Down-regulation of MSMO1 promotes the development and progression of pancreatic cancer

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Abstract

**Background:** Methylsterol monooxygenase 1 (MSMO1), as a completely unique tumor biomarker, plays a vital role in the malignant progression of various cancer. Until now, the potential function and pathway of MSMO1 in the development of pancreatic cancer (PC) has not been explored yet, to our knowledge.

**Methods:** We systematically explored the detail function of MSMO1 in Epithelial-mesenchymal transition (EMT) and cell proliferation of PC in vitro and in vivo.

**Results:** MSMO1 expression was much lower in PC tissues than that in paired normal pancreas. MSMO1 positive expression was negatively associated with T stage, lymph node metastasis and vascular permeation of PC patients. Meanwhile, positive MSMO1 expression indicated a significantly better prognosis and an independent favorable prognostic factor. MSMO1 silencing promoted cell invasion and migration via activating EMT and PI3K-AKT-mTOR pathway [p-PI3K (Tyr458), p-AKT (Ser473) and p-mTOR (Ser2448)] in Capan-2, Panc-1 and SW1990 cells. In vivo, subcutaneous tumor size was enhanced by MSMO1 silencing following with the consistent change of EMT and PI3K/AKT signaling shown in vitro. The motivation of EMT and PI3K-AKT-mTOR pathway was also demonstrated in MSMO1 silencing mouse PANC02 cells.

**Conclusion:** Down-regulation of MSMO1 in PC was associated with advanced progression and poor prognosis of PC patients. MSMO1 acts as a tumor suppressor via inhibiting the aggressive malignant biology of PC accompanying with regulating EMT and PI3K/AKT signaling.

Key words: MSMO1, Pancreatic Cancer, EMT, PI3K-AKT-mTOR, Progression

Introduction

Pancreatic cancer (PC) is one of the most lethal tumors that attributes to aggressive malignant biological behavior [1]. The 5-year survival rate of PC keeps going down and the incidence rate remains continuously rising [2]. Until 2030, PC is forecast for the second leading cause of death associated with cancer in the world [3]. It is urgent to discover the new biomarkers for targeting the malignant biology for PC patients. Epithelial-mesenchymal transition (EMT) is a cellular process initiated from cellular micro environment following the acquisition of mesenchymal phenotypes and the repression of epithelial features, which finally contributes to the cell invasion and metastasis of cancer, including PC [4, 5]. The key characteristic of EMT is the deficiency of epithelial markers, such as E-cadherin and the gain of interstitial markers, such as Vimentin and MMPs [6]. Increasing evidence confirms the significant role of PI3K-AKT-mTOR signaling pathway and it’s downstream targets (p-AKT, p-PI3K and p-mTOR) in regulating EMT process [7, 8]. Methylsterol monooxygenase 1 (MSMO1), as a synonym for sterol-C4-methyl oxidase analog (SC4MOL), displays critical function in the normal synthesis of cholesterol [9]. Recently, MSMO1 has been reported to promote the development of various cancers including liver [10], breast [11], and oligodendroglioma [12]. However, the mechanism and function of MSMO1, especially in PC, is still unknown, to our knowledge. In current study, we systematically investigated the potential role and mechanism of MSMO1 in the progression of PC in vitro and vivo.
Materials and methods

Cell Lines and Culture

Capan-2, Panc-1, and SW1990 cell lines were offered by General Surgery Laboratory of China Medical University (Shenyang, China) and Panc02 cell lines was obtained from Cell Biology Institute of China Medical University. All cell lines were cultured in temperate situation according to the recommendation from ATCC, such as suitable culture medium within one percent penicillin-streptomycin combination (HanHeng, China) added ten percent fetal calf serum (Gibco Invitrogen, Carlsbad, CA) and maintained in 37 °C humidified incubators filled with five percent CO2. All cell lines were detected for mycoplasma free before experiments.

Tissue Samples

92 pairs of fresh pancreatic cancer samples and paired adjacent normal pancreatic tissues were collected from surgical treatment patients at the Gastrointestinal Surgery Department of the First Hospital of China Medical University and the General Surgery Department of the People’s Hospital of Liaoning Province between 2010/9 and 2020/12. All the patients were with complete follow up data and pathologically diagnosed as PC. 8th AJCC Cancer Staging Manual was utilized to classify staging standard. Meanwhile, 22 pairs of PC fresh samples were collected under -80 °C condition for qRT-PCR assays.

