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Original Research Article

HMGB1-mediated-TLR4/MyD88 signaling regulates cell proliferation, migration and invasion in esophageal squamous cell carcinoma

Xuyang Liang[1,2], Yanqin Ma[2], Zhimei Zhang[2,3], Dazhou Xu[2], Shengxiang Lv[2], Yanling He[4], Shuxian Zhang[2], Ling Ren[2], Ping Xu[1]*

[1]Department of Gastroenterology, Shanghai Songjiang Clinical Medical College of Nanjing Medical University, Shanghai 201600, [2]Department of Gastroenterology, Lianyungang Clinical Medical College of Nanjing Medical University; [3]Phase I Clinical Research Center, [4]Department of Pathology, The First People's Hospital of Lianyungang, Lianyungang, Jiangsu 222061, China

For correspondence:* ***Email:* *sjzxxp@yeah.net*

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Abstract

Purpose: *To study the effect of high mobility group box protein 1 (HMGB1)-mediated toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88) on esophageal squamous cell carcinoma (ESCC).*
Methods: *Cell viability was determined using CCK-8 test and colony formation assay, while cell migration and invasion were assessed by Transwell assay. Western blotting was used to determine protein and mRNA expression levels. The effect of TLR4/MyD88 signaling pathway on proliferation of ESCC cells was investigated via in vitro knockdown of HMGB1.*
Results: *Data from in vitro experiments indicated that HMGB1 knockdown significantly decreased the proliferation, migration, and invasion of EC9706 cells in siHMGB1 group, when compared with siNC group (number of invasive cells in siHMGB1 vs. corresponding number in siNC: 26.7 ± 4.5 vs. 68.7 ± 2.5; $p < 0.01$), and also decreased proliferation, migration and invasion of TE-9 cells in siHMB1, relative to siNC group (number of invasive cells in siHMGB1 vs. corresponding number in siNC: 29.3 ± 3.5 vs. 55.7 ± 3.1; $p < 0.01$). Moreover, MGB1 knockdown via siTLR4 transfection significantly down-regulated the expressions of TLR4/MyD88 and epithelial-mesenchymal transition (EMT), but over-expression of TLR4 reversed the inhibition by HMGB1 knockdown on ESCC cells ($p < 0.01$).*
Conclusion: *These findings suggest that HMGB1-mediated TLR4/MyD88 signal pathway is a potential treatment route for ESCC.*

Keywords: *HMGB1-mediated TLR4/MyD88 signaling, Esophageal squamous cell carcinoma, Cell migration and invasion*

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INTRODUCTION

Esophageal squamous cell carcinoma (ESCC), a subtype of tumor, is characterized by frequent occurrence of esophageal lesions, early metastasis, and late diagnosis, and it is one of the cancers with the highest mortality rate globally [1]. Currently, the main therapeutic

approaches, i.e., surgical resection for early-stage ESCC, radiotherapy, and chemotherapy for patients in advanced stage of ESCC, have made some progress in clinical trials. However, with a five-year survival rate of 5 %, the prognosis of ESCC is still very poor [2]. Therefore, there is need for a greater understanding of the molecular processes that regulate tumor cell functions so as to enhance the identification of potential therapeutic targets for ESCC.

High mobility group box 1 (HMGB1), a non-histone nuclear protein known for its remarkable stability, is extensively distributed in the nucleus of each cell type, and it is present in small quantities in the cytoplasm and cytomembrane [3]. Studies have shown that HMGB1 is an essential factor in several diseases, such as sepsis, ischemia-reperfusion, neurodegeneration, and in particular, malignancy [4]. Recently, it was reported that HMGB1 is an early marker in ovarian carcinoma, non-small cell lung cancer [5], and colorectal carcinoma. Additionally, some researchers have found that HMGB1 mediates radio resistance and chemoresistance. For example, downregulation of HMGB1 via siRNA-mediated knockdown enhanced the radiosensitivity of breast cancer and bladder cancer. Moreover, HMGB1 is involved in chemoresistance in pancreatic ductal adenocarcinoma and colorectal cancer [6].

