

Original Article

Lgr5 over-expression is positively related to the tumor progression and HER2 expression in stage pTNM IV colorectal cancer

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Abstract: Recent studies display that Leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) appears to involve the initiation of colorectal cancer (CRC). However, its role in the progression of CRC is not clear at present. In the present study, the expression of Lgr5, HER2, VEGF, and Ki-67 was detected by immunohistochemistry in primary cancer tissue and the matched normal mucosa, metastatic lymph node and distant metastatic tissues in 42 CRC cases staged as pTNM IV. The correlation of Lgr5 over-expression with the CRC progression, survival time, and expression of HER2, VEGF, and Ki-67 was evaluated. Moreover, the Lgr5 expression at the invasive front or residual cancer cells around coagulation necrosis was compared with that at the center of CRC in 51 paraffin embedded tissues. The results revealed that Lgr5 over-expression was more frequently found in the metastatic tissues of both lymph nodes and distant area when compared with primary CRC tissue ($P < 0.05$). Additionally, cancer cells in the invasive front and residual cancer cells around or among the coagulation necrosis presented stronger Lgr5 immunoreactivity than that at tumor center ($P < 0.05$), and strong positive staining was often observed in tumor budding cells. While, HER2 over-expression was detected in 28.9% (IHC 3+) and 42.1% (IHC 3+/2+) of CRC patients, neither Lgr5 nor HER2 expression was significantly related to the prognosis of CRC patients, though there was a positive correlation between Lgr5 and HER2 ($P < 0.05$) or Ki-67 expression ($P < 0.05$). In conclusions, Lgr5 over-expression might involve the proliferation, invasion, and distant and regional metastasis of CRC cells, and has potential positive relation to HER2 expression.

Keywords: Colorectal cancer, Lgr5, HER2, progression, prognosis

Introduction

Leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) is a member of the G protein coupled receptor super-family and has been proven to be a downstream target gene of Wnt/ β -catenin pathway [1]. Recent studies suggested that Lgr5 was involved in the pathogenesis of colorectal adenocarcinoma (CRC), potentially via the Wnt/ β -catenin signaling pathway [2-7]. At present, however, the association of Lgr5 expression in CRC tissue with the progression and prognosis of CRC patients is still controversial [2-8]. Thus, further studies are required to elucidate this relationship in depth. In the present study, the Lgr5 expression was examined by immunohistochemistry (IHC) in

primary cancer tissue and the matched normal mucosa, metastatic lymph node and distant metastatic tissues in 42 CRC cases staged as pTNM IV, aiming to evaluate the role of Lgr5 in the proliferation, invasiveness and metastasis of CRC cells, and the prognostic prediction in patients with the end stage CRC.

Materials and methods

Cases

Forty-two cases of CRC staged as pTNM IV according to the pTNM staging system of 7th AJCC, dating from 2009 to 2011, were retrieved from the archives of the Department of Pathology, the Affiliated Drum Tower Hospital of

Table 1. Primary antibodies used for IHC in this study

Antibodies	No	Dilution	Company
Rabbit anti-human Lgr5 monoclonal antibody	EPR3065Y	1:400	Lifespan biosciences
Rabbit anti-human HER2 polyclonal antibody	A0485	1:2500	Dako
Mouse anti-human VEGF monoclonal antibody	VG1	1:400	Dako
Mouse anti-human Ki-67 monoclonal antibody	MIB1	1:50	Dako

Nanjing University Medical School. The collection and use of these samples were in accordance with the Guideline for Use of Human Specimens developed by the Ethics Committee of Drum Tower Hospital, and the private information related to these samples was removed completely.

Histology and immunohistochemistry

All samples were fixed in 4% formaldehyde and embedded in paraffin. Routine HE staining was performed, and histopathological examination independently done again by two experienced pathologists. The clinicopathological information was collected, including age, gender, tumor size, cancer grade, and TNM stage of CRC. The EnVision method was used for IHC. The EnVision IHC kit and DAB solution were purchased from Dako Company. Other reagents and antibodies used in this study are shown in **Table 1**.

