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The transcription factor CREB is involved in sorafenib-inhibited renal cancer cell proliferation, migration and invasion

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Our previous reports showed that the cyclic-AMP-response element-binding protein (CREB) served as a proto-oncogene in the process of tumorigenesis and mediated the growth and metastatic activity of renal cancer cells. Our study, therefore, explored the role of CREB in sorafenib--inhibited cell proliferation, migration and invasion. Renal cancer cells were cultured in medium containing sorafenib for 12, 24, 48 and 72 h. The MTT assay was used to study the cytotoxic effects of sorafenib. Cell invasion and migration were assayed in wound healing and transwell experiments, respectively. Protein expression levels were evaluated by western blotting. The results show that sorafenib treatment decreased cell viability in a dose- and time-dependent manner. Sorafenib inhibited cell migration and invasion and decreased the expression of MMP-2 and MMP-9. Moreover, addition of the recombinant plasmid pCI-neo/CREB (PN) reversed the sorafenib-induced inhibition of cell proliferation, migration and invasion. These results show that CREB is associated with the sorafenib-induced inhibition of proliferation, migration and invasion.

Keywords: CREB, renal cancer, sorafenib, migration, invasion

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Renal cell carcinoma (RCC) is the most frequent malignancy of the kidney. RCC is a highly vascularized cancer associated with metabolic diseases. Due to frequent metastases, only 1/3 of RCCs can be effectively managed by resection, chemotherapy and radiotherapy (1–3). Unlike previous therapies, anti-angiogenic targeted therapies are effective for the advanced or late stage RCC. Among these, sorafenib is a novel molecular inhibitor of multi-tyrosine kinases (PDGFR and VEGFR) and the RAF/MEK/ERK cascade pathway (4, 5). This effect causes inhibition of growth signaling and angiogenesis (6–8). Unfortunately, in previous clinical uses, sorafenib functioned with variable efficacy for individuals (9, 10). More efforts need to be focused on the changes in proto-oncogenes after sorafenib treatment.

The cyclic-AMP-response element-binding (CREB) protein, a proto-oncogenic transcription factor, plays an important role in regulating tumorigenesis (11–12). In response

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to diverse extracellular signals, kinases such as MAPK and PKA are involved in the phosphorylation of CREB at the Ser-133 residue, thus increasing expression levels of its target genes (13). Increasing evidence has demonstrated that CREB phosphorylation is necessary for cell survival and for promoting tumorigenesis (14–16). Our previous reports showed that a CRE decoy could block the CREB binding site of its targeted genes (cyclins and bcl-2), thus abrogating the anti-tumor drug-induced cell apoptosis and cell cycle arrest (3, 17–18). Nonetheless, the possible role of pCREB in the progression of sorafenib-treated RCC growth and metastasis has not been investigated. In this study, we used the recombinant plasmid pCI-neo/CREB (PN) (constitutively active CREB at Ser-133) (19) to investigate the role of CREB in sorafenib-treated RCC cell lines and demonstrate its effects on cell proliferation and migration under CREB signaling.

EXPERIMENTAL

Cell culture and treatment

We obtained the human renal carcinoma cell lines ACHN, 786-O and OS-RC-2 from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). 786-O and OS-RC-2 cells were cultured in RPMI 1640 medium, and ACHN cells were cultured in DMEM (HyClone, UT, USA). Both media were supplemented with 10 % heat inactivated fetal bovine serum (HyClone, Auckland, NZ), 100 U mL^{-1} streptomycin and 100 mg mL^{-1} penicillin (HyClone, Auckland, NZ). The cultures were incubated at 37 °C in 5 % CO_2.

Cells were treated with 0.50 μmol L^{-1} sorafenib (Bayer Healthcare, Leverkusen, Germany) for 24 h for transwell and wound healing experiments. PN was transfected using the Lipofectamine 2000 transfection reagent (Invitrogen, USA) following the manufacturer's instructions.

