Clinical Evidence on the Interaction Between MLK4, KRAS and Microsatellite Instability to Determine the Prognosis of Early-Stage Colorectal Carcinoma

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Key Words
MLK4 • KRAS • MSI • MSS • Colorectal carcinomas • Prognosis

Abstract
Background/Aims: MLK4 (KIAA1804) is the second most frequently mutated kinase in microsatellite stable (MSS) colorectal carcinomas (CRC). This molecule is known to regulate different physiological cellular processes, including cell cycle, senescence and apoptosis, and mechanistic evidence has been provided that MLK4 plays a role in carcinogenesis. However, whether this kinase exerts a tumor suppressive role or an oncogenic function is still an object of debate. This study aims to elucidate the role of MLK4 in the pathogenesis of CRC by investigating human tumor specimens. Methods: This study assessed MLK4 expression levels by immunohistochemistry in surgical tumor samples from 204 early-stage CRC patients and their correlation with various clinical-pathological features and patients' outcomes. In addition, MLK4 mRNA transcription was analysed in an independent cohort of 786 colon cancer samples. Results: Loss of MLK4 staining was associated with poor overall (OS) and progression free survival (PFS) in CRC patients during a univariate analysis (OS: 101 vs 164 months, p=0.0002; PFS: 85 vs 125 months, p=0.0001), as well as in multivariate analysis (OS: HR=1.70; p=0.001; PFS: HR=1.61; p=0.001). This was confirmed by analysis of MLK4 mRNA in the second independent cohort. A subgroup analysis according to KRAS mutation status showed that MLK4 staining was associated with better OS and PFS in KRAS mutated cases (HR=2.77; p=0.0001 and HR=2.31; p=0.0003, respectively) and microsatellite stable tumors.
(HR=1.87; p=0.002 and HR=1.06; p=0.006) but not in KRAS wildtype and microsatellite unstable tumors. **Conclusion:** By providing the first report from clinical specimens on the prognostic significance of MLK4, we define an oncogenic loss-of-function of this kinase and suggest a possible role in the interaction with KRAS signaling in determining an aggressive phenotype of CRC. These findings warrant the further investigation of MLK4 in wider cohorts and various clinical settings.

**Introduction**

Colorectal cancer is a heterogeneous tumor. However, in recent years, efforts to establish a molecular classification of colorectal carcinomas (CRC) led to the definition of biological, prognostic and clinical relevance, of several signaling pathways and gene mutations including, among others, APC, KRAS, BRAF, and microsatellite instability (MSI) [1-3]. In particular, determining the KRAS and MSI status of tumors has acquired a specific clinical and therapeutic relevance.

Activating KRAS mutations are found in approximately 40% of colorectal carcinomas. Constitutive activation of KRAS is known to induce phosphorylation of β-catenin, which in turn, causes its dissociation from E-cadherin, thus enabling the transcriptional activity of β-catenin [4-7]. Determination of KRAS mutational status has become standard clinical practice as a predictor of response to the administration of EGFR-blocking compounds, such as cetuximab and panitumumab [8, 9].

MSI defines a subset of tumors frequently exhibiting a CpG island methylator phenotype (CIMP) and a hyper-mutation phenotype (for review see [4]); such tumors are characterized by a better outcome in comparison to microsatellite-stable (MSS) tumors, and it has most recently been shown that the higher mutation burden and tumor-specific neo-antigen formation typical of MSI tumors underlies the excellent response rates and outcomes observed during treatment with immune-checkpoint inhibitors [10, 11].

MLK4 (MAP3K21 or KIAA1804), a member of the mixed-lineage serine/threonine kinase (MLK) family, activates c-Jun amino-terminal kinase (JNK) and p38 through its downstream targets MKK4/7 and MKK3/6 [12]. Within this signaling pathway, MLK4 is known to regulate several different cellular functions, including cell cycle, proliferation, senescence and apoptosis (for review see [13, 14]); thus, a role of this kinase in the development of CRC has been postulated. Specifically, mutations of MLK4 have been reported in around 3% of CRC [15, 16], which makes this gene the second most frequently mutated kinase in MSS tumors [1, 2]. MKK4/7 and JNK have been reported to have a tumor suppressive function in different tumor types [17-21], and multiple loss-of-function (LOF) mutations for MLK4 have been described in breast and pancreatic cancer [22, 23].