Experimental Animals

All C57BL/6 mice used in this study, weighting 20 to 25 g, were purchased from the company of Beijing Vital River Laboratory Animal Technology (Beijing, China). The animals were kept in the specific pathogens free (SPF) Animal Experimental Ministry of China Medical University with standard conditions (temperature 24±2 °C, humidity 55±10%, and 12-hour day/night cycle). Free access to rodent chow and water were applied for experimental animals. All animal experiments were performed strictly according to institutional regulations in facilities approved by the Animal Care Committee of China Medical University in accordance with Chinese government guidelines for animal experiments.

Immunohistochemistry

Paraffin embedded PC tissues fixed by neutral formaldehyde. Tissues were used to cut 4 µm slices and dewaxing. Antigen retrieval was next done for 3 minutes under high pressure. Three percent H2O2 and goat non-immune serum were utilized to block tissues for 30 minutes. Primary antibody incubated overnight with at 4 °C. Sections were incubated with the secondary antibody for 30 minutes at room temperature and then observed color reaction via using 3,3’-diaminobenzidine (DAB). Immunohistochemistry (IHC) score was conducted as previously described [13]. Four grades: 0 (negative), 1 (weak), 2 (medium), and 3 (strong) was described the positive staining intensity. The 5 grades: 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%) were used to calculate according to the stained positive areas. The final scores were calculated by three professional pathologists. The final score, ranging 0-12, was the multiplication of two grades and >6 was regarded as high expression.

Hematoxylin-Eosin Staining

Briefly, xylene I and II each for 10 minutes was used to dewax the slices. 100%, 90%, 80%, 70% alcohol for 5 minutes each, hematoxylin for 5 minutes, 5% acetic acid differentiation for 1 minute, tap water for 1 minute, eosin for 3 minutes, 70%, 80%, 90%, 100% alcohol each for 10 seconds, xylene I and II for 5 minutes.

EMT Construction

We transfected Capan-2, PANC-1 and SW1990 cell lines with negative control and MSMO1 siRNA. Recommended growth media (RPMI 1640 for Capan-2 and SW1990 cells, DMEM for Panc-1 cells) containing 1% FBS was used to better induce EMT phenotype in transfected cell lines. The EMT construction model was confirmed by the alteration involving EMT-like cell morphology (a spindle-shaped and fibroblast-like morphology), EMT enhanced cell motility, and the changes of EMT-related markers, which were regarded to reflect the EMT process.

Western Blot

RIPA lysis buffer containing 1% PMSF (Beyotime, China) was used to collect total protein from cell lines. 10% SDS-PAGE was used to separate protein and PVDF membranes were loaded with separate protein. All membranes were blocked with 5% degreasing milk and incubated with primary antibodies: MSMO1 (Abcam), E-cadherin (Abcam), β-catenin (Proteintech), Vimentin (Proteintech), AKT (Bimake), PI3K (Bimake), m-TOR (Bimake), p-AKT (Ser473) (Cell Signaling Technology), p-PI3K (Tyr458) (Cell Signaling Technology), p-mTOR (Ser2448) (Cell Signaling Technology), GAPDH (Proteintech) overnight at 4 °C. Next day, membranes were incubated with secondary antibodies for two hours. Finally, blots were detected by ECL kit (Beyotime, China). The experiments were done thrice.
Real-time Quantitative PCR

TRIZOL reagent (Takara, Japan) was used to extract total RNA from tissues samples, following the instructions. RNA level was maintained to the same level in each sample by using nucleotide test. GenePharma (Soochow, China) provided the primers for qRT-PCR. The primer sequences were manifested in Table 1. The total RNA and TaKaRa RNAiso Reagent (Takara, Japan) was used to synthesize cDNA. Finally, the QuantStudio 7 Pro Real-Time PCR System (Thermo Fisher Scientific, USA) was used to measure the mRNA expression of MSMO1 and GAPDH under the conditions as below: 95 °C for 30s, 45 cycles of 95 °C for 5s and 60 °C for 45s. The ΔΔCt method with melt-curve dissociation was used to evaluate the quality of amplification products. The experiments were repeated three times.