The innate immune response is believed to rely significantly on Toll-like receptor (TLR), a transmembrane protein and one of the pattern recognition receptors (PPRs) [7]. The most important member of TLR family is the well-studied TLR4 which is expressed in various tumor tissues, including prostate cancer and melanoma. On receiving information from tumor-associated antigens, myeloid differentiation factor 88 (MyD88) is activated by TLR4, resulting in nuclear translocation of nuclear transcription factor kappa B (NF-κB) and activation of its transcriptional activity. Accumulating evidence has indicated that TLR4 regulates NF-κB through MyD88 protein, and promotes tumor cell proliferation [8]. In epithelial ovarian cancer patients, activation of TLR4/MyD88 pathway leads to low overall patient survival [9]. Moreover, down-regulation of TLR 4 and MyD88 is a promising treatment for patients with epithelial ovarian cancer and hepatocellular carcinoma [10]. In addition, HMGB1 has been identified as an endogenous ligand for TLR4 which has been demonstrated to promote radio-resistance in ESCC [11]. Interestingly, emerging evidence indicates that HMGB1-mediated TLR4/MyD88 signaling is involved in persistence of papillomavirus and neuroinflammation [12]. However, not much is known about the function of HMBG1-mediated TLR4/MyD88 signaling in ESCC progression.

By conducting loss-and gain-of-function analysis, the effect of HMGB1 on ESCC proliferation, migration, and invasion was investigated in this study. Furthermore, the involvement of TLR4/MyD88 signaling pathway in the effect of HMGB 1 knockdown on ESCC cells, was studied.

EXPERIMENTAL

Cell culture

The Human ESCC cell lines EC9706 and TE-9 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were maintained on RPMI-1640 supplemented with 10 % fetal bovine serum (FBS) in a 5 % CO_2 incubator at 37 °C.

Cell transfection

The SiRNA targeting HMGB1 (siHMGB1), TLR4 (siTLR4) and scramble negative control (siNC) were obtained from Gene Pharma (Shanghai, China). Full-length TLR4 cDNA was cloned into mammalian expression vector pcDNA3.1 (Invitrogen, USA) so as to construct a TLR4 over-expressing plasmid. The EC9706 and TE-9 cells were plated in a 6-well plate, each at a density of 3×10^5 cells per well, and cultured overnight. When the cells reached 80 % confluency, transfection was performed for 48 h using Lipofectamine 2000 transfection reagent (Invitrogen) kit in line with manufacturer's instructions.

Western blotting

Total protein was extracted from ESCC cells in each group using RIPA lysis buffer (Beyotime, Jiangsu, China), and the protein content of the extract was measured utilizing BCA kit (Beyotime). Then, 30 µg of each protein extract was subjected to 12 % SDS polyacrylamide gel electrophoresis, prior to transfer onto polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The membranes were blocked by incubation with a mixture of tris-buffered saline-Tween-20 (TBST) and 5 % non-fat dried milk at normal temperature at room temperature for 1 h. Thereafter, the membranes were incubated overnight at 4℃ with specific primary antibodies (HMGB1, TLR, MyD88, E-cadherin, N-cadherin, Vimentin, and GAPDH). After washing, the membranes were incubated with

horseradish peroxidase-conjugated secondary antibody (1:5000, Santa Cruz) at 20℃ for 60 min. Finally, the protein signals were subjected to enhanced chemiluminescence (ECL) using ECL reagents (Amersham Biosciences Corp., USA). The relative protein expressions were calculated with reference to GAPDH which served as internal standard.

CCK-8 assay

Cell proliferation was determined with Cell Counting Kit-8 assay (CCK-8; Dojindo Laboratories, Kumamoto, Japan) as described by the kit manufacturer. After transfection, about 3000 cells were seeded in 96-well plates and cultured for 1, 2, and 3 days. Then, 10 µL CCK-8 reagent was added to each well, and incubation was continued for the indicated durations, after which optical density value of each well was read at 450 nm in a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Growth curves were plotted for each group, based on the absorbance at each time point.

Colony formation assay

To assess the cell multiplication potential, a colony formation test was carried out. The transected cells were cultured at a density of 500 cells per well and grown for a further 2 weeks. After rinsing the naturally-formed colonies with PBS, they were fixed in 4 % formaldehyde for 30 min at ambient temperature and stained with a 0.1 % crystal violet solution for 15 min. Finally, after air-drying, colonies from three random light fields were counted under a microscope.