In spite of this putative function during cancer development, MLK4 is the least characterized member of the MLK family and its role in carcinogenesis is poorly understood. At present, the function of MLK4 in tumor formation has been addressed only by two preclinical studies providing contradictory results. The first characterized MLK4 mutations as activating and promoting tumorigenesis in KRAS mutated colorectal tumors [15]; the second described the same mutations as loss-of-function mutations causing impairment of the enzymatic activity of MLK4 [24]. However, to our knowledge no study has yet assessed the relevance of MLK4 in determining the prognosis of colorectal cancer patients.

Due to these contradictory reports, we investigated the correlation between MLK4 protein levels in colorectal carcinomas and overall survival (OS) or progression free survival (PFS) in relation to KRAS and MSI status.
Materials and Methods

Clinical samples for survival analyses

Surgical specimens from patients with early-stage colorectal adenocarcinomas exhibiting moderate differentiation (G2 according to WHO, T-categories T2 and T3 and with no nodal or distant metastasis), undergoing surgery in curative intention at the hospital of the Ludwig-Maximilians-University Munich (LMU Munich) between 1994 and 2004 were considered. Follow-up data were provided by the Munich Tumor Registry (TZM; Tumorregister München). To minimize biases related to the postoperative morbidity and comorbidity, patients who died within six months since surgical resection were not considered for analysis. The final case collection comprised tissue from 204 patients, 94 (46%) of whom died as a consequence of CRC within 5 years of diagnosis. The survival data of 158 cases (77%) was censored as case follow-up was discontinued or patients died due to other reasons than colorectal cancer. The characteristics of this collection are summarized in Table 1. The study was conducted in agreement with the requirements of the ethics committee of the University of Munich.

Tissue microarray technique

As previously noted, tissue microarrays (TMA) from CRC were generated [25]. In brief, representative areas of viable carcinoma tissue were determined on 5 μm sections of formalin fixed, paraffin embedded carcinoma samples which were stained with hematoxylin-eosin. By using a tissue-arraying instrument (Beecher Instruments, Sun Prairie, WI, USA), 1 mm needle core-biopsies were taken from appropriate areas of the corresponding paraffin-embedded carcinoma blocks. They were then positioned in recipient paraffin array blocks at specified coordinates. To ensure representative sampling, six probes were taken from each tumor, three from central carcinoma areas and three from the invasive front. To enhance adherence between cores and paraffin, the recipient blocks were incubated for 30 min at 37°C.

Immunohistochemistry

Immunohistochemical staining was performed on 5 μm sections of TMA blocks. As the primary antibody, MLK4 polyclonal rabbit antibody (Acris, dilution 1:40, Herford, Germany) was used. Pre-Treatment for antigen retrieval was performed by microwaving for 2 x 15 min at 750 W in Enhancer (Linaris, Cat.No. E7000, Dossenheim, Germany). Detection was performed using SignalStain Boost IHC Detection Reagent HRP, Rabbit, (Cell Signaling, Cat.No. B114). DAB+ (Dako, Cat.No. K3468, Hamburg, Germany) was used as a chromogen. Finally, slides were counterstained with hematoxylin Gill’s Formula (Vector Laboratories, Cat. No. H-3401, Eching, Germany). To verify staining specificity, system controls without primary antibodies, as well as immunoglobulin isotype control antibodies were employed.

Analyses of KRAS mutations

Analyses of KRAS exon 2 codon 12/13 were done as previously described [4, 6, 26]. Briefly, genomic DNA was extracted from micro-dissection carcinoma lesions using QIAamps DNA FFPE Tissue kit (Qiagen, Hilden, Germany). Pyro-sequencing was performed using the Pyro-Gold kit (Qiagen) and HotStar Taq-Polymerase (Qiagen). To identify anti-sense sequences, the PF2 primer (5’-tgt ggt agt tgg agc-3’) was used. For sequencing and sequence analyses, the PyroMark Q24 device (Qiagen) and the PyroMark™ Q24 software were applied [27, 28].

