Int J Clin Exp Pathol 2018;11(5):2613-2620
www.ijcep.com /ISSN:1936-2625/IJCEP0072888

Original Article

MST1 inhibits cell proliferation and invasion of non-small-cell lung cancer by regulating YAP phosphorylation and Hippo pathway

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Received January 16, 2018; Accepted February 23, 2018; Epub May 1, 2018; Published May 15, 2018

Abstract: Mammalian ste20-like kinase 1 (MST1) is a Ser/Thr kinase involved in cell proliferation, apoptosis, and embryonic development. MST1 is also an important member of the Hippo pathway which can regulate organ size and cell proliferation. Growing evidence indicates that MST1 influences cell proliferation through different pathways including Wnt, Akt, JNK, and Hippo pathways in different cells respectively. However, little is known about the role and mechanism of MST1 in lung cancer. In this study, we aimed to assess the biological functions of MST1 in non-small-cell lung cancer (NSCLC). We investigated expression of MST1 in lung cancer cell lines and one human bronchial epithelium cell line (HBE) by Western blot. The results confirmed that MST1 expression was higher in HBE cells, but significantly lower in NSCLC cells. Moreover, MST1 was either overexpressed or depleted in NSCLC cell lines, and the results confirmed that MST1 could markedly inhibit cell proliferation and invasion through regulating the Hippo pathway and Yes-associated protein (YAP) phosphorylation. These results indicate that MST1 may play an important role in NSCLC and may serve as a potential therapeutic target for NSCLC.

Keywords: Mammalian Sterile 20-like kinase 1, Yes-associated protein, non-small-cell lung cancer

Introduction

Mammalian ste20-like kinase 1 (MST1) is kinase which belongs to a serine/threonine family of sterile 20 (Ste20) proteins [1, 2]. It was reported that MST1 is involved in diverse functions including apoptosis, autophagy, and cellular proliferation in different organs or tissues [3-11]. MST1 deficient or mutant mice have been utilized to show increased proliferation or tumorigenesis of liver and colon [12, 13]. In addition, Lin et al. reported that loss of MST1/2 kinases in the epithelium can lead to neonatal lethality caused by lung defects, indicating the important role of MST1 in development [14]. Lange AW et al. also found Mst1/2 deletion in the fetal lung enhanced proliferation of bronchiolar epithelial cells [15]. Increasing reports also demonstrated that MST1 has important roles in tumors [5, 16-19]. Lin et al. reported decreased expression of MST1 was detected in breast cancer, and the patients with negative expression of MST1 had poor overall survival [16]. Similar results were also reported in colorectal cancer, gastric cancer, and astrocytoma [5, 17-19]. Furthermore, Seidel C et al. found frequent hypermethylation of the CpG island promoter of MST1/2, leading to reduction of MST1 expression as determined by RT-PCR in human soft tissue sarcomas [20].

MST1 was also identified as an important member of Hippo signaling pathway which is involved in regulating organ size and organism homeostasis [9]. The core component and downstream effector of the pathway in mammals is Yes-associated protein (YAP) which has been shown as a potent oncogene [21]. Nuclear location of YAP, which has been observed in a variety of tumors [22-24], might drive tumor cell proliferation. As the upstream kinases, MST1 reportedly could induce YAP phosphorylation and cytoplasmic location [8, 12].

Interestingly, increasing evidence indicates that MST1 may influence cell proliferation through

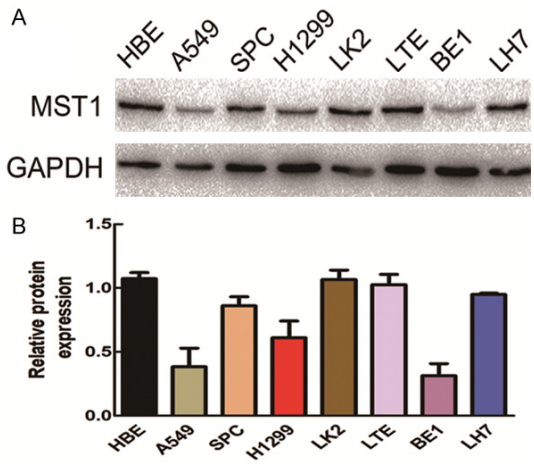

Figure 1. A, B: The protein level of MST1 in seven lung cancer cell lines (A549, BE1, SPC, LK-2, H1299, LH7, and LTE) and one human bronchial epithelium cell line (HBE). MST1 was high expressed in HBE cells, whereas loss or low expression of MST1 was found in, A549, H1299, and BE1 cells.