Transwell assay

Transwell cell test was employed to determine cell invasion and migration. After 48 h of transfection, harvested cells were re-suspended in medium without FBS. For the migration experiment, the upper chamber contained approximately 5×10^4 cells, while the lower compartment contained the same media with 10 % FBS. Cells that migrated to the bottom compartment were incubated for 24 h before fixation in methanol and staining with 0.1 % crystal violet. The stained cells were counted in five randomly-selected fields using IX71 photomicroscope. Then, utilizing Image-Pro Plus 6.0, the total number of migrated cells was calculated. A similar procedure was used for the invasion experiment, except that Matrigel (BD Biosciences, USA) was used to pre-coat the transwell chambers.

Statistical analysis

The GraphPad Prism 6.0 Software (GraphPad Inc., California, CA, USA) was applied for graphical presentation of quantitative data from the aforementioned experiments. Each experiment was done at least three times. Differences between groups were assessed using unpaired and two-tailed Student's *t*-test. Comparison amongst groups was done with one-way ANOVA and Tukey's posthoc test. Values of $p < 0.05$ indicated statistically significant differences.

RESULTS

HMGB1 knockdown inhibited proliferation, migration, and invasion of ESCC cells

As shown in Figure 1 A, transfection of siHMGB1 was performed on EC9706 and TE-9 cells, and western blotting analysis demonstrated that HMGB1 was significantly down-regulated after siHMGB1 transfection. Results from CCK-8 assay revealed the HMGB1 knockdown significantly decreased the viability of EC9706 cells (Figure 1 B) and TE-9 cells (Figure 1 C) after 48 and 72 h. Moreover, data from colony formation experiments confirmed that the proliferation of EC9706 and TE-9 cells were significantly suppressed, as evidenced by fewer colonies in siHMGB1 group, relative to siNC group (Figure 1 D). Transwell chamber experiments showed marked reductions in the migrations of EC9706 and TE-9 cells, with numbers of migrated cells being 33.0 ± 4.6 and 39.7 ± 4.5, respectively, after siHMGB1 transfection, when compared with the corresponding numbers of 90.7 ± 5.1 and 101.0 ± 3.0, respectively, in siNC group (Figure 1 E). Furthermore, the number of invasive cells dropped significantly, following HMGB1 knockdown in EC9706 cells (26.7 ± 4.5 *vs.* 68.7 ± 2.5) and TE-9 cells (29.3 ± 3.5 *vs.* 55.7 ± 3.1), as shown in Figure 1 F.

HMGB1 knockdown suppressed TLR4/MyD88 signaling and epithelial-mesenchymal transition in ESCC cells

The HMGB1 knockdown significantly down-regulated the protein expressions of TLR4 and MyD88, and suppressed the protein expressions of epithelial-mesenchymal transition markers, as depicted in E-cadherin enhancement and reductions in levels of N-cadherin and Vimentin in EC9706 cells (Figure 2 A) and TE-9 cells (Figure 2 B). These data revealed that TLR4/MyD88 signaling might be implicated in the

suppressive effects of HMGB1 knockdown on ESCC cells.

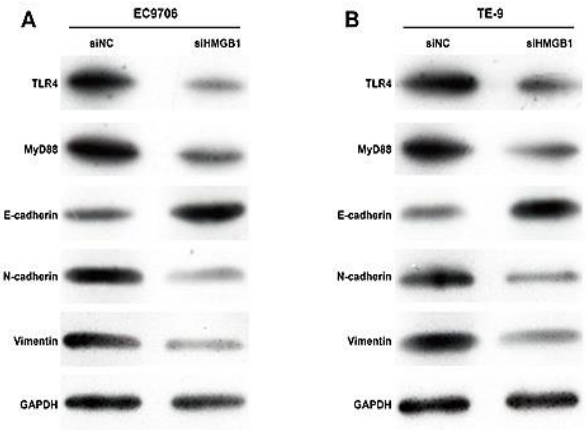

Figure 2: Effect of HMGB1 knockdown on TLR4/MyD88 signaling and EMT. siHMGB1 or siNC was transfected into EC9706 and TE-9 cells for 48 h. The protein expression levels of TLR4, MyD88, N-cadherin, E-cadherin, and Vimentin in EC9706 cells (A) and in TE-9 cells (B) were assayed using Western blotting. EMT: epithelial-mesenchymal transition