| Gene name | Primer sequences |
|-----------|------------------|
| MSMO1     | sense: 5’-TGCTTTGGTTGCCAAGCCTCAT-3’; anti-sense: 5’-TTCAAAATGAGGCCCTGAAA-3’ |
| GAPDH     | sense: 5’-TGACTTCACACGGCACACCAA-3’; anti-sense: 5’-CACCCCTGTGCTGTACGAAA-3’ |

RNA Interference

The MSMO1 siRNA and negative control (NC) were designed by OriGene (OriGene Technologies, Inc. USA). The verified sequence (OF) of MSMO1 siRNA were as followed: Sense: GAAGCCCUUAAUUUCUUUAUTT. Anti-sense: AUUAGAAAAUUAGGGCUUCACTT. Capan-2, Panc-1, and SW1990 cells were transiently transfected with MSMO1 siRNA and NC for 24 hours with lipo3000 (Invitrogen, Carlsbad, CA, USA) following the instruction. In the result section, the silencing effect of MSMO1 was testified.

Cell Migration and Invasion Assays

Based on our previous study [14], for cell migration assay, 2×10^5 cells/ml of Capan-2 cells, 3×10^5 cells/ml of Panc-1 cells and 1.5×10^6 cells/ml of SW1990 cells were implanted into chamber inserts with serum-free growth media in 24-well plates within growth media containing 10% FBS at the bottom of each well as a stimulus after transfected for 24 hours. 24 hours later, the per-cooling methanol was used to fix the migrated cells and the Crystal Violet (Sigma) was used for dyeing. The invasion assay was similar to migration that the top of membrane coated with Matrigel (BD Biosciences, USA). A microscope (Nikon Microphot-FX, Japan) was used to calculate the final migrating and invading cells at 20× magnification in five random fields each chamber. Each independent experiment was performed thrice.

In vivo Cell-line-Derived Tumor Xenograft

To detect the function of MSMO1 in cell proliferation, total six C57B6 mice were used to construct subcutaneous tumor model by injecting PANC-02 cells resuspended in PBS which were transfected with MSMO1 siRNA and negative control respectively (1×10^6/ml, 100 ul). The selected mice are generally 4 weeks old and the middle of the armpit was chosen as the planting site. After injection, cotton swab was used to reduce bleeding and the overflow of the cell suspension from the injection site. Four weeks later, the mice were sacrificed and tumors were resected. The longest and shortest parts of the tumor are measured using a vernier caliper with the following formula: V=1/2*a*b^2 (a is the long axis, b is the short axis). After the subcutaneous tumor is removed, a part of tumors was used for later extraction of protein and other was used for hematoxylin and eosin (HE) and IHC staining.

Statistical Analysis

Statistical analysis was emerged from SPSS 20.0 software (Chicago, IL, USA). Three independent experiments results were presented by mean ± SD. Discrepancies between two groups were compared through independent t test. MSMO1 and clinicopathological parameters were compared by chi-square test. To deal with survival curve, Kaplan-Meier method was utilized and significance was compared via log-rank test. Independent prognostic factors were evaluated by Cox’s proportional hazards regression model. P<0.05 was considered as statistically significant.

Results

MSMO1 is Down-Regulated in PC and Associated with The Aggressive Clinical Stage and Prognosis of PC Patients

In our study, IHC showed MSMO1 expression in human PC tissues (43/92,46%) was lower compared with normal tissues (38/45, 84%) (P<0.0001) (Figure 1A). MSMO1 was mainly expressed in the cytoplasm with a small amount of nuclear expression in human PC tissues (Figure 1B). Meanwhile, As the degree of tumor differentiation enhancing, the expression of MSMO1 increases (Figure 1C). In current study, high MSMO1 expression was negatively relevant with T stage (P=0.025), lymph-node metastasis (P=0.046) and vascular permeation (P=0.009) of 92 PC patients (Table 2), patients with low MSMO1 expression had a poor prognosis shown by Kaplan-Meier curve (P=0.01) (Figure 1D). In addition, the low MSMO1 expression independently causes poor prognosis was observed by univariate and multivariate Cox’s
regression analysis results (Table 3). Compared with the PC tissues, higher mRNA expression of MSMO1 in normal pancreatic tissues were shown by qRT-PCR assay (P=0.009) (Figure 1F).