different pathways including Wnt, Akt, JNK, and Hippo pathways in different cells respectively [5, 25-27]. Chao et al. reported that MST1 regulates proliferation of glioma cells via AKT/mTOR signaling pathway rather than YAP phosphorylation [5]. Overexpression and nuclear location of YAP in non-small-cell lung cancer (NSCLC) has been well documented [23]. However, little is known about the role and mechanism of MST1 in lung cancer. In this study, we aimed to assess the biological functions of MST1 NSCLC, and further confirmed whether MST1 functioned through regulating YAP phosphorylation.

Materials and methods

Cell culture

Human bronchial epithelium cell line (HBE) and human NSCLC cell lines A549, BE1, SPC, LK-2, H1299, LH7, and LTE were cultured in RPMI-1640 (Gibco, Invitrogen, NY, USA) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. For transfections, cells were seeded in a six-well plate 24 h before the experiment.

Plasmid and transfection

The plasmid of pJ3H-Mst1 (Plasmid #12203) was purchased from addgene. pJ3H-Mst1 or control pJ3H plasmid was transfected into cells using Lipofectamine Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The protein level was assessed 72 h later by Western blotting.

Small-interfering RNA experiment

MST1 siRNA (sc-39570) was purchased from Santa (Santa Cruz, USA). For the siRNA transfection experiments, cells were seeded in six-well plates according to the manufacturer's instructions. Twenty-four hours later, the siRNA was transfected into the cells using Lipofectamine RNA iMAX reagent (Invitrogen, Carlsbad, CA, USA). Transfected cells were incubated for another 48 h and subjected to various analyses. Following transfection, the protein level was assessed 72 h later by Western blotting.

Western blotting

Total protein from cells were extracted in lysis buffer (Pierce, Rockford, IL, USA) and quantified by the Bradford method. The same amount of protein was separated by 10% SDS-PAGE and then electophoretically transferred to a PVDF membrane (Millipore, Bedford, MA). The membrane was blocked with 5% dry milk and incubated overnight at 4°C with rabbit polyclonal antibodies against MST1 (1:500, #3682, Cell Signaling Technology), p-YAP (p-Ser 127 YAP, 1:500, #4911, Cell Signaling Technology), YAP (1:500, Cell Signaling Technology, #14074), CTGF (1:100, #sc14940, Santa Cruz Technology, Inc. CA, USA); CyclinD1 (1:500, #2922, Cell Signaling Technology) and GAPDH (1:2000, #G8795, Sigma, St. Louis, MO, USA). After washing, the membrane was incubated with a secondary antibody for 2 h. Protein bands were visualized with the enhanced chemiluminescence (Pierce) and detected using BioImaging Systems (UVP, Upland, CA, USA). The relative protein levels were calculated based on β-actin protein as a loading control.

MTT assay

Twenty-four hours after transfection, cells were plated in 96-well plates in medium containing 10% FBS at about 2000 cells per well, and quantitation of cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Briefly, 20 μl of 5 mg/mL MTT (Sigma, St. Louis, MO, USA) solution was added to each well and incubated for 4 h at 37°C, then the media was removed from