Cell proliferation assay

Cell proliferation was calculated using the CellTiter96® Aqueous One Solution Cell Proliferation Assay (Promega, USA). In short, 2000 cells were plated in each well of a 96-well plate and received treatment with sorafenib at different concentrations (0.05, 0.10, 0.20, 0.50, 1.0 and 2.0 μmol L^{-1}) or different times (12, 24, 48 and 72 h). Four hours before the end of the study, 20 μL of CellTiter 96® Aqueous One Solution Reagent was added to each well. The absorbance at 492 nm was measured using a spectrophotometer.

Western blotting

Cells were lysed with RIPA buffer (Solarbio, Beijing, China) containing the 1 % protease inhibitor PMSF (Solarbio, Beijing, China). Total protein concentrations were measured using a BCA protein assay kit (Beyotime, Beijing, China). For western blot analysis, each sample (35 μg) was loaded and separated using 12 % SDS-PAGE, transferred onto PVDF membranes, blocked with 5 % non-fat dry milk (diluted with TBST), incubated with specific primary antibodies overnight at 4 °C as follows: rabbit monoclonal anti-CREB (Cell Signaling, Boston, catalog no. 9197, 1:1000), rabbit monoclonal anti-CREB (Ser-133) (Cell Signaling, Boston, catalog no. 9198, 1:1000), rabbit monoclonal anti-MMP-2 (Abcam, Cambridge, catalog no. 7033, 1:1000), rabbit monoclonal anti-MMP-9 (Abcam, Cambridge, cata-

log no. 137651, 1:1000) and rabbit monoclonal GAPDH (Cell Signaling, Boston, catalog no. 5174, 1:2000). After washing with TBST, the blots were incubated with horseradish peroxidase-labelled secondary antibody (Boster, Wuhan, catalog no. 1054, 1:5000) and visualized using an enhanced chemiluminescence reagent.

Wound healing assay

After the cells had grown to 70 % confluence, we made linear scratches with a micropipette tip in the middle of the 24-well plate, washed the dislodged cells with PBS and replaced the culture medium with fresh serum-free medium. After 24 h, we quantified cell migration by measuring the wound width.

Transwell assay

The cell invasion assay was carried out in transwell inserts fitted with polycarbonate filters (8 μm pore size, Corning, NY, USA). Upper sides of filters were pre-coated with Matrigel (BD Biosciences, NJ, USA). Two days after transfection, the cells were plated at a density of 5.0×10^4 cells per upper well in 100 μL of culture medium (10 % FBS) and the lower chamber was filled with 500 μL of medium (20 % medium). Cells were allowed to invade for 24 h at 37 °C in 5 % CO_2, following which, the non-invading cells in the upper surface of the membrane were removed by scrubbing with a cotton-tipped swab. Bottom cells were fixed in 4 % polyoxymethylene for 30 min and stained with 0.1 % crystal violet. The mean number of invading cells was counted under five preselected microscopic fields.

Statistical analysis

All experiments were repeated in triplicate. Data were expressed as means ± standard deviation using SPSS, version 18.0 (SPSS, Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant for ANOVA and STD *t*-test.

RESULTS AND DISCUSSION

Clinical development of the targeted protein kinase inhibitor, sorafenib, offers hope for future treatment of patients with advanced RCC whose prognoses are poor. Studies have shown that sorafenib disrupted microvasculature and inhibited tumor proliferation through anti-angiogenic, anti-proliferative and/or pro-apoptotic effects (20–22). Its widely accepted preclinical and clinical activity occurs because of its multiple molecular targets (receptor tyrosine kinases and serine/threonine kinase Raf) (4–5). In this study, we investigated the cytotoxic effect of sorafenib on RCC cells (ACHN, 786-O and OS-RC-2) using the MTT assay. As shown in Fig. 1, sorafenib inhibited RCC cell growth in a time- and dose-dependent manner. The IC_{50} values for sorafenib after 12, 24, 48 and 72 hours of treatment were calculated for each cell line (Table I). ACHN displayed relatively low cytotoxicity towards 786-O and OS-RC-2 cells. We, therefore, decided to further research 786-O and OS-RC-2 cells. To explore the effect of sorafenib on 786-O and OS-RC-2 cell migration and invasion, we treated cells with 0.50 μmol L^{-1} sorafenib in wound-healing and transwell experiments. In comparison with the control group, the sorafenib-treated group displayed significantly reduced migra-