HMGB1 knockdown-mediated suppressive effects on ESCC cells

Since TLR4/MyD88 expression was significantly increased in ESCC tissues and down-regulated after HMGB1 knockdown in ESCC cells, it was speculated that TLR4/MyD88 signaling might be an important regulator in HMGB1 knockdown-mediated suppressive effects on ESCC cells. The EC9706 cells were transfected with siTLR4 or siHMGB1, with or without TLR4 in order to test the veracity of this speculation. At the molecular level, TLR4 knockdown significantly down-regulated the expression levels of TLR4, MyD88, and EMT parameters (E-cadherin level was raised, while levels of N-cadherin and Vimentin were reduced). Besides, TLR4 overexpression reversed the suppressive effects of HMGB1 knockdown or TLR4 knockdown on TLR4/MyD88 signaling and EMT markers. These data are presented in Figure 3.

Subsequently, the impacts of TLR4 expression variations on the proliferation, migration, and invasion of EC9706 cell were investigated. Results from CCK-8 test demonstrated that cell viability was reduced following TLR4 knockdown, while the impaired cell viability induced by HMGB1 knockdown or TLR4 knockdown was reversed by TLR4 overexpression in EC9706 cells (Figure 4 A). In addition, colony formation assay revealed that TLR4 knockdown inhibited cell proliferation, but overexpression of HMGB1 or TLR4 promoted cell proliferation (Figures 4 B and C).

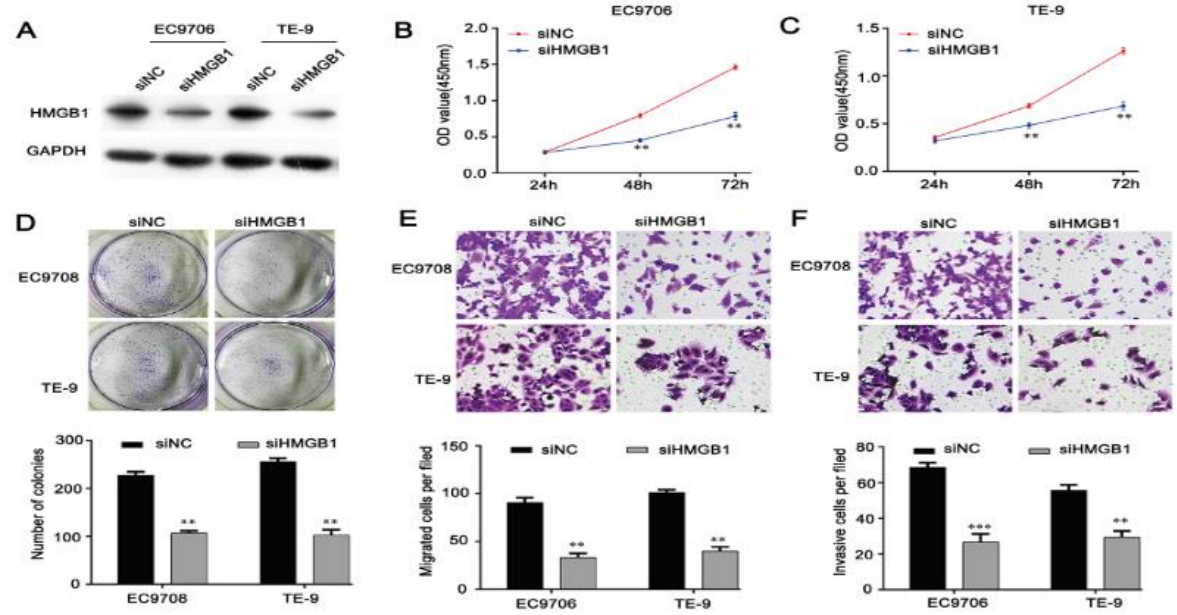

Figure 1: The impact of HMGB1 knockdown on proliferation, migration and invasiveness of ESCC cells. (A) Relative protein expression levels, as assayed using western blotting. (B and C) At 24, 48, and 72 h, CCK-8 assay revealed growth suppression effects in both EC9706 and TE-9 cells. (D) Cell proliferation in each group, as determined using cell proliferation experiments. (E) Cell migration, and (F) cell invasion, as determined using transwell chamber assays. Data are presented as mean ± SD of at least three replicate assays. $^{**}P < 0.01$; $^{***}p < 0.001$, *vs.* siNC