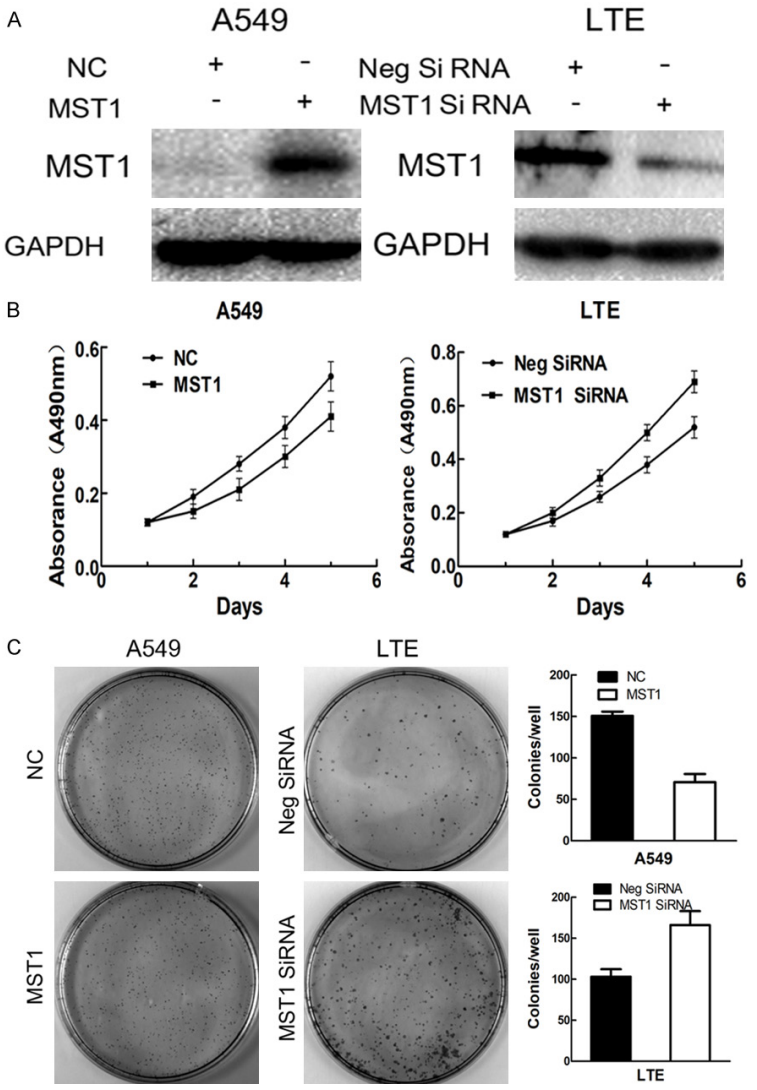

Figure 2. A: Western blot analyses of the protein expression of MST1 in NSCLC cells transfected with MST1 or MST1 specific siRNA. Left, A549 cells transfected with pJ3H-Mst1 and empty vector. Right, LTE cells transfected with specific MST1 siRNA and negative control siRNA. B: MTT assay indicates that overexpression of MST1 in A549 cells suppressed proliferation and that downexpression of MST1 in LTE cells promoted proliferation (Points, mean results of three independent experiments; bars, SD, $P<0.05$). C: Colony formation assay indicates that MST1 overexpression significantly suppressed cell proliferation in A549 cells and that MST1 downexpression markedly promoted proliferation in LTE cells. Columns, mean for three experiments; bars, SD ($P<0.05$).

each well, and the resultant MTT formazan was solubilized in 150 μl of DMSO. The results were quantitated spectrophotometrically by using a test wavelength of 490 nm.

Colony formation assay

Forty-eight hours after transfection, cells were plated into 6-cm cell culture dishes (1000 cells per dish) and incubated for 14 days. Cells were then stained with Giemsa and the number of colonies with more than 50 cells were counted.

Cell invasion assay

A 24-well Transwell chamber was used with a pore size of 8 μm (Corning, NY, USA) and the inserts were coated with Matrigel (BD Bioscience) in serum-free medium. Forty-eight hours after transfection, cells were trypsinized and transferred to the upper Matrigel hamber in serum-free medium containing $3*10^5$ cells and incubated for 16 h. Non-invading cells on the upper membrane surface were then removed with a cotton tip, and the cells which passed through the filter were fixed with 4% paraformaldehyde and stained with hematoxylin. The number of invaded cells was counted in 10 randomly selected high power fields under microscope. Data presented are representative of three individual wells.

Statistical analysis

Statistical software SPSS 13.0 (SPSS, Chicago, IL, USA) was used for all analyses. The Kaplan-Meier method was used to estimate t differences in the survival of subgroups of patients were compared by using Mantel's log-rank test. A p value of <0.05 was considered to indicate statistical significance.

