skp2) proteasome complex. Interestingly, by the knockdown of Skp2 or the inhibition of p38MAPK, functional ER $\alpha$  protein levels could be restored and then control of gene expression and proliferation by estrogen and antiestrogen in ER $\alpha$ -negative breast cancer cells [24]. Knockdown of Cul1 inhibits melanoma cell growth while overexpression of Cul1 enhances cell proliferation. Cul1 regulates melanoma cell growth and cell cycle progression through degradation of p27 by functional SCFSkp2 complex [25]. CAC1 that contains cullin1 is capable of promoting cell proliferation by regulating CDK2 [26]. This observation raises the possibility that the levels of Cul1 may be intimate with cancer therapy.

Our previous research had confirmed that the Cul1 may be an important marker of human gastric cancer prognosis [9]. Here we provide evidences that Cul1 might be a biomarker in gastric cancer postoperative adjuvant chemotherapy. Patients with low Cul1 expression will

with longer survival time if receive post-operational adjuvant FLO treatment. A reasonable explanation of the mechanism behind of the synergistic effects on gastric cells by knock-down cul1 and FO treatment might be on FAD signal pathway. However, the exact molecular events between cul1 and FO on apoptosis of GC cells need to be further elucidated.

The mechanism of Cul1 as an oncoprotein to exert tumorigenic role needs to be explored. It is worthy to invent a small molecular inhibitor of Cul1 and to investigate the synergetic role of the inhibitor in chemotherapy on other cancers. Cullin1 is expected as the indicators of early diagnosis, malignant degree and prognosis, and may become a new target for cancer gene therapy.

Finally, we have to emphasis that the present data on how Cul1 enhances the sensitivity of GC cells to FO is preliminary and very limited, however, it gives us more expectation that the strategy might be useful in development of personalized medicine in cancer therapy.

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## Disclosure of conflict of interest

None.

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