**MSMO1 Inhibits Epithelial-Mesenchymal Transition, The Capacities of Cell Malignant Migration and Invasion in vitro**

The essential key in cancer metastasis is EMT [15]. To verify the effect of MSMO1 in PC cell function, we transfected RNA interference or negative control (NC) in Capan-2, Panc-1 and SW1990 cells. In results, the EMT-like cell morphology was stimulated by MSMO1 down-regulation, the cells lost their epithelial features and presented a spindle/fibroblast-like morphology, compared with the NC group, (Figure 2A). As demonstrated in Transwell assays, the capacities of invasion and migration were significantly enhanced in MSMO1 siRNA transfected cells group in Capan-2, Panc-1 and SW1990 cells (Figure 2B).

**MSMO1 Silencing Agitated EMT Activation via PI3K-AKT-MTOR Signal Pathway in PC Cells**

For the purpose of exploring the potential mechanism of MSMO1 in PC cell function. Capan-2 cells, Panc-1 cells and SW1990 cells was used for MSMO1 silencing construction, and protein was then extracted from transfected cells to conduct Western blot. We found MSMO1 silencing inhibited EMT related protein (E-cadherin) and enhanced others (β-catenin and Vimentin). Some EMT markers showed no change, such as C-myc (Figure 3A-C). Simultaneously, several proteins of PI3K-AKT-mTOR signal pathway [p-PI3K (Tyr458), p-AKT (Ser473) and p-mTOR (Ser2448)] was enhanced in MSMO1siRNA group (Figure 3D-F). Based on the above results, the EMT induction and PI3K-AKT-mTOR signal pathway were both stimulated by MSMO1 silencing in PC cells in vitro.

**MSMO1 Down-Regulation Promotes Cell Proliferation of Pancreatic Tumors in vivo**

We constructed the models of subcutaneous tumor by PANC02 cells which were transfected with MSMO1 siRNA and negative control (NC), respectively. Tumor volumes in MSMO1 siRNA group were much larger (P=0.0138) (Figure 4A, B). In histology, tumor tissues were observed by HE staining (Figure 4C), besides, MSMO1 and E-cadherin expression were decreased but β-catenin, Vimentin, p-PI3K (Tyr458) and p-AKT (Ser473) expression were increased in Tumor was further confirmed by IHC (Figure 4D). By western blot analysis, at protein level, Panc02 cell line exhibited E-cadherin expression was down-regulated, β-catenin, Vimentin, p-PI3K and p-AKT expression was up-regulated (Figure 4F).
**Figure 2.** In vitro, MSMO1 silencing promoted PC cells EMT, migration and invasion. (A) The EMT-like cell morphology of Capan-2, PANC-1 and SW1990 cells in MSMO1 siRNA and NC groups. (B) Migration and invasion in Capan-2, PANC-1, and SW1990 cells after MSMO1 silencing. Bars indicate means ± SD, *P < 0.05, **P < 0.01.

**Table 2.** The Clinical Significance of MSMO1 Expression in Human PC Samples

| Characteristics                  | No. of Patients | MSMO1 expression | χ²  |
|----------------------------------|----------------|------------------|-----|
| Case                             | 92             | Low 49/High 43   | .910|
| Gender                           |                |                  |     |
| Male                             | 54             | Low 33/High 21   |     |
| Female                           | 38             | Low 16/High 22   |     |
| Age (y)                          |                | Low 12/High 11   | 1.000|
| ≤65                              | 23             | Low 12/High 11   |     |
| >65                              | 69             | Low 37/High 32   | .817|
| Tumor location                   |                |                  |     |
| Bead                             | 66             | Low 36/High 30   | .765|
| Body and tail                    | 26             | Low 13/High 13   |     |
| Tumor size (cm)                  |                |                  |     |
| <1                               | 79             | Low 43/High 36   |     |
| ≥1                               | 13             | Low 6/High 7     | .951|
| Differentiation                  |                |                  |     |
| Well                             | 30             | Low 16/High 14   |     |
| Moderate                         | 57             | Low 30/High 27   |     |
| Poor                             | 5              | Low 3/High 2     |     |
| T stage                          |                |                  |     |
| T1+T2                           | 64             | Low 29/High 35   | .025*|
| T3+T4                           | 28             | Low 20/High 8    |     |
| Lymph nodes metastasis           |                |                  |     |
| Negative                         | 63             | Low 29/High 34   | .046*|
| Positive                         | 29             | Low 20/High 9    |     |
| UICC staging                     |                |                  |     |
| IA + IIA                         | 69             | Low 34/High 35   | .231|
| IIB + III + IV                   | 23             | Low 15/High 8    |     |
| Vascular permeation              |                |                  |     |
| Negative                         | 59             | Low 25/High 34   | .009*|
| Positive                         | 23             | Low 23/High 9    |     |
| Neural permeation                |                |                  | .078|
| Negative                         | 79             | Low 39/High 40   |     |
| Positive                         | 13             | Low 10/High 3     |    |
| Pretherapeutic CA19-9 level      |                |                  |     |
| <37 U/mL                         | 54             | Low 26/High 28   | .291|
| ≥37 U/mL                         | 38             | Low 23/High 15   |     |