Results

MST1 expression was lower in NSCLC cells

To investigate expression of MST1 in NSCLC cells, we examined expression in seven lung cancer cell lines (A549, BE1, SPC, LK-2, H1299, LH7, and LTE) and one human bronchial epithelium cell line (HBE). As shown in **Figure 1**, MST1

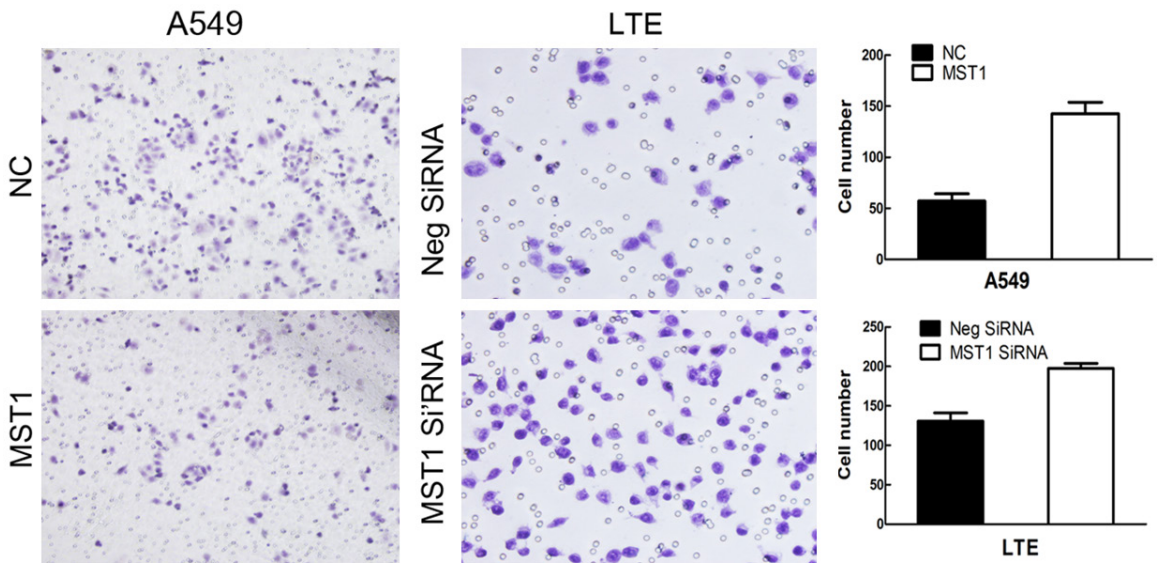

Figure 3. Effects of MST1 on the invasion of NSCLC cell lines. Transwell assay indicates that overexpression of MST1 in A549 cells significantly suppressed cell invasion. Knockdown of MST1 in LTE cells greatly promoted tumor cell invasion. Graphs show the number of cells invaded through the transwell after 16 h of incubation. The number of invaded cells was counted in 10 randomly selected high power fields under microscope. Columns, mean for three experiments; bars, SD (P<0.05).

was high expressed in HBE cells, whereas low expression of MST1 was found in A549, H1299, and BE1 cells.

MST1 inhibited proliferation of lung cancer cells

To evaluate the biological influence of MST1 on NSCLC, we overexpressed MST1 in A549 cells, and the protein levels of MST1 were significantly upregulated compared with control empty vector. We also down-regulated MST1 in LTE cells, and the protein levels of MST1 were significantly depleted (**Figure 2A**). We then performed MTT assay and colony formation assays in overexpressed and depleted cells. By MTT assay, the proliferation rate in A549 cells after transfecting with pJ3H-Mst1 was significantly reduced compared with control groups (**Figure 2B**). Also, silencing of MST1 by siRNA knockdown elicited a significant increase of the proliferation rate, as compared with control groups (**Figure 2B**). Consistent with the results of MTT assay, overexpression of MST1 in A549 cells (control versus MST1, 131±9 versus 70±6, P<0.05) led to a significant reduction in foci numbers as well as sizes (**Figure 2C**). Depletion of MST1 in LTE cells (control versus MST1 siRNA, 130±10 versus 197±6, P<0.05) led to a significant increase in foci numbers as well as sizes (**Figure 2C**).