MSS analysis

As previously described, the status of MSS or high-grade microsatellite instability (MSI-H) was determined by analyzing the two-monomonucleotide repeat markers BAT-25 and BAT-26 [29-32]. DNA was
amplified in a duplex PCR (Qiagen DNA Multiplex PCR kit, 100 nM BAT25 and 100 nM BAT26-specific primers) with the following cycle profile: denaturation at 95°C for 15 min, 34 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 90 sec and extension at 72°C for 60 sec, with a final extension step at 60°C for 30 min. One ml of the PCR product was mixed with 18.5 ml of highly deionized formamide (HiDi formamide) and 0.5 ml DNA Size Standard LIZ 500 / (2250) (both Applied Biosystems, Darmstadt, Germany). This mixture was denatured for 3 min at 94°C, instantly put on ice, and separated using an ABI 3130 Genetic Analyzer. Results were evaluated applying GeneMapper Software (Applied Biosystems).

**Evaluation of MLK4 by immunohistochemistry**

Sections were examined using light microscopy. As expected, because MLK4 stained positive in the cytoplasm of carcinoma cells, cytoplasmic staining was defined dichotomically according to the presence (score 1) or absence (score 0) of a staining signal (Fig. 1A, B). To exclude intraobserver variability, an observer who had no prior knowledge of prognosis or other clinicopathological variables, evaluated the specimens thrice.

**Analysis of gene expression microarray data sets**

Publicly available colorectal cancer gene expression datasets which matched tumor transcriptome and clinical data were available and retrieved from the Gene Expression Omnibus (GEO - accession codes GSE14333 and GSE39582). Both datasets were generated on Affymetrix HG-U133 Plus2.0 microarrays and normalized simultaneously in R (www.r-project.org) by Robust Multi-array Average (RMA) [33] using custom brainarray CDF (v19, ENTREZG) [34], which yielded one optimized probe set per gene [35, 36].

**Statistical analyses**

Cross-tabulations were calculated using Fisher’s exact test. Kaplan-Meier analysis was employed to estimate cancer specific survival by the log-rank test. Optimal cutoffs for continuous variables were selected by receiver operating characteristic (ROC) curve analyses and Youden’s index. Multivariate analysis was performed using the multivariate Cox regression model. P-values < 0.05 were considered statistically significant. Statistics were performed using SPSS statistical software (version 25.0; SPSS Inc., Chicago, IL).
Results

**MLK4 protein levels in colorectal carcinomas**
A first analysis was conducted to evaluate MLK4 expression and cellular localization by immunostaining in CRC and matched normal colonic mucosa samples. As expected, staining for MLK4 was observed only in the cytoplasm of cells. In CRC, as defined by a dichotomic assessment according to the presence or absence of staining signal, MLK stained positive in 146 cases (72%) and negative in 58 cases (28%) (Fig. 1A and 1B). In contrast, MLK4 staining was evident with no exception in all matched non-tumor colon mucosa tissue adjacent to tumor lesions. In particular, although a pattern of continuous expression of MLK4 could be seen along the whole length of the crypts, its staining intensity was accentuated on their apical portion (Fig. 1C-E). Therefore, these data suggest that the loss of MLK4 staining is a frequent feature of CRC.

**Loss of MLK4 in colorectal carcinomas correlates with patient’s survival**
To assess the prognostic significance of MLK4 staining in determining the outcome of CRC patients, a Kaplan-Meier analysis according to the presence or absence of MLK4 staining was performed. Loss of MLK4 was associated with poorer OS and PFS in comparison to patients with positive MLK4 staining (p=0.0002 and p=0.0001, respectively; Fig. 2A and 2B). Age (p=0.019) but not gender (p=0.55) was significantly associated with patient outcomes. A multivariate Cox regression analysis including age, gender, T-category, KRAS mutational status and MSI status showed that loss of MLK4 staining was independently associated to a relative risk of 1.70 [confidence interval: 1.24 – 2.34] of poor overall survival and to a relative risk of 1.61 of disease progression [confidence interval: 1.22 – 2.11 – p = 0.001 each, Table 2 and 3].