Immunostaining for Lgr5 was performed on the tissue of primary cancer and their matched normal mucosa, metastatic lymph nodes and distant metastatic tissues of 42 cases of CRC, and on the tissue of the cancer center, invasive front and residual cancer tissue of necrosis in 51 matched blocks of primary cancer of 42 CRC cases. And immunostaining for HER2, VEGF, Ki-67 were done in primary cancer tissue of 42 CRC cases. IHC was performed according to manufacturer's instructions. Findings from IHC were scored semiquantitatively by two experienced pathologists independently in a blind manner. Where there was disagreement between pathologists, the consensus was reached by a joint reading. Determination of Lgr5 expression and VEGF expression was done according to the staining intensity and proportion of positive cells: staining intensity: 0, no staining; 1, light yellow; 2, yellow and 3, yellow; proportion of positive cells: 0, <5%; 1, 6-25%; 2, 26-50%; 3, 51-75% and 4, >76%. The final score was the product by multiplying the staining intensity and quantity of positive cells,

and the total score ranged from 0 to 12. The immunostaining was considered 0 or negative when the score was 0; 1+ or weak

positive, 1-3; 2+ or moderately positive, 4-7; 3+ or strong positive, 8-12. The evaluation of HER2 expression was done according to previously criteria reported in gastric cancer [9]: 0, no staining; 1+, $\geq 10\%$ cells with weak staining; 2+, $\geq 10\%$ cells with moderate staining; 3+, $\geq 10\%$ cells with strong staining. HER2 IHC 2+ and 3+ are regarded over-expression. In the evaluation of Ki-67 expression, only the proportion of positive cells was determined and divided into 4 grades: $\leq 25\%$, 25-50%, 50-75% and $>75\%$. In each experiment, positive control and negative control were included.

Statistical analysis

The immunoreactivity of Lgr5, VEGF, HER2 and Ki-67 detected in the primary cancer and the matched normal mucosa, metastatic lymph nodes, distant metastatic tissues, and cancers at the center, invasive front and residuals surrounding the necrotic cancers were graded four expression level of 0, 1+, 2+ and 3+. Comparisons of expression levels were done with Chi square test. The Kaplan-Meier method was used to evaluate the survival time, and log-rank test was employed to compare the survival among groups. Values of $P < 0.05$ were considered statistically significant. Statistical analysis was done with SPSS version 16.0 (SPSS, Chicago, IL).

Results

Clinicopathological information of CRC patients

Among 42 cases of CRC patients, there were 22 males and 20 females, with the median age of 59 years (ranged from 30 to 86 years old). In addition, tumor located at the right colon, transverse colon, and left colon in 18, 2 and 22 cases with CRC, respectively. CRC metastasis was found in the liver (n=32), omentum (n=6; 3 from left colon and 3 from right colon), unilateral ovary (n=1; from sigmoid colon), bilateral

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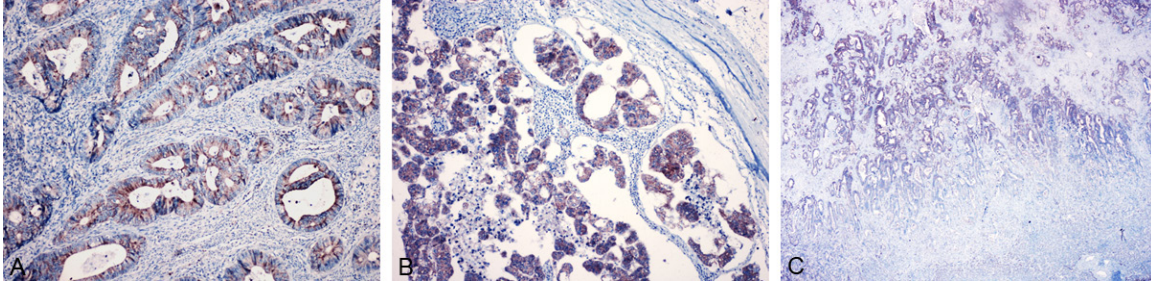


Figure 1. Primary cancer tissue (A), metastatic lymph nodes (B) and distant metastatic lesion in the liver (C) of CRC presented Lgr5 protein expression variably.