*P<0.05.

**Table 3.** The Univariate and Multivariate Analysis of Clinicopathological factors For Survival

| Characteristics                  | Median Survival (Days) | Univariate Analysis P (Log Rank) | Multivariate Analysis Hazard Ratio (95% CI) | P   |
|----------------------------------|------------------------|---------------------------------|-------------------------------------------|-----|
| Gender (Male /Female)            | 708/432                | .910                            | -                                         |     |
| Age (≤65 />65y)                  | 474/800                | 1.000                           | -                                         |     |
| Tumor location (Bead/Body and tail) | 615/317               | .817                            | -                                         |     |
| Tumor size (<4/≥4 cm)            | 629/321                | .765                            | -                                         |     |
| Differentiation (Well/Moderate/Poor) | 629/474/200           | .951                            | -                                         |     |
| T stage (T1+T2/T3+T4)            | 800/317                | .025                            | 2.218 (1.120-4.393)                       | .022*|
| Lymph nodes metastasis (Negative/Positive) | 660/418               | .046                            | 1.212 (0.637-2.305)                       | .588|
| Vascular permeation (Negative/Positive) | 184/76                | .009                            | 1.223 (0.654-2.920)                       | .528|
| Neural permeation (Negative/Positive) | 629/365               | .078                            | -                                         |     |
| CA19-9 level (<37 U/mL/≥37 U/mL) | 423/615                | .291                            | -                                         |     |
| MSMO1 expression (low/high)      | 356/915                | .001                            | 0.431 (0.200-0.931)                       | .032*|

*P<0.05.

**Discussion**

MSMO1, as a typical target in cholesterol biosynthesis, catalyzes the demethylation of C4-methylsterol in the cholesterol synthesis pathway and plays a key role in the normal synthesis of cholesterol [9, 16]. Recently, MSMO1 is considered as a novel and critical gene target in various solid cancers, including breast cancer [11], oligodendroglia cells [12], hypopharyngeal cancer [17], gastric cancer.
MSMO1 expression in various cancers. In breast cancer patients, targeting MSMO1 with siRNA increases the resistance to endocrine therapy [11]. Down-regulation of MSMO1 was significantly associated with poor prognosis in liver cancer [20] and worse 5-years overall survival rates in gastric cancer [18]. Currently, we first found that MSMO1 expression in normal pancreatic tissues was much higher than that in paired PC tissues. Meanwhile, low expression of MSMO1 was associated with advanced clinical stage (T stage, vascular permeation, lymphatic metastasis and poor prognosis) of PC patients, which is consistent with previous studies. The close relationship of low MSMO1 expression with the advanced clinical stage of PC patients drives us to investigate the potential role of MSOM1 in PC development in vitro.

EMT (Epithelial-mesenchymal transition) is considered as the initiating factor that converts benign carcinoma to the malignance with the high metastatic growth dissemination involving various signal pathways [23, 24]. Our data implied a novel and specific role of MSMO1 in EMT here. EMT-like cell morphology and cell mobility were stimulated by MSMO1 silencing in vitro. Nowadays, the potential role of MSMO1 in cancer cells is still controversial. MSMO1 overexpression suppressed malignant cytological functional behavior in oligodendroglioma cells [12]. The high propensity for metastatic spread of uveal melanoma is the results of the transformation of MSMO1 [19]. Bioinformatics analysis presents the same trend in hypopharyngeal cancer [17] and cervical squamous cell carcinoma [21]. However, MAGEA6 positively regulates MSMO1 and facilities the capacity of migration and invasion in esophageal cancer cells [25]. Due to the different kinds of cancer and the related cellular environment, the function and mechanism of MSMO1 would be different and even contrary in various cancers.