To validate these findings, we tested for clinical correlations of MLK4 mRNA expression levels in a simultaneously normalized dataset comprising 786 CRC cases, which had follow-up data on tumor progression and in a subset of 562 patients within this collective with available data on OS. We identified ideal cutoff at the MLK4

**Fig. 2.** Significance of MLK4 expression on overall survival (OS) and progression free survival (PFS). Kaplan-Meier plot of OS (A) and PFS (B) according to the presence or absence of MLK4 staining (n = 204; in this and the following figures, the log-rank test was used to estimate the indicated p values).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases</th>
<th>Relative risk (95% confidence interval)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLK4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>146/204 (72%)</td>
<td>1.00</td>
<td>0.001</td>
</tr>
<tr>
<td>Negative</td>
<td>58/204 (28%)</td>
<td>1.70 (1.24 – 2.34)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>112/204 (55%)</td>
<td>1.00</td>
<td>0.698</td>
</tr>
<tr>
<td>Female</td>
<td>92/204 (45%)</td>
<td>1.14 (0.59 – 2.23)</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 70</td>
<td>144/204 (71%)</td>
<td>1.00</td>
<td>0.947</td>
</tr>
<tr>
<td>≥ 70</td>
<td>60/204 (29%)</td>
<td>1.64 (0.84 – 3.18)</td>
<td></td>
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<tr>
<td>T-category</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>31/204 (15%)</td>
<td>1.00</td>
<td>0.438</td>
</tr>
<tr>
<td>T3</td>
<td>173/204 (85%)</td>
<td>1.43 (0.58 – 3.55)</td>
<td></td>
</tr>
<tr>
<td>KRAS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT mutated</td>
<td>118/191 (62%)</td>
<td>1.19 (0.62 - 2.27)</td>
<td>0.603</td>
</tr>
<tr>
<td>MSI-Status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stable</td>
<td>64/183 (35%)</td>
<td>1.27 (0.66 - 2.44)</td>
<td>0.467</td>
</tr>
</tbody>
</table>
expression intensity of 233 and 189 (natural scale) for PFS and OS respectively, using ROC curve analyses and Youden's index (Fig. 3A). Dichotomous categorization of cases by means of these cutoffs exhibited a highly significant positive correlation between MLK4 mRNA expression and PFS (p=0.000003) and OS (p=0.02) by using the Kaplan-Meier method (Fig. 3B). Applying a proportional hazards regression analysis, PFS was shown to be a prognostic factor independent of other key clinical and pathological variables (Table 4), whereas
OS showed a trend toward statistical significance (p = 0.053). Collectively, these findings suggest that preserved expression of MLK4 is associated with a favorable outcome in patients with CRC.

**MLK4 staining correlates with patient outcomes in KRAS mutated but not in KRAS wild-type (WT) tumors**

It has been recently proposed that MLK4 interacts with the RAS pathway to increase tumorigenicity in CRC [15]. To assess a possible interaction between MLK4 and KRAS mutation status in determining prognosis, a survival analysis was repeated by stratifying patients according to the presence or absence of KRAS mutations. In line with the expected incidence of KRAS mutations, 73 (38%) out of the 191 cases with available KRAS mutational status had an exon 2 codon 12 or codon 13 mutation. KRAS mutational status was not associated with age (p = 0.447), gender (p = 0.229), T-category (p = 0.228) or MSI Status (p = 0.223) and, as expected [37], did not correlate with OS (p = 0.57) or PFS (p = 0.07). Positive MLK4-staining was detected in 52 (71%) of KRAS-mutated and in 86 (73%) of KRAS WT patients (Table 5).

