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LncRNA TUC338 promotes invasion of lung cancer by activating MAPK pathway

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Abstract. – OBJECTIVE: Lung cancer is highly heterogeneous and the 5-year survival rate is less than 15%. It is currently difficult to determine the heterogeneity of lung cancer and the underlying pathogenetic of metastasis. We aimed to investigate the effect of long non-coding RNA (lncRNA) TUC338 on the invasion of lung cancer by activating MAPK pathway and to understand the heterogeneity and metastasis mechanism of lung cancer.

PATIENTS AND METHODS: The expression of lncRNA TUC338 in 42 samples of lung cancer and paracancerous tissues were accessed by RT-qPCR. The relationship between the expression of lncRNA and clinicopathological parameters was analyzed. After overexpressing and interfering with lncRNA TUC338, effects of lncRNA TUC338 on cell proliferation and invasion were determined by cell counting kit-8 (CCK8) and transwell assay. The protein expression was evaluated by Western blot.

RESULTS: Higher expression of TUC338 in lung cancer was observed in comparison with that in paracancerous tissues. The survival time of TUC338 was correlated with the expression of TUC338. Clinical data analysis revealed that the expression of TUC338 was correlated with the overall survival, tumor size and lymph node metastasis in patients, but not with age and gender. After interfering and overexpressing TUC338, it was found that the activity of lung cancer cells was decreased, as well as the invasion ability after interference with TUC338. After overexpression of TUC338, we found that lung cancer cell activity increased, as well as the invasion ability. By Western blot, we found that TUC338 can promote the development of lung cancer through regulating MAPK pathway.

CONCLUSIONS: TUC338 was overexpressed in lung cancer, and its expression may have a relationship to the prognosis of lung cancer. MAPK pathway was involved in the invasion of lung cancer regulated by TUC338.

Key Words:
lncRNA TUC338, MAPK pathway, Lung cancer, Invasion.

Introduction

Lung cancer is one of the most common malignant tumors, whose morbidity and mortality rank first in the world[1,2]. It has been reported that in the next 30 years, the number of lung cancer deaths in China will reach as high as 18 million. Since 2025, the number of people dying from lung cancer in China will be over 1 million each year[3]. Due to the insidious onset of lung cancer, most of them are in advanced stage when diagnosed, resulting in treatment difficulties, thus shortening the survival of patients. At present, the common clinical markers of lung cancer are carcino-embryonic antigen (CEA), neuron-specific enolase (NSE), Cy-fra21-1, etc. However, the sensitivity of the diagnosis of lung cancer and its specificity are not high, which cannot meet the clinical requirements.

Long non-coding RNA (lncRNA) has no function in encoding protein. The transcript is over 200 nt, it can regulate gene expression through multiple levels, including transcription and post-transcriptional regulation levels. With the deepening of research, more and more studies reported that lncRNA was greatly involved in the development of various diseases[4]. In tumor research, lncRNA can be served as a marker of early diagnosis, as well as the target of treatment[5]. LncRNA TUC338 functioned a lot in cancers such as liver cancer and tongue cancer, however, the relationship between lncRNA TUC338 and lung cancer has not been reported[6,7].

MAPK mitogen-activated protein kinases (MAPKs) are intracellular class of serine/threonine protein kinases. MAPK signaling pathway is divided into three major categories, namely, extracellular signal-regulated kinase (ERKs), c-Jun N-terminal kinase (JNKs) and P38 kinase pathway. The activation of MAPK signaling pathway happened through chemical substances, oxidative

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stress, cytokines, neurotransmitters and other factors; thereby, phosphorylation occurred. Transient activation of ERKs promotes cell survival and proliferation, and continuous activation of ERKs promotes cell differentiation. For JNKs, transient activation of which promote cell proliferation and differentiation, continuous activation promote cell apoptosis. For P38, it activates downstream molecules, proto-oncogene c-myc and nuclear factor-KB (NF-kB), to promote cell death. Activated MAPKs pathway promote the occurrence of oxidative stress, inhibit the expression of anti-apoptotic protein Bcl-2, promote the expression of apoptotic protein Bax, and stimulate the release of cytochrome C, finally upregulate Caspase-3, 8, 9, thus inducing cell death[8-10]. However, there is no report on the effect of MAPK signal transduction pathway in lung cancer. Therefore, our study investigated the function of MAPK signaling pathway in lncRNA TUC338 in promoting lung cancer invasion. It further revealed the MAPK signal transduction pathway in the development of lung cancer and provided a foundation for the development of clinical diagnostic reagents and diagnostic targets of lncRNAs.