**Figure 3. Discrepancy of EMT-induced targets and PI3K-AKT-mTOR signal pathway in vitro.** (A) The protein level of MSMO1 and EMT features in Capna-2 cells. (B) The protein level of MSMO1 and EMT features in Panc-1 cells. (C) The protein level of MSMO1 and EMT features in SW1990 cells. (D) The changes of PI3K-AKT-mTOR signal pathway in Capan-2 cells. (E) The changes of PI3K-AKT-mTOR signal pathway in Panc-1 cells. (F) The changes of PI3K-AKT-mTOR signal pathway in SW1990 cells. Bars indicate means ± SD, *P < 0.05, **P < 0.01, ***P < 0.001.
Numerous evidences reveal E-cadherin is a critical EMT-related element [26]. As previously, Numb-PRRL amplifies a set of EMT activators in PC, including E-cad and Vimentin [27]. GATA6, the down-stream of Wnt-β-catenin signaling pathway, promotes EMT associated with E-cadherin downregulation [28]. At the same time, we found MSMO1 down-regulation inhibited E-cadherin but enhanced β-catenin and Vimentin expression. To this context, the down-regulation of MSMO1 provided a potential unique point to influence EMT.

PI3K-AKT pathway plays a significant role in the malignant cellular function and the initiation of EMT [29]. AKT regulates cell-cycle following the down-regulation of E-cadherin in EMT transmission [30, 31]. mTOR, as the downstream target of PI3K-AKT signal pathway, also relates to regulate EMT[32]. Besides, AKT is the key down-stream effector of MSMO1 and inter-crossing protein between EGFR and PI3K/AKT/mTOR signaling pathway [33]. However, MSMO1 has not been previously analyzed in the context of PI3K/AKT/mTOR signaling. In our study, WB shown that MSMO1 silencing increased p-PI3K (Tyr458), p-AKT (Ser473) and p-mTOR (Ser2448) expression in vitro. Thus, MSMO1 down-regulation facilitated EMT processing by mediating the PI3K/AKT/mTOR signaling pathway in PC, which is not reported yet. It is worthy noted that mTOR catalyze two multiprotein complexes, mTORC1 and mTORC2 [34]. mTORC1 promotes biosynthesis [35], while mTORC2 plays seminal functions in cell metabolism and survival through the p-AKT (Ser473) [36]. In current study, we did not distinguish these two multiprotein complexes and the mTOR complexes will be a worthwhile point for us to explore in the future. Finally, MSMO1 down-regulation promoted the formulation of subcutaneous tumors accompanied with the promotion of EMT and PI3K/AKT/mTOR signaling pathway in vivo.

In conclusion, down-regulation of MSMO1 takes an important part in the aggressive clinical stage of PC patients. MSMO1 mediates EMT through activating PI3K-AKT-mTOR pathway in vitro and in vivo, which facilitates a novel diagnostic and therapeutic facility for PC. However, the corresponding mechanism is not further investigated in current study, which would be investigated in future.
Abbreviations

MSMO1: Methylsterol monooxygenase 1; PC: Pancreatic cancer; EMT: Epithelial-mesenchymal transition; qRT-PCR: Quantitative real-time polymerase chain reaction; IHC: Immunohistchemistry; HE: Hematoxylin-Eosin Staining; RIPA: Radio Immunoprecipitation Assay; PMSF: Phenylmethylsulfonyl Fluoride; PVDF: Poly(vinylidene fluoride); ECL: Enhanced Chemiluminescence; FBS: Fetal Bovine Serum.

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Ethics statement

Human participants and animal study in this research was authorized by The Ethics Committee of The First Hospital of China Medical University (No. AF-SOP-07-1.1-01). Informed consent was accepted by each participating patient for this essay.

Author contributions

All authors contributed to conception and design, data, statistical analysis and interpretation; participated in drafting and revising the article; allowed to submit for publication and be responsible to all aspects of the work.

Competing Interests

The authors have declared that no competing interest exists.

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