Table 2. Lgr5 expression in primary CRC and their matched metastatic lymph nodes and distant metastatic tissues (IHC)

	IHC	n	Primary cancer			
			0	1+	2+	3+
Normal mucosa	0	42	42	0	0	0
metastatic lymph nodes*		34	3	13	12	6
	0	3	0	2	0	1
	1+	7	0	5	2	0
	2+	12	1	5	6	0
	3+	12	2	1	4	5
distant metastatic tissues#		42	3	18	13	8
	0	4	0	2	1	1
	1+	8	0	6	1	1
	2+	9	1	5	3	0
	3+	21	2	5	8	6

Note: *P<0.05; #P<0.05.

ovaries (n=1; from sigmoid colon), sigmoid colon from right colon (n=1) and liver and ligament lymph nodes of the duodenum from the rectum (n=1). Moreover, stage T2, T3 and T4 CRC was found in 1, 38 and 3 patients, respectively; stage N0, N1, N2 and N3 CRC in 7, 21, 12 and 2 patients, respectively. Moderately differentiated CRC was noted in 29 patients and poorly differentiated CRC in 13 patients.

Immunohistochemistry

The immunoreactivity of Lgr5 and VEGF located both at the cytoplasm and Ki-67 in the nucleus. HER2 immunostaining was mainly found on the cell membrane with/without cytoplasm. According to the HER2 expression pattern in the gastric cancer, only CRC cells with HER2 expression on cell membrane were counted. In stage IV CRC, the positive rate of Lgr5 was 92.9 (39/42), and 50% of CRC (21/42) showed

strong positivity (IHC 2+ or 3+). The positive rate of Lgr5 on stage IV CRC was higher than that reported in our previous study on stage I~IV CRC (54%, 55/102) (P<0.01) [2]. Statistical analyses revealed that the Lgr5 expression of variable extents was observed in the primary CRC (**Figure 1A**), and their matched metastatic lymph nodes (**Figure 1B**) and distant metastatic tissues (**Figure 1C**), and the proportion of strong positive samples increased in the above order (50% (21/42), 68.6% (24/35) and 71.4% (30/42)). The positive rate of Lgr5 expression in metastatic lymph nodes and distant metastatic tissues was both higher than it in the matched primary CRC (P<0.05) (**Table 2**). Of 51 paraffin embedded tissues, Lgr5 expression in the invasive front (n=50) (**Figure 2A**) and the residual tissues surrounding the necrotic cancer (n=41) (**Figure 2B**) were both markedly higher than that in tumor center (n=51) (P<0.001). In addition, the tissues at the budding site of cancer were also strong positive for Lgr5 (**Figure 2C**). Of patients with stage IV CRC, the rate of HER2 over-expression was as high as 42.1% (16/38, IHC: 2+ or 3+), and 28.9% (11/38) cases presented IHC 3+ (**Figure 3**). Spearman correlation analyses displayed that Lgr5 expression was positively associated with the HER2 over-expression and Ki-67 expression (P<0.05), but had no relationship with gender, age, cancer site, histological grade and VEGF over-expression (P>0.05; **Table 3**). Kaplan-Meier analyses showed the mean survival time of patients with Lgr5 negative or weakly positive CRC was 35.22±3.97 months, and that of patients with Lgr5 strong positive CRC was 28.74±3.57 months. Although the latter had shorter survival time, significant difference was not observed from the Log-rank test (P>0.05)

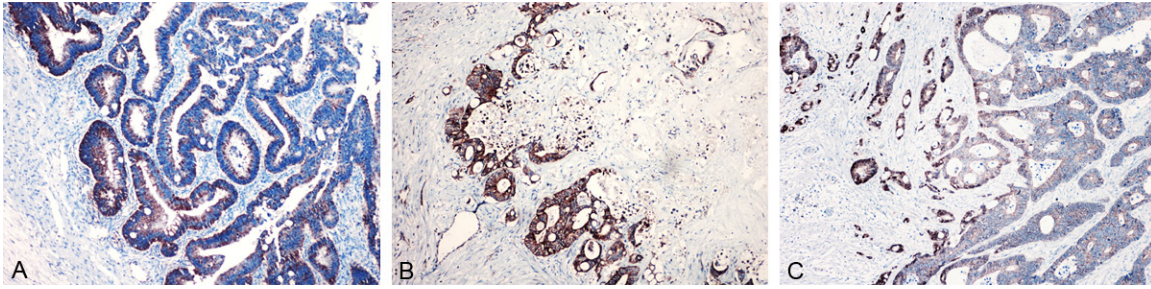


Figure 2. Strong immunoreactivity of Lgr5 was found at invasive front (A), residual tissues surrounding the necrotic cancer (B) and tumor budding (C) in CRC.