Patients and Methods

Clinical Samples

Patients who underwent lung cancer surgery from July 2013 to July 2016 in our hospital were collected. Lung cancer and paracancerous tissues excised from the surgery were immediately stored in a liquid nitrogen tank for long-term preservation. All patients included in this work or their relatives signed informed consent forms. This study was approved by the Ethics Committee of the First Affiliated Hospital of Dalian Medical University.

Cell Culture and Transfection

A549, H1755, SK-MES-1, H1299 lung cancer cells were purchased from Shanghai Chinese Academy of Sciences Cell Bank (Shanghai, China). All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2 under the recommended conditions. Glioma cells in logarithmic growth phase were seeded in 6-well plates. When the cells grew to a density of 80%, plasmids and interference sequences carrying the target gene were transferred into the cells according to the instructions of Lipofectamine 2000 reagent.

Real-time Fluorescence Quantitative PCR

We used TRIzol to extract total RNA following the manufacturer's protocol and the resulting RNA molecules were reverse transcribed into cDNA using the ReverTra Ace qPCR RT kit. QPCR assay was performed using a Taq-Man 2 × Universal polymerase chain reaction (PCR) Master Mix reagent and a CFX96™ Real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA).

TUC338 forward, 5'-GCAGCGACAGTGCGAGCT-3' and reverse, 5'-TCCGAGTGAGTTAGGAAG-3'. The internal reference GAPDH primer sequence: 5'-ACCACAGTCCATGCCATCAC-3' for the upstream primer and 5'-TCCACCCTGTTGCTGTA-3' for the downstream primer.

Cell Counting Kit-8 (CCK8)

Transfected cells were seeded in 96-well plates and cultured for 24, 48, 72, and 96 h, respectively, and serum-free medium was replaced. 10 μL of CCK8 were added to each well for incubation at 37°C with 5% CO_2 for 1 h. OD value was measured at 450 nm wavelength. The proliferative activity of A549 cells after overexpressing TUC338 and SK-MES-1 cells after interference with TUC338 were detected at 6, 24, 48, 72, and 96 h, respectively.

Transwell Assay

A549 and SK-MES-1 cells transfected with TUC338 siRNA or pcDNA-TUC338 and their corresponding control cells were seeded onto pre-treated Matrigel. 600 μL and 100 μl of culture medium were added into the upper and lower chambers, respectively. 24 h later, the cells were stained with 0.1% crystal violet. Then, cell staining was observed under a microscope. Infiltrate cells were randomly selected in 10 regions for counting (× 200).

Western Blotting

After extraction of total proteins, they were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.45 μm nitrocellulose membrane. After blocking with phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA), primary antibodies of MMP2, MMP9, MAPK, p-MAPK, ERK, (1:1000) and GAPDH (1:2000) were incubated, followed by incubation of nitrocellulose membrane with horseradish peroxidase (HRP)-labeled secondary antibody, and finally exposed with enhanced chemiluminescence

(ECL) Development (Shanghai Beyotime Biotechnology Co., Ltd. Shanghai, China), gel imaging analysis system was utilized to determine the integral optical density (IOD) values of each band to GAPDH as an internal reference.

Statistical Analysis

We used Statistical Product and Service Solutions (SPSS) 18.0 statistical software (SPSS Inc., Chicago, IL, USA) for statistical analysis, Graphpad Prism 5 (La Jolla, CA, USA) for graph editing, and Image-Pro 6.0 (Silver Springs, MD, USA) for the gray-scale analysis of protein bands. The Student's *t*-test was used to analyze data between two groups that obeyed a normal distribution. x^2-test was used for categorical data analysis. $p<0.05$ was considered statistically significant; $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$.