Analysis of survival showed that preserved MLK4 staining correlates with a better OS and PFS of patients with KRAS mutations (p = 0.0001 and p = 0.0003, respectively; Fig. 4A and 4B). Loss of MLK4 staining was associated with an independent relative risk of 2.77 [CI: 1.64–4.69] for OS and 2.31 [CI: 1.50–3.56] for PFS in a multivariate Cox regression analysis including gender, age and T-category (p = 0.0001). In contrast, no correlation was found between MLK4 levels, OS and PFS in patients with KRAS WT (p = 0.10 and p = 0.17, respectively; Fig. 4C and 4D). The prognostic relevance of MLK4 in KRAS WT tumors points to a functional interaction between the loss of this kinase and KRAS mutations to determine an aggressive phenotype.

**MLK4 staining in MSS colorectal carcinomas correlates with patient outcomes**

In a subsequent analysis, MLK4 was assessed according to the microsatellite stability status of patients, which was available in 183 cases. Out of the 64 (35%) MSI tumors, 20 (31%) had no MLK4 staining. In patients with MSS tumors, absence of MLK4 staining was found in 32 (27%) cases (Table 6).

In MSS cases, no correlation was found between MLK4 and different clinical-pathological variables such as age, gender, T-category and KRAS mutation status (Fisher’s exact test; data not shown). However, MLK4 positivity was associated with better OS and PFS (p = 0.002 and p = 0.006 respectively; Fig. 5A and 5B). This was confirmed by a multivariate Cox regression analysis including gender, age, T-category, KRAS mutational status and MLK4 staining, indicating an independent relative risk of 1.87 [CI: 1.24 – 2.82, p = 0.003] for OS and of 1.60 [CI: 1.14 – 2.26, p = 0.007] of tumor progression in this subgroup.

In MSI cases no significant correlation was found between cytoplasmic MLK4 levels and clinicopathological variables or patient survival (Fig. 5C). Although a significant correlation

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**Table 4. Multivariate analysis of MLK4 expression and clinical variables for disease free survival (PFS) and overall survival (OS). CI: confidence interval**

<table>
<thead>
<tr>
<th>Variables</th>
<th>HR (95% CI)</th>
<th>P</th>
<th>HR (95% CI)</th>
<th>OS (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (≥ vs &lt; median)</td>
<td>1.00 (0.77 - 1.13)</td>
<td>0.973</td>
<td>1.87 (1.39-2.53)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Gender (F vs M)</td>
<td>0.74 (0.57 - 0.96)</td>
<td>0.025</td>
<td>0.68 (0.50-0.91)</td>
<td>0.010</td>
<td></td>
</tr>
<tr>
<td>AJCC stage</td>
<td>2.62 (2.25 - 3.19)</td>
<td>&lt;0.0001</td>
<td>2.09 (1.70-2.50)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>MLK4</td>
<td>0.59 (0.45 - 0.77)</td>
<td>0.0001</td>
<td>0.75 (0.56-1.00)</td>
<td>0.053</td>
<td></td>
</tr>
</tbody>
</table>

**Table 5. MLK4 expression in relation to KRAS mutational status**

<table>
<thead>
<tr>
<th></th>
<th>KRAS wt</th>
<th>KRAS mut</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLK4 neg</td>
<td>32 (27%)</td>
<td>21 (29%)</td>
<td>53 (28%)</td>
</tr>
<tr>
<td>MLK4 pos</td>
<td>86 (73%)</td>
<td>52 (71%)</td>
<td>138 (72%)</td>
</tr>
<tr>
<td>Total</td>
<td>118 (62%)</td>
<td>73 (38%)</td>
<td>191 (100%)</td>
</tr>
</tbody>
</table>
between MLK4 staining and PFS (p = 0.043; Fig. 5D) was observed in the univariate analysis, this could not be confirmed in a multivariate Cox regression analysis including age, gender, T-category and KRAS mutational status (p = 0.127).
Discussion