MST1 inhibited the invasion of lung cancer cells

To further address the impact of MST1 on NSCLC cell invasion, we then performed Matrigel invasion assay in A549 and LTE cells with overexpression and downregulation of MST1 respectively. As shown in **Figure 3**, overexpression of MST1 in A549 cells significantly inhibited cell invasion compared with control empty vector (control versus MST1, 150±9 versus 70±17, P<0.05). In contrast MST1-depleted LTE cells showed significantly stronger invasion ability compared with control siRNA transfected cells (control versus MST1 siRNA, 103±16 versus 166±29, P<0.05).

MST1 regulated the Hippo signaling pathway via influencing YAP phosphorylation

To explore the mechanism by which MST1 inhibited cell proliferation and invasion in NSCLC cells, we performed overexpression and downregulation of MST1 to investigate its influence on the Hippo pathway. By Western blot, overexpression of MST1 in A549 cells significantly inhibited the expression of CTGF and CyclinD1 (**Figure 4A**). Conversely, depletion of MST1 in LTE cells significantly increased the expression of CTGF and CyclinD1 (**Figure 4B**). The results indicated MST1 could regulate the Hippo pathway in NSCLC cells.

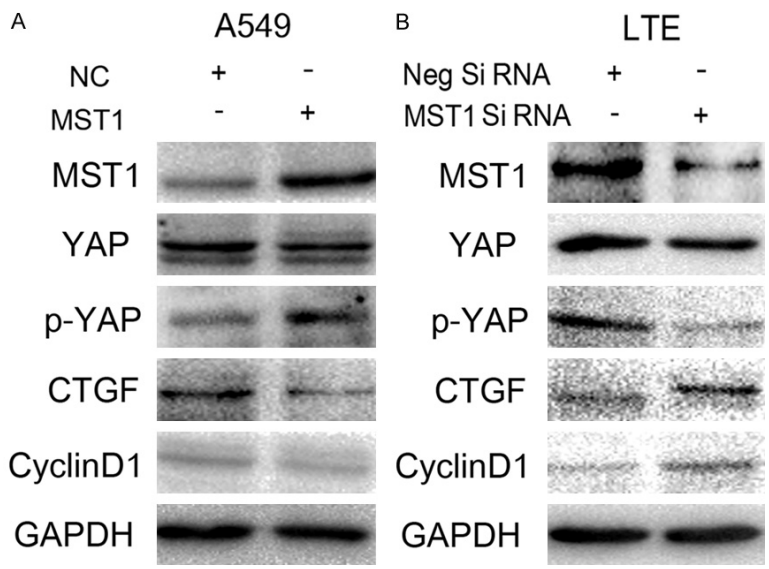

Figure 4. A: Western blot showing overexpression of MST1 in A549 cells and significantly inhibited expression of CTGF, CyclinD1, and increased phosphorylated YAP (p-Ser 127) compared with total YAP. B: Depletion of MST1 in LTE cells significantly increased expression of CTGF and CyclinD1 and decreased phosphorylated YAP (p-Ser 127) compared to total YAP.

Further study demonstrated that overexpression of MST1 in A549 cells could increase the level of phosphorylated YAP (p-Ser 127) rather than the level of total YAP (**Figure 4A**). Additionally, depletion of MST1 in LTE cells could significantly decrease the level of phosphorylated YAP (p-Ser 127) rather than the level of total YAP (**Figure 4B**). These results indicate that MST1 could inhibit proliferation and invasion of lung cancer through its regulation of YAP phosphorylation and Hippo pathway.

Discussion

Lung cancer has been the leading cause of death among the malignant tumors worldwide [28]. In recent years, targeted molecular therapies on NSCLC especially on adenocarcinoma have made great progress. However, since the incidence of NSCLC is increasing and the long-term survival rate is not improved, it is still an important goal to explore more novel therapeutic targets.

MST1 was first identified as a homolog of the ste20 kinase from Saccharomyces cerevisiae in 1995 [1]. Subsequently, MST1 was shown to respond to cellular stress and lead to cellular death [29]. Further studies indicated that MST1 is involved in autophagy and cellular proliferation [6-8]. Consequently, MST1 may also function as a tumor suppressor because of its regulation of cellular homeostasis and cellular apoptosis. MST1 deficient or mutant in mice has been proven to induce proliferation or tumorigenesis of liver and colon [12, 13]. Decreased expression of MST1 has been reported in a variety of tumors including breast cancer colorectal cancer, gastric cancer, astrocytoma, and soft tissue sarcomas [15-20]. These results demonstrated that MST1 might function as a tumor suppressor.