Results

TUC338 is Highly Expressed in Lung Cancer and Clinical Data Analysis

Higher expression of TUC338 expression in 52 glioblastoma tissues was found in comparison with that in paracancerous tissues by RT-PCR analysis (Figure 1A). Further analysis of the relationship between TUC338 and lung cancer prognosis found that lower overall survival was observed in lung cancer patients with high expression of TUC338 than those in low expression group (Figure 1B). Clinical data analysis revealed that TUC338 expression in patients was correlated to the overall survival rate, tumor size and lymph node metastasis, but not with age and gender (Table I).

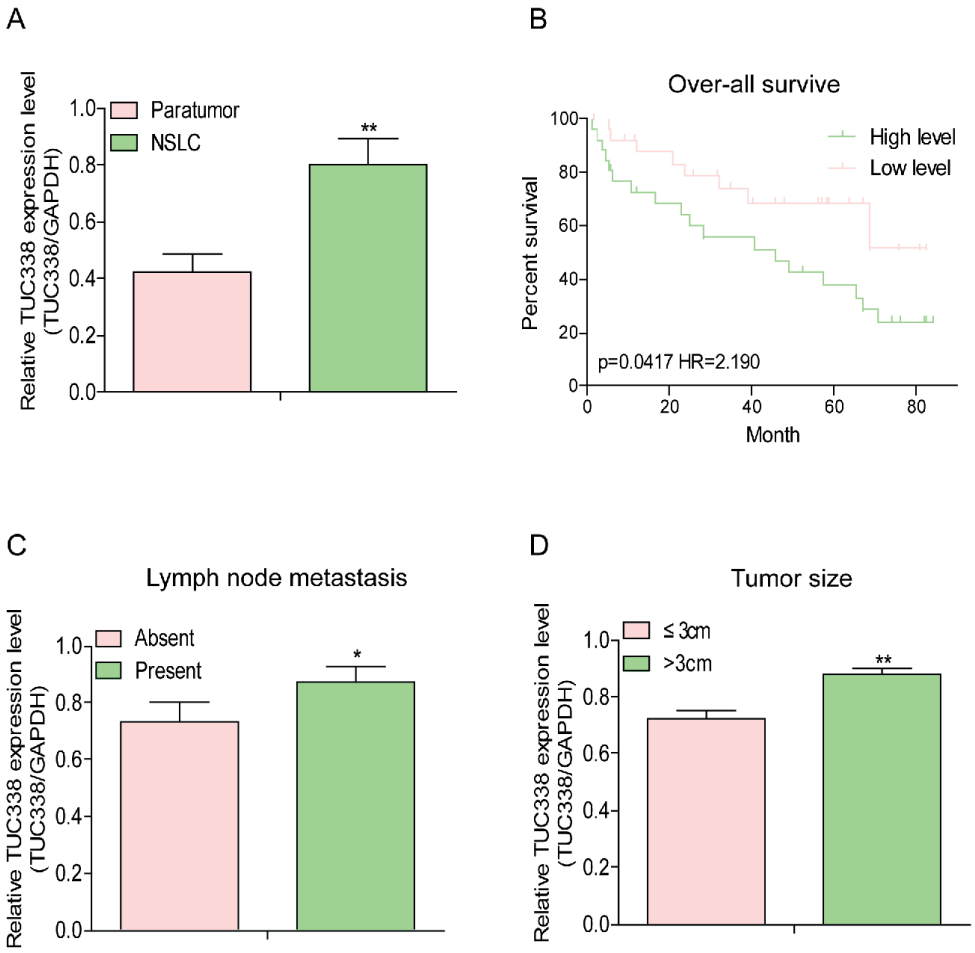

Figure 1. LncRNA TUC338 is highly expressed in lung cancer. ***A,*** TUC338 in 52 cases of lung cancer tissues was significantly higher than in 52 paracancerous tissues. ***B,*** The overall survival rate of lung cancer patients with high TUC338 expression was significantly lower than those with low expression. ***C***, The expression of TUC338 in patients with lymph node metastasis was significantly higher than that in patients without lymph node metastasis. ***D,*** The expression of TUC338 in patients with tumor size greater than 3 cm was significantly higher than that less than or equal to 3 cm.