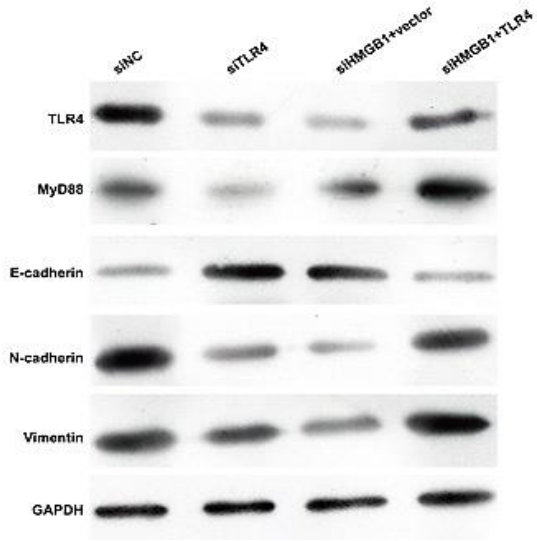

Figure 3: HMGB1 knockdown mediated suppressive effects on EMT by regulating TLR4/MyD88 signaling. EC9706 cells were transfected with siNC, siTLR4 or siHMGB1, with or without TLR4. The protein expression levels of TLR4, MyD88, E-cadherin, N-cadherin, and Vimentin were estimated using Western blotting

In addition, the inhibitory effect of siHMGB1 on cell migration (Figures 4 D and E) and invasion (Figures 4 F and G) in EC9706 cells were repeated by TLR4 transfection but were reversed by TLR4 overexpression. Collectively, these data demonstrate that TLR4 knockdown inhibited, while TLR4 overexpression enhanced cell proliferation, migration, invasion, and EMT in ESCC cells. Thus, HMGB1-mediated TLR4/MyD88 signaling may have a significant role in the pathogenesis of ESCC.

DISCUSSION

This research first showed that the expression of HMGB1 is apparently associated with increased inter-relationship with adjacent ESCC tissues. This is in good agreement with previous studies which reported that HMGB1 was highly expressed in various tumor tissues, and was interrelated to TNM stage and poor prognosis of esophageal carcinoma, lower rectal cancer, and epithelial ovarian cancer [13]. Furthermore, it was determined that knockdown of HMGB1 significantly decreased ESCC cell proliferation, migration, invasion, and levels of EMT markers. Consistently, HMGB 1 knockout by shRNA suppressed blood vessel formation and aggressiveness of breast cancer cells [14]. Suppression of MiR-218-5p expression decreased the proliferation, migration, and invasion of prostate cancer cells through downregulation of HMGB 1 [15].