However, little is known about the expression and function of MST1 in NSCLC. So far, the only report from Xu et al. showed that ectopic expression of MST1 in lung cancer cell A549 could inhibit cell growth and induce apoptosis [30]. By Western blot, we also observed high expression of MST1 in normal bronchial epithelium cell but low expression in cancer cells. Our result further demonstrated that down-regulation of MST1 might involve tumorigenesis of NSCLC.

MST1 is reportedly involved in the control of proliferation, invasion, apoptosis and/or differentiation of various types of cells [5, 6, 30]. Next, we further investigated the role MST1 in regulating the proliferation and invasion of lung cancer cells. MTT assay showed that the proliferation rate was reduced by MST1 overexpression and increased after MST1 knockdown. Consistent with results of the MTT assay, overexpression of MST1 led to a significant decrease, whereas depletion of MST1 led to a significant increase in foci numbers. These results demonstrate that MST1 can inhibit the proliferation of lung cancer cells. In contrast, loss of MST1 expression can promote proliferation and colony formation in lung cancer cells. Our results were in agreement with the reports by Xu et al. [30] that overexpression of MST1 could suppresses cell growth and cell progression. Moreover, in the present study, Matrigel invasion assay showed that overexpression of MST1 significantly inhibited cell invasion compared with control empty vector, whereas depletion of MST1 significantly induced inva-

sion compared with control empty vector in lung cancer cells. These results also demonstrated that MST1 could significantly suppress migration and invasion of NSCLC.

More recently, MST1 was also identified as a member of the Hippo pathway which can regulate organ size and cell proliferation. As an important downstream effector, YAP has been thought as a potent oncogene [31]. When the pathway inactivating, YAP failed to be phosphorylated and entered the nuclei. Then the nuclear location of YAP functions as a transcription co-activator and can induce the transcription of genes including CTGF, CyclinD1, and promote cell proliferation. Thus far, overexpression and nuclear location of YAP has been observed in liver, ovarian, prostate cancer, and lung cancer [22, 23, 32, 33]. Expression of YAP in NSCLC was significantly correlated with p-TNM stage, lymph node metastasis, and poor prognosis. Moreover, overexpression or depletion of YAP confirmed that YAP markedly promoted cell proliferation and invasion [22].

MST1 was the upstream kinase of Hippo pathway, reportedly could lead to YAP phosphorylation and cytoplasmic location [12]. However, in addition to Hippo pathway, MST1 may also influence cell proliferation through different pathways including Wnt, Akt, and JNK pathway [25-27]. Chao et al. reported that MST1 regulates proliferation of glioma cells via AKT/mTOR signaling pathway rather than YAP phosphorylation and Hippo pathway [5]. Thus, the present study aimed to evaluate whether MST1 influences proliferation and invasion of NSCLC by regulating YAP. In the present study, overexpression of MST1 in A549 cells significantly inhibited expression of CTGF and CyclinD1. Conversely, depletion of MST1 in LTE cells significantly increased expression of CTGF and CyclinD1. The results indicated that MST1 could regulate the Hippo pathway in NSCLC cells. Therefore, overexpression of MST1 could activate Hippo signaling pathway, and depletion of MST1 could inactivate Hippo signaling pathway. Further study indicated that overexpression of MST1 could significantly increase the level of phosphorylated YAP, depletion of MST1 could significantly decrease the level of phosphorylated YAP. The results indicate that MST1 could inhibit the proliferation and invasion of lung cancer through its regulation of Hippo signaling pathway and YAP phosphorylation.

In conclusion, the present study identified MST1 as a candidate tumor suppressor which was downregulated in NSCLC cells. In addition, further study in NSCLC cell lines confirmed that MST1 markedly inhibited cell proliferation and invasion and could negatively regulate YAP oncoprotein. These results indicate that MST1 may play a potential role in NSCLC and MST1 might serve as a candidate target protein for future therapeutics of NSCLC.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81401885) and the funding from the Basic Scientific Project of University, Liao Ning Province, China (LQNK201705).

Disclosure of conflict of interest

None.

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