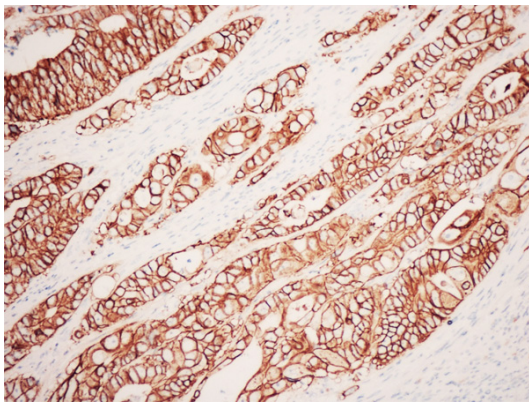


Figure 3. HER2 IHC 3+ was found in CRC.

(**Figure 4A**). Kaplan-Meier analyses showed the mean survival time was 33.77 ± 3.97 months in patients with low HER2 expression (0 or 1+) and 28.6 ± 2.83 months in patients with high HER2 expression (2+ or 3+). This suggested that patients with HER2 over-expression had a shorter survival time, but no marked difference was observed from the log-rank test ($P > 0.05$) (**Figure 4B**).

Discussion

In the present study, the expression of Lgr5, VEGF, HER2 and Ki-67 in the primary CRC and the matched normal mucosa, metastatic lymph nodes and distant metastatic tissues were detected by the immunostaining method. Our results showed that Lgr5 protein over-expression was not only involved in the tumorigenesis, proliferation, local invasion and lymph node and distant metastasis of CRC, but associated with the HER2 protein expression.

In recent years, research about the relationship between Lgr5 over-expression and CRC progression is in its infancy and this issue is still

controversial. Our previous study results by TMA and IHC methods revealed that 54% (55/102) of CRC demonstrated clustered or patchy expression of Lgr5, which was more frequently found in females, and Lgr5 expression was positively associated with β -catenin. However, the correlation of Lgr5 expression with CRC metastasis was not observed in that study [2]. In another study by Ziskin et al [8], the results showed that Lgr5 expression was not associated with the invasiveness of CRC by the FISH method in 891 patients with CRC. Nevertheless, other groups concluded that Lgr5 expression was involved in the CRC progression. In 2010, a study from Japan found that higher mRNA expression level of Lgr5 was found in metastatic CRC cell lines than in primary CRC cell lines. In addition, their clinical study also revealed that increased Lgr5 expression was involved in not only the pathogenesis of CRC (positive in 35 of 50 CRC patients and all the patients with sporadic adenoma) but the progression of CRC (being related with vasculature invasion, depth of invasion, lymph node metastasis and several pTNM stages [IIIC vs. IIIB]) [4]. A study from Norway also found that the proportion of CRC with positive expression against Lgr5 at the budding site, vasculature involvement and nerve involvement of primary CRC was 12.9%, 14.8% and 26.7%, respectively, and the ratio was as high as 51.6% at distant metastatic tissues [5]. Moreover, the Lgr5 expression level in metastatic tissues from primary CRC with positive Lgr5 expression was 6-11.5 times higher than it in metastatic tissues from negative cases. Amsterdam et al [10] found that the number of Lgr5 positive cells at the center of cancer nest was 1-4 in well differentiated CRC (stage 1-4) and 9-18 in poorly differentiated CRC by immunostaining method, and the cluster distribution of Lgr5

Table 3. Correlation analysis of Lgr5 expression and clinicopathological characteristics in patients with stage IV CRC

		n	Lgr5 IHC Score			
			0	1+	2+	3+
Gender	F	20	0	11	4	5
	M	22	3	7	9	3
Age	≤60	21	1	7	9	4
	>60	21	2	11	4	4
Grade	G2	29	2	10	11	6
	G3	13	1	8	2	2
HER2*	IHC 0	16	3	7	4	2
	1+	6	0	4	2	0
	2+	5	0	3	1	1
	3+	11	0	3	5	3
VEGF	IHC 0	2	0	0	1	1
	1+	9	1	5	2	1
	2+	15	1	7	5	2
	3+	7	1	3	2	1
Ki-67#	≤25%	2	0	1	1	0
	25%~50%	22	2	12	6	2
	50%~75%	12	1	5	3	3
	75%~100%	6	0	0	3	3