Mutations of MLK4 have been reported to occur in 3% of CRC and in two out of 24 colorectal cancer cell lines [15, 16, 38]. In our assessment of human CRC, we found a positive correlation between the presence of MLK4 staining, OS and PFS, which was confirmed by a multivariate analysis. Our findings were validated by data on mRNA expression levels from a publicly available gene expression microarray cohort of 786 colon cancers, which confirmed the strong positive correlation between high MLK4 levels expression and PFS (Fig. 3). Therefore, these data, which focus on patients with early-stage tumors, support the hypothesis that MLK4 has a tumor suppressive function in CRC. This is corroborated by the fact that, while MLK4 loss was found in 28% of samples, staining for MLK4 was invariably present in normal colonic mucosa, where it stained positive along the full length of the crypts. The higher prevalence of MLK4 loss in tumor samples vs. normal tissue, points to the fact that this kinase might play a more important and frequent role in the pathogenesis of CRC than suggested by the reported prevalence of its mutations (3%). This is likely due to the fact that epigenetic alterations, like aberrant methylation of the KIAA1804 gene, might contribute to the loss of MLK4 together with less frequently observed mutations of this gene [39].

Our subgroup analysis showing that MLK4 has a prognostic significance in KRAS mutated and in MSS tumors, sheds light on how MLK4 mechanistically interferes with the biology of CRC in determining an aggressive phenotype. KRAS has been described to contribute to the pathogenesis of CRC by causing oncogene-induced senescence (OIS), a process which recent evidence has shown to be counteracted by JNK and p38 [40-42], both of which are downstream targets of MLK4. Consistently, preclinical investigation has shown that selectively restoring the function of MLK4 leads to activation of JNK and its downstream targets, cJUN and ATF in colon cancer cells [24]. Therefore, escape from OIS by preserved MLK4-JNK signaling might be one mechanism by which MLK4 counteracts the oncogenic function of KRAS in CRC.

Patients with MSS CRC have a poorer prognosis in comparison to patients with MSI tumors. This is thought to be due to several factors, comprising the higher immunogenicity of MSI tumors, which is responsible for a higher effectiveness of mechanisms of immunemediated elimination of cancer cells [43-48]. Our data showed that MLK4 positivity was associated with better OS and PFS in MSS cases, but not in MSI tumors (Fig. 5A). The lack of prognostic significance in MSI tumors might be due to the fact that a possible beneficial effect of MLK4 expression could be attenuated by the overall prognosis in MSI patients and may not be captured in our analysis due to the better small size of this patient subgroup in our collective. However, this may have a mechanistic cause: although no data are available on the significance of MLK4 in MSS tumors, MLK3, a closely related member of the MLK family was shown to function as a repressor of WNT signaling by reducing the transcriptional activity of the β-catenin/TCF complex. Since loss-of-function mutations of APC or stabilizing mutations of β-catenin are frequently found in MSS tumors, MLK4 might act to counteract the oncogenic effect of WNT signaling in these tumors. The favorable effect of MLK4-expression in MSS patients observed by us and the high mutation frequency reported for MLK4 in MSS tumors by The Cancer Genome Atlas (TCGA) consortium might reflect this function [2].

Conclusion

By analyzing human specimens, we provide the first evidence on the fact that loss of MLK4 is a determinant in the prognosis of CRC patients. We contribute to the debated question on the function of MLK4 in tumorigenesis by suggesting that MLK4 exerts a tumor suppressive function. Our results also shed light on possible mechanisms of action of MLK4 in CRC and other tumors by postulating an interaction with KRAS signaling in determining an aggressive phenotype. These findings warrant the further investigation of MLK4 in wider
coHORTS and different clinical settings. In particular, we propose that MLK4 is assessed in vitro to detect a possible interaction with RAS-RAF-MEK-K-ERK signaling and that its role in relation to β-catenin signaling is assessed in MSS tumors. In addition, MLK4 might play a role in determining whether and to what extent patients respond to treatment with EGF-receptor antagonists.

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The study protocol has been approved by the research institute's committee on human research.

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Disclosure Statement

No conflicts of interest exist.

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