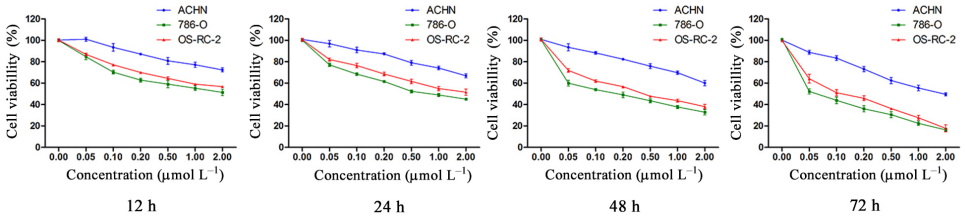

Fig. 1. Effect of sorafenib on ACHN, 786-O and OS-RC-2 cell proliferation. Cell viability was measured using the MTT assay. Data are presented as means ± SD of three independent experiments.

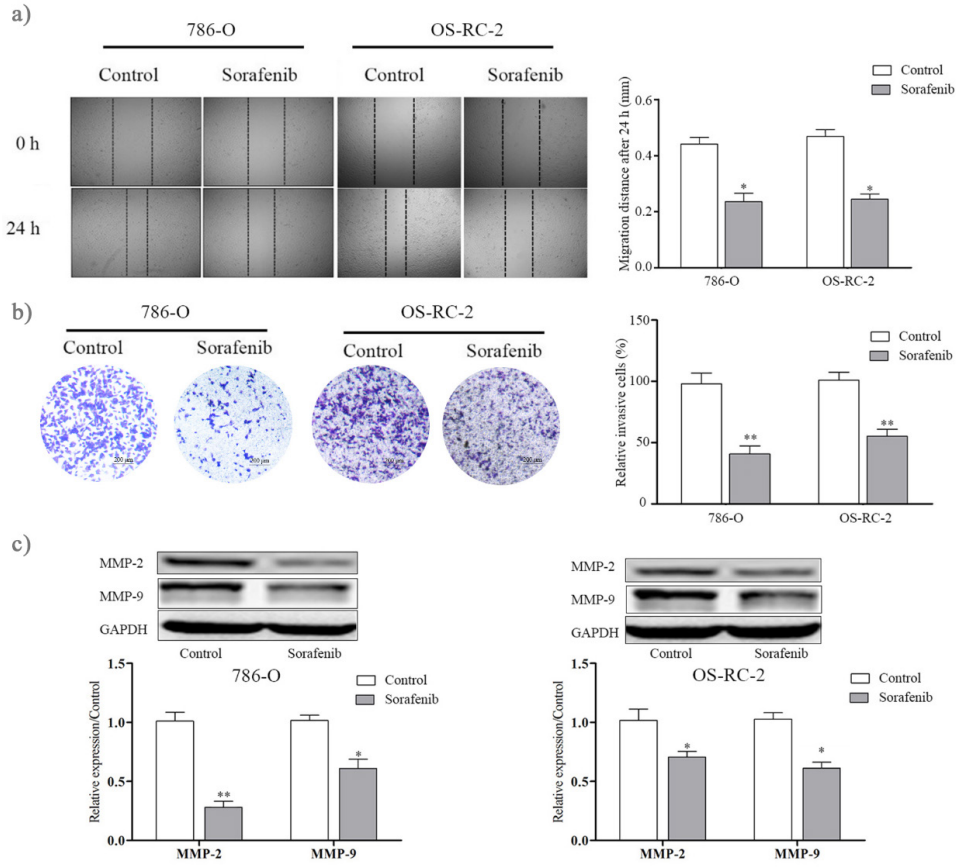

Fig. 2. Sorafenib inhibited 786-O and OS-RC-2 cell migration and invasion. Sorafenib decreased the migration (a) and invasion (b) capacities of 786-O cells. Representative graphs were derived from data obtained 24 h after treatment with 0.5 μmol L^{-1} sorafenib. Sorafenib significantly reduced the expression levels of MMP-2 and MMP-9 proteins compared to that in the controls (c). Data are presented as mean ± SD from three independent experiments. *$p < 0.05$, **$p < 0.01$ *vs.* the control group.

Table I. The concentration producing 50 % growth inhibition (IC_{50}) of sorafenib on renal cancer cells

Cell line	IC_{50} (μmol L^{-1})			
	12 h	24 h	48 h	72 h
ACHN	9.238	6.421	4.318	1.601
786-O	1.727	0.885	0.185	0.059
OS-RC-2	3.057	1.898	0.463	0.138

tion distances and fewer invading cells, as well as downregulation of the MMP-2 and MMP-9 expression levels (Fig. 2). These results indicated that sorafenib could inhibit the proliferation, migration and invasion of RCC cells, thereby substantiating its tumor-suppressive role in tumorigenesis.