Table I. The correlation between the expression of TUC338 and clinicopathological features in patients with lung cancer (n = 52).

		LncRNA TUC338 expression		
Clinicopathologic features	Number of cases	Low (n=26)	High (n=26)	*p*-value
Age (years)				0.7813
≤ 56	27	13	14	
> 56	25	13	12	
Gender				0.7774
Male	31	15	16	
Female	21	11	10	
Tumor size				0.0054*
≤ 3 CM	28	9	19	
> 3 CM	24	17	7	
TNM stage				0.0261*
I-II	24	8	16	
III-IV	28	18	10	
Lymph node metastasis				0.0112
Absent	23	16	7	
Present	29	10	19	

Screening of Cell Lines

The total RNA was extracted from lung cancer cell lines (A549, H1755, SK-MES-1, H1299) and the relative expression of TUC338 in cells was accessed by RT-PCR. As Figure 2A showed, the highest level of TUC338 was found in SK-MES-1 cell line, the lowest was found in A549 cell line, so we selected SK-MES-1 cell line for the subsequent interference experiments, and A5448 cell line for overexpression experiments. The corresponding interference sequences and plasmids were constructed and transfected into these cell lines. The transfection results were shown in Figure 2B. The interference effect of si-TUC338 1 # was the best. After transfection with pcDNA-TUC338, TUC338 level in A549 cells was significantly increased (Figure 2C). The results demonstrated that both knockdown and overexpression of TUC338 were successful.

TUC338 Promotes Proliferation of Lung Cancer Cells

To determine the biological function of TUC338 on the proliferation of lung cancer cells, CCK8 assay was performed in this report. The results pointed out that the activity of SK-MES-1 cells was significantly decreased after knockdown of TUC338 (Figure 2D), whereas overexpression of TUC338 significantly increased the viability of A549 cells (Figure 2F). This indicated that TUC338 can promote the proliferation of lung cancer cells.

TUC338 Promotes Invasion of Lung Cancer Cells

For clarifying the effect of TUC338 on invasion of lung cancer cells, matrigel gel was used to mimic the basement membrane to construct an invasive detection system based on the transwell plate. The results suggested that inhibition of TUC338 expression could significantly inhibit the invasion ability of SK-MES-1 cells (Figure 2E), and overexpression of TUC338 significantly increased invasion of A549 cells (Figure 2G). This demonstrated that TUC338 can promote invasion of lung cancer cells.

TUC338 Regulates Invasion of Lung Cancer Cells by Activating MAPK Pathway

We carried out Western blot to explore the mechanism of TUC338 in promoting lung cancer invasion, and we found that the expression of MAPK pathway-related proteins, MMP2, MMP9, p-MAPK and p-ERK in lung cancer cells decreased after interference with TUC338; but increased after overexpressing TUC338 (Figure 3A-B). These results suggested that TUC338 regulated the progression of lung cancer by regulating the MAPK pathway.

Discussion

Recently, the incidence and mortality of lung cancer have presented a rapid upward trend, and chemoresistance has become a major prob-