Note: *P<0.05; #P<0.05.

positive cells was closely related to the cancer stage. Occasionally, Lgr5 positive cells were found at the interface between cancer and stroma. In the present study, the Lgr5 expression was detected in stage IV CRC. Our results showed that Lgr5 expression was related to the proliferation of cancer cells (proportion of Ki-67 positive cells), and the Lgr5 expression in metastatic lymph nodes and distant metastatic tissues was significantly higher than that in primary CRC of the corresponding cases. In addition, we also found the heterogenous expression of Lgr5 protein by IHC in 51 tissue blocks of CRC. The results showed the invasive front was strongly positive against Lgr5 and the Lgr5 expression in the invasive front was markedly higher than that at the cancer center. This finding was consistent with that reported by Wu et al [11]. Moreover, the cancer at the budding site was also strongly positive against Lgr5. We also accidentally found that the Lgr5 expression was at a higher level at the residual tissues surrounding the necrotic cancer and significantly different from that at the cancer center. These results suggested that the increased Lgr5 expression was involved in the course of the

proliferation, local invasion and death regulation of CRC cells. Unfortunately, the number of surgically resected CRC cases at stage IV was very small, and there were only 42 samples were studied in this cohort. Further studies with large samples are required to confirm the above findings. In any case, the above findings provided the evidence that Lgr5 over-expression was involved in the death, local invasion, lymph node metastasis and distant metastasis of CRC cells. Although the discrepancies about this issue were found in the previous and present studies because of the difference in methodology, sample sizes and research subjects, increasing studies showed that Lgr5 positive CRC cells might have potent biological capacity and was possibly involved in the progression of CRC.

As in the correlation between Lgr5 over-expression and CRC progression, the relationship between Lgr5 and prognosis of CRC patients is still controversial. Wu et al [11] found that Lgr5 expression in primary CRC was significantly higher than that at the adjacent mucosa, and the Lgr5 expression was positively related to the cancer grade, invasion depth, lymph node metastasis, distant metastasis, pTNM stage and Ki-67 expression. These findings together with those from in vitro experiments suggested that the Lgr5 expression was closely associated with the prognosis of CRC patients, and Lgr5 was a possible marker of stem cells of CRC. Besides in CRC, studies also report that Lgr5 over-expression was also involved in the progression of gastric cancer [12] and glioblastoma [13], and patients with Lgr5 over-expression usually had a poor prognosis. Ziskin et al [8] employed FISH to detect the Lgr5 expression in 891 CRC samples. However, they did not found the correlation between Lgr5 over-expression and prognosis of CRC patients. In the present study, although CRC patients with Lgr5 over-expression had a shorter survival time, statistically significant difference was not noted. However, the number of CRC at stage IV is small in clinical practice, and studies with large sample sizes are required to elucidate this issue.

In 2010, ToGA III study showed patients with advanced gastric cancer with HER2 IHC 2+/FISH+ or HER2 IHC 3+ could benefit from traditional chemotherapy in combination with Herceptin [14]. In past 3 years, advanced gastric