It, however, remains unknown which of the downstream nuclear effectors participate in this process. Emerging evidence showed that CREB acted as a proto-oncogene in regulation of numerous physiological processes, including tumor growth and metastasis (11, 23–24). It is not clear whether CREB is involved in the sorafenib-induced inhibition of cell growth and metastasis. We, therefore, investigated CREB and pCREB modifications in sorafenib-treated 786-O and OS-RC-2 cells. Western blot analysis was used to observe protein changes in cells treated with 0, 0.05, 0.10, 0.20, 0.50 and 1.0 μmol L^{-1} sorafenib for 24 h. Interestingly, the results showed that sorafenib incubation inhibited pCREB levels in a concentration-dependent manner, with no obvious changes in total CREB expression (Fig. 3).

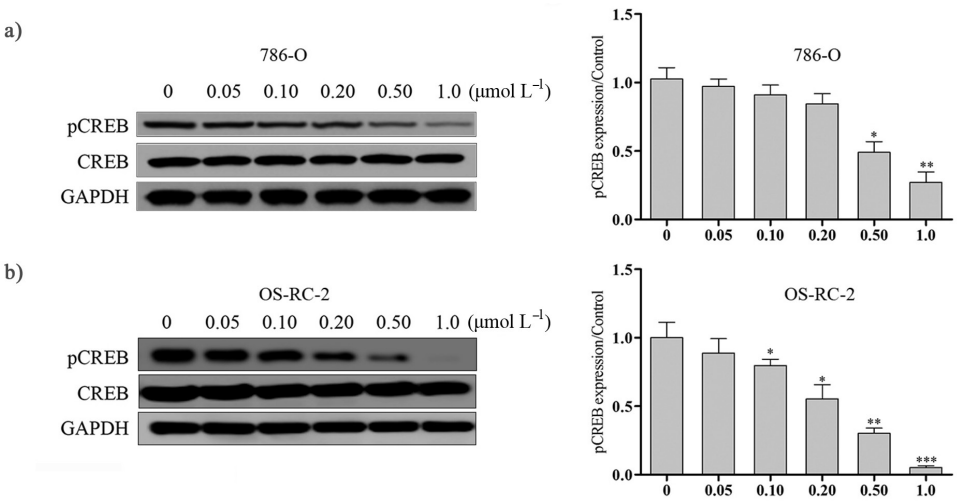

Fig. 3. Effect of sorafenib on pCREB and CREB expression. 786-O and OS-RC-2 cells were treated with 0, 0.05, 0.10, 0.20, 0.50 and 1.0 μmol L^{-1} sorafenib for 24 h, and pCREB and CREB were assayed by western blotting. Results are represented as means ± SD from three independent experiments. *p < 0.05, **p < 0.05, ***p < 0.001 *vs.* the control group.