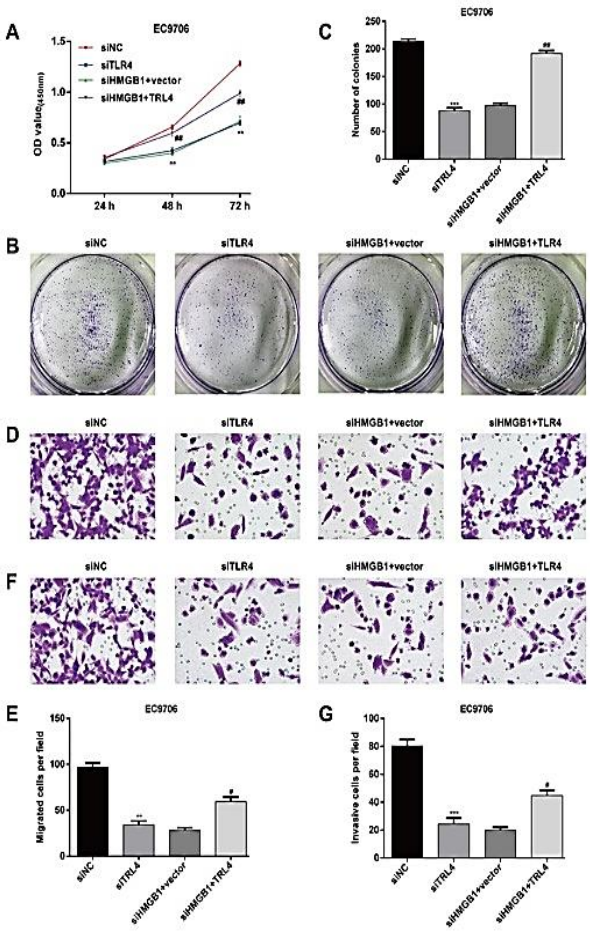

Figure 4: HMGB1 knockdown mediated suppressive changes in cell proliferation, migration and invasion by regulation of TLR4/MyD88 signaling. EC9706 cells were transfected with siNC, siTLR4, or siHMGB1, with or without TLR4. (A) Cell viability, as was measured using CCK-8 assay. (B and C) Cell proliferation, as was determined using colony formation test. Cell migration (D and E) and invasion (F and G), as were measured using Transwell chamber test. Data are presented as mean ± SD of at least three assays. **P* < 0.01; ****p* < 0.001, *vs.* siNC; #*p* < 0.05; ##*p* < 0.01, *vs.* siHMGB1 + vector

Thus, HMGB 1 silencing might greatly reduce lung cancer migration and invasiveness. Migration and invasion of tumor cells are the major contributors to cancer progression, with negative impact on prognosis. Epithelial-mesenchymal transition (EMT) is widely considered as a driver of cancer metastasis, as it primarily provides cancer cells with migratory and invasive potential. Data from this study showed that HMGB1 knockdown increased E-cadherin levels and decreased levels of N-cadherin and Vimentin in ESCC cells. Several studies have indicated positive effects of HMGB1 on tumor cell invasion. For example, HMGB1 induced high expression of p62, which facilitated SNAIL-mediated EMT in glioblastoma cells [16].

Moreover, a study showed that HMGB2 promoted EMT in prostate cancer PC3 cells [17].

In the present study, it was also discovered that HMGB1 up-regulation increased the protein expression levels of TLR4 and MyD88 in ESCC tissues. In contrast, HMGB1 knockdown significantly down-regulated the expressions of TLR4/MyD88 and epithelial-mesenchymal transition (EMT), and similar effects were produced by siTLR4 transfection. However, overexpression of TLR4/MyD88 signaling reversed the suppressive impacts of HMGB1 knockdown on ESCC cells. These results indicate that high TLR4/MyD88 expression is positively correlated with tumor cell invasiveness, which is similar with observations about other forms of cancer, e.g. colorectal cancer and lung cancer. In addition to cancer metastasis, inflammation-related EMT promotes transcription factor expression through inflammatory mediator-induced NF-κB activation [18]. The TLR4/MyD88 signal route is activated during inflammation. This may explain the elevation of E-cadherin, whereas the levels of N-cadherin and Vimentin were decreased following inhibition of TLR4/MyD88. Currently, not much is known about the direct interaction between HMGB1 and TLR4/MyD88 signaling in the regulation of ESCC cell behavior. Emerging evidence indicate that HMGB1 mediated TLR4/MyD88 signaling in papillomavirus persistence. This might support the finding that HMGB1 knockdown suppressed the proliferation, migration and invasion of ESCC cells by inhibiting TLR4/MyD88 signaling.

CONCLUSION

This study has demonstrated that HMGB1 promotes EMT of ESCC cells via upregulation of TLR4/MyD88 signaling, resulting in enhanced proliferation, migration, and invasion of the cells. Thus, this research sheds fresh light on the function of TLR4/MyD88 signaling in HMGB1-induced EMT of ESCC cells. Therefore, targeting HMGB1-mediated TLR4/MyD88 signaling may be a strategy for treating human ESCC.

DECLARATIONS

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None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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