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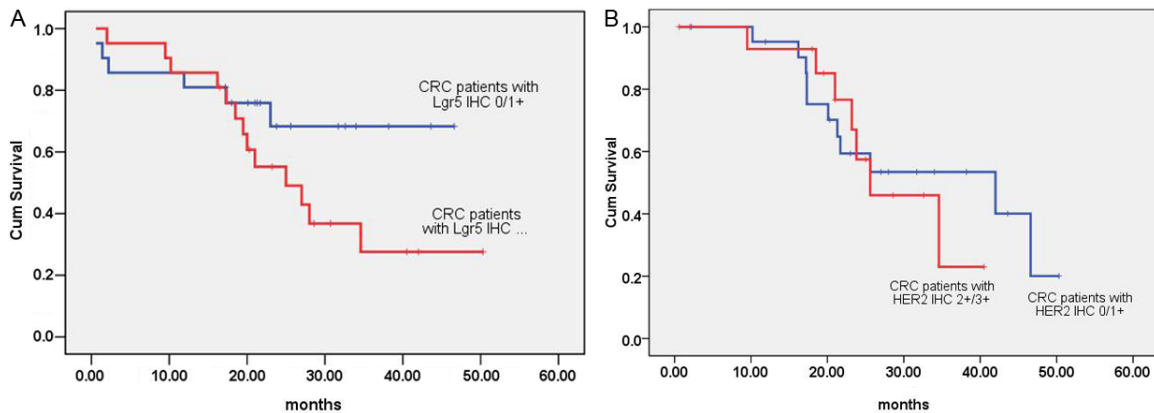


Figure 4. Survival curves of CRC patients with low (IHC 0/1+) and high (IHC 2+/3+) expression of Lgr5 (A) and HER2 (B).

cancer has been regarded an indication for Herceptin therapy which has been used in Europe, America, Japan, and China. This significantly prolong the overall survival time of advanced gastric cancer patients. Previous studies reported that the positive rate of HER2 in gastric cancer was 6.8-26.8% by the IHC method, and gastric cancer with HER2 expression was usually intestinal type gastric cancer [14, 15]. To date, few studies have been conducted to investigate the HER2 over-expression in CRC. In the present study, we examined the HER2 expression status in 42 end stage CRC by immunostaining, and the Lgr5 expression status was evaluated with the assessing criteria used in gastric cancer. And the results showed that 28.9% of advanced CRC showed HER2 IHC 3+ and 42.1% showed IHC 3+ or 2+. However, Wei et al [16] found that only 1.9% (1/53) of stage IV CRC was strongly positive against HER2. Another study from Taiwan revealed that 6% (4/67) of CRC was positive against HER2 on cell membrane [17]. Thus, the positive rate of HER2 over-expression in advanced CRC was significantly higher in the present study than that reported in above two studies. In gastric cancer, although there was controversy on the relationship between HER2 over-expression and prognosis of patients, most of the studies showed that patients with HER2 over-expression had a poor prognosis. In our study, end stage CRC patients with HER2 over-expression (similar to Lgr5 expression) had a shorter survival time when compared with patients whose tumors were negative or weakly positive for HER2, but marked difference was not observed. This finding was in accordance to that from the

study of Kruszewski et al [18]. Of note, the positive ratio of HER2 detected by IHC in stage IV CRC (3+ in 28.9% of CRC by IHC [cell membrane]) in our study was still higher than that in the study of Kruszewski et al [18] (27% of CRC positive on cell membrane; 66% of CRC positive in cytoplasm; overall, 3+ in 15% and 2+ in 32% of CRC).

It is well known that members of GPCR [19] and HER [20, 21] families are important targets in the treatment of malignancies. Currently, available studies showed that there was an interaction between these two families which might promote the growth and metastasis of breast cancer [22-25]. Lgr5 and HER2 are two important representatives of above two families, respectively. No study suggested the possible crosstalk between Lgr5 and HER2. In the present study, the close relationship between Lgr5 and HER2 expression in CRC was found by IHC and Spearman analysis in 42 CRC at stage IV. Thus, further study on the relationship between Lgr5 and HER2 might be helpful for elucidation of the role of two proteins in the progression and the targeted therapy of CRC.

Taken together, our findings showed that the Lgr5 protein expression in CRC was significantly higher than that in normal mucosa, but markedly lower than that at distant metastatic tissues and metastatic lymph nodes. Moreover, the Lgr5 protein expression at the invasive front, including tumor budding, and residual tissues surrounding the necrotic cancer was significantly higher than that at cancer center. These results suggested that Lgr5 expression was involved in the pathogenesis, growth, local

invasion and metastasis of CRC. In the primary CRC at stage IV, the proportion of HER2 over-expression on cell membrane was at a high level (IHC: 26.2% for 3+; 38.1% for 3+ or 2+), and Lgr5 expression was positively related to the HER2 expression and Ki-67 expression. Thus, there might be potential relationship among the proteins in the progression of CRC.

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Disclosure of conflict of interest

None.

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