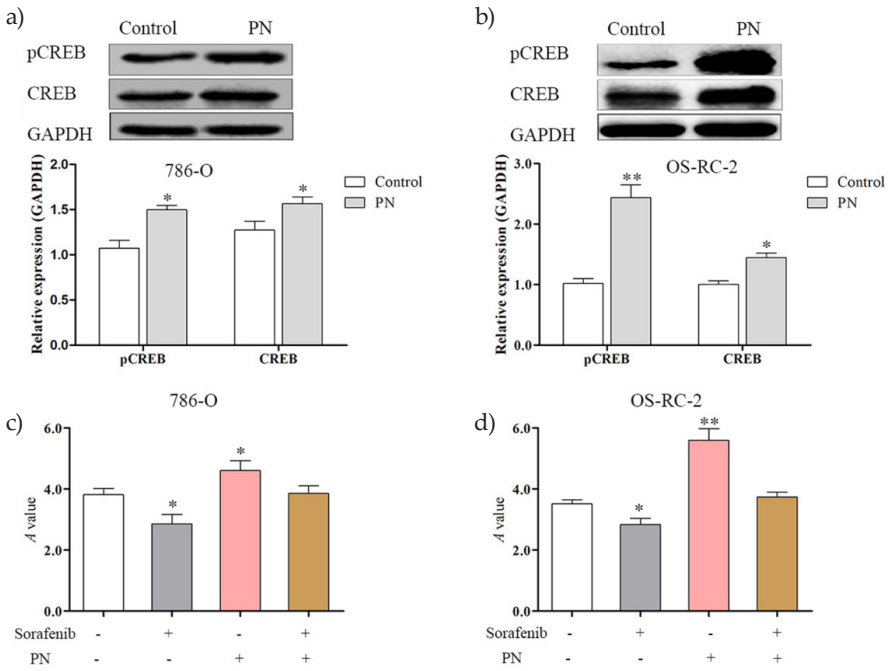

Fig. 4. Sorafenib regulated cell proliferation through CREB signaling. After 786-O and OS-RC-2 cells were treated with PN for 24 h, the expressions of pCREB and CREB were assayed by western blotting (a, b), and the cell viability was assayed by MTT (c, d). Data are presented as means ± SD, all experiment were repeated three times. *$p < 0.05$, **$p < 0.01$ *vs.* the control group.

These data suggested that sorafenib was capable of reducing the activation of CREB in RCC cells.

To further confirm the role of CREB in sorafenib-regulated cell proliferation, we transfected cells with the CREB recombinant plasmid PN to enhance the pCREB levels within 786-O and OS-RC-2 cancer cells (Fig. 4a and b). MTT analysis showed that PN markedly increased the proliferation at 24 h, while co-treatment of sorafenib and PN reversed the sorafenib-inhibited cell proliferation (Fig. 4c and d). These results indicated that CREB signaling was involved in sorafenib-inhibited cell proliferation.

Furthermore, in the process of RCC tumorigenesis, two pivotal features of metastatic malignancies, migration and invasion, are thought to provide the metastatic potential of cancer cells (25). During this process, the extracellular matrix and components of the basement membrane are degraded by the concerted action of MMPs, allowing tumor cells to escape from the primary site more easily (26–27). Proteins such as MMP-2, MMP-9 degrade laminin, gelatin and collagen, eventually disrupting the extracellular matrix structure and contributing to EMT progression and facilitating metastasis (28–29). In previous studies, we explored the changes of EMT-related proteins (E-cadherin, vimentin, N-cadherin, fibronectin) and MMP-2/9 when CREB was diminished. The results showed that reduction of pCREB could suppress RCC cell migration and regulate the expressions