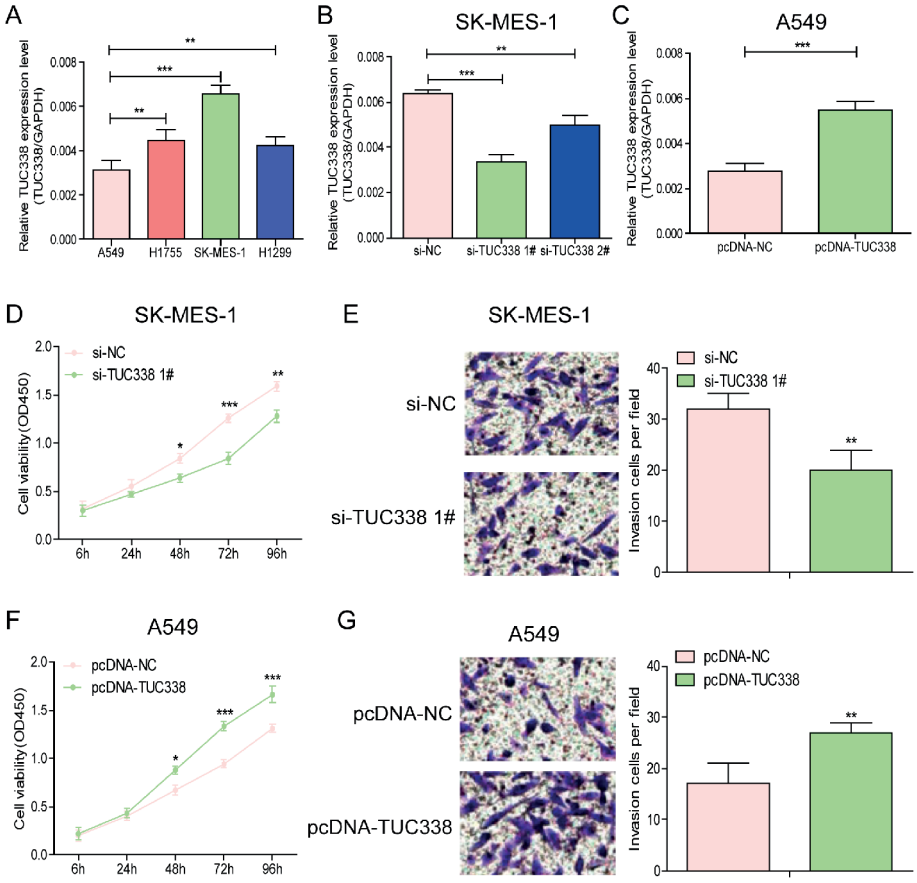

Figure 2. TUC338 inhibits cell viability and promotes invasion of lung cancer cells. ***A,*** A549 cells had the lowest expression of TUC338 and SK-MES-1 cells had the highest one. ***B,*** Transfection of si-TUC338 significantly decreased the expression of TUC338 in SK-MES-1 cells, which was significantly reduced by si-TUC338 1 #. ***C,*** After overexpression of TUC338, TUC338 expression was significantly increased in A549 cell line. ***D,*** After TUC338 interference, SK-MES-1 cell viability was significantly enhanced. ***E,*** The invasion ability of SK-MES-1 cells was significantly reduced after TUC338 interference. ***F,*** After overexpression of TUC338, A549 cell viability was significantly reduced. G. After overexpression of TUC338, A549 cell invasion was significantly enhanced.

lem that needs to be solved urgently for treating lung cancer[11]. The 5-year survival rate of patients with advanced lung cancer in the past 30 years was still around 20%. Despite the cytoreductive surgery and chemotherapy, about 80% of patients still presented drug resistance and reoccurrence[12,13]. With the development of molecular biology, urgent need is required for new molecular targeted therapy strategies to enhance the survival rate of patients with advanced lung cancer.

LncRNA is an emerging field of research in recent years. Although early studies suggested that lncRNAs had no definite function, lncRNAs have recently been reported to exert an important role in growth and pathology. In particular, lncRNA also had a great effect on tumor research[14,15]. Human TUC338 had 590 bp in length, the gene was located on chromosome 12. Li et al[16] found that TUC338 can target the binding of TIMP1 to promote migration and invasion cervical cancer cells. Wang et al[17] found that TUC338 can also target TIMP-1 in colorectal cancer, thereby increasing the cancer cell invasion and metastasis. In addition, TUC338 has also been reported in hepatocellular carcinoma and tongue squamous cell carcinoma[6,7,18]; however, the function of TUC338 in the occurrence and progression of lung cancer is not clear yet.

This study firstly performed RT-PRC in 52 pairs of lung cancer tissues and their paracancerous tissues. Results showed that TUC338 was highly expressed in lung cancer, indicating that it may play a carcinogenic effect on the patho-

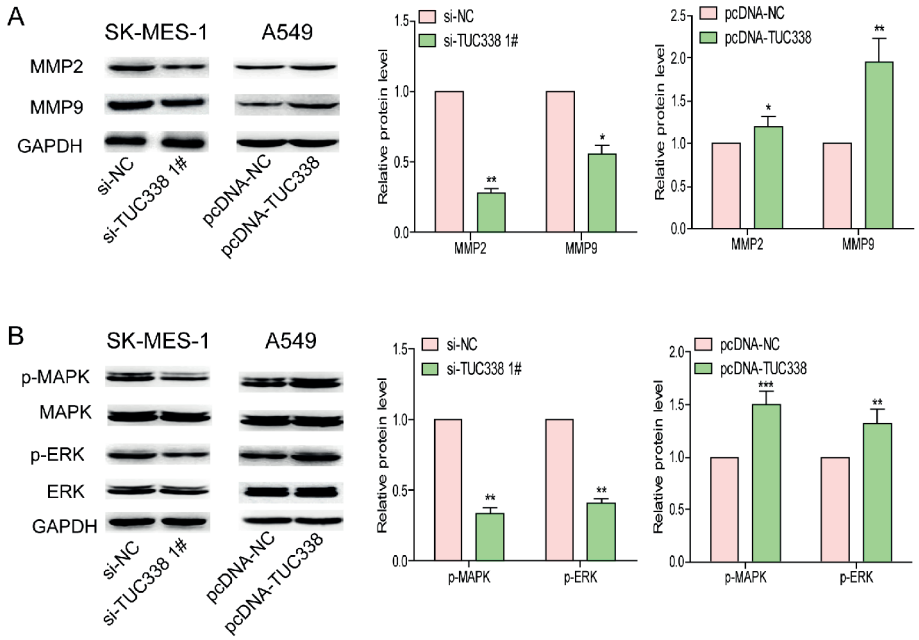

Figure 3. TUC338 regulates the MAPK pathway. ***A,*** After interference with TUC338, the protein expressions of MMP2 and MMP9 were significantly decreased in SK-MES-1 cells. After overexpression of TUC338, the protein expression of MMP2 and MMP9 was significantly increased in A549 cells. ***B,*** The protein expressions of p-MAPK and p-ERK were significantly decreased in SK-MES-1 cells after interference with TUC338. The protein expressions of p-MAPK and p-ERK were significantly increased in A549 cells after overexpression of TUC338.

genesis of lung cancer. Further study found that the expression of TUC338 was related to lung cancer patients' survival time, tumor size and lymph node metastasis, suggesting that TUC338 could have a certain relation to the prognosis of lung cancer. Next, we studied the action of TUC338 on cells. Overexpression of TUC338 showed that the proliferation ability of lung cancer cells was significantly increased as well as the cell invasion. Knockdown TUC338 led to decreased proliferation and invasion of lung cancer. MAPKs are a kind of important signal transduction system that mediates cell response widely in eukaryotic cells. Activated MAPK is involved in physiological processes, including cell proliferation, differentiation, migration, apoptosis, and stress response[19]. Currently, more and more studies have found that MAPK was involved in the development and progression of many tumors. Zhao et al[19] found that benzidine can activate EMT in bladder mucosal epithelial cells by activating ERK1/2 in the MAPK signaling pathway. It further exerted an essential role in the process of epithelial cells derived from malignant cells. EMT was an important biological process in invasion and migration. ERK5 was an important member of the MAPK family. Studies have shown that the expression of MEK5, an upstream kinase of ERK5, was significantly increased in prostate cancer cells, whereas MEK5 was highly expressed in bone metastases of prostate cancer. The activation of ERK5 signaling pathway can promote the metastasis of prostate cancer cells *in situ*. Compared with benign prostatic hyperplasia cells and prostatic adenocarcinoma cells, the expression of ERK5 was also significantly increased in prostate cancer cells[20]. Our paper found that the expression of MAPK-related protein was significantly decreased after interference with TUC338. On the contrary, the expression of MAPK-related protein was significantly increased after overexpression of TUC338. These findings indicated that TUC338 was greatly involved in the regulation of MAPK pathway and thus modulating the progression of lung cancer invasion.

In summary, *in vitro* we found that TUC338 may activate the ERK in MAPK pathway to promote lung cancer invasion, which provided a theoretical basis for the pathogenesis and treatment of lung cancer, it opened up a way for further research on the disease.

Conclusions

TUC338 was overexpressed in lung cancer tissues, and it was capable of improving the invasion of lung cancer cells by activating the MAPK pathway, it provided a new basic for the targeted therapy of lung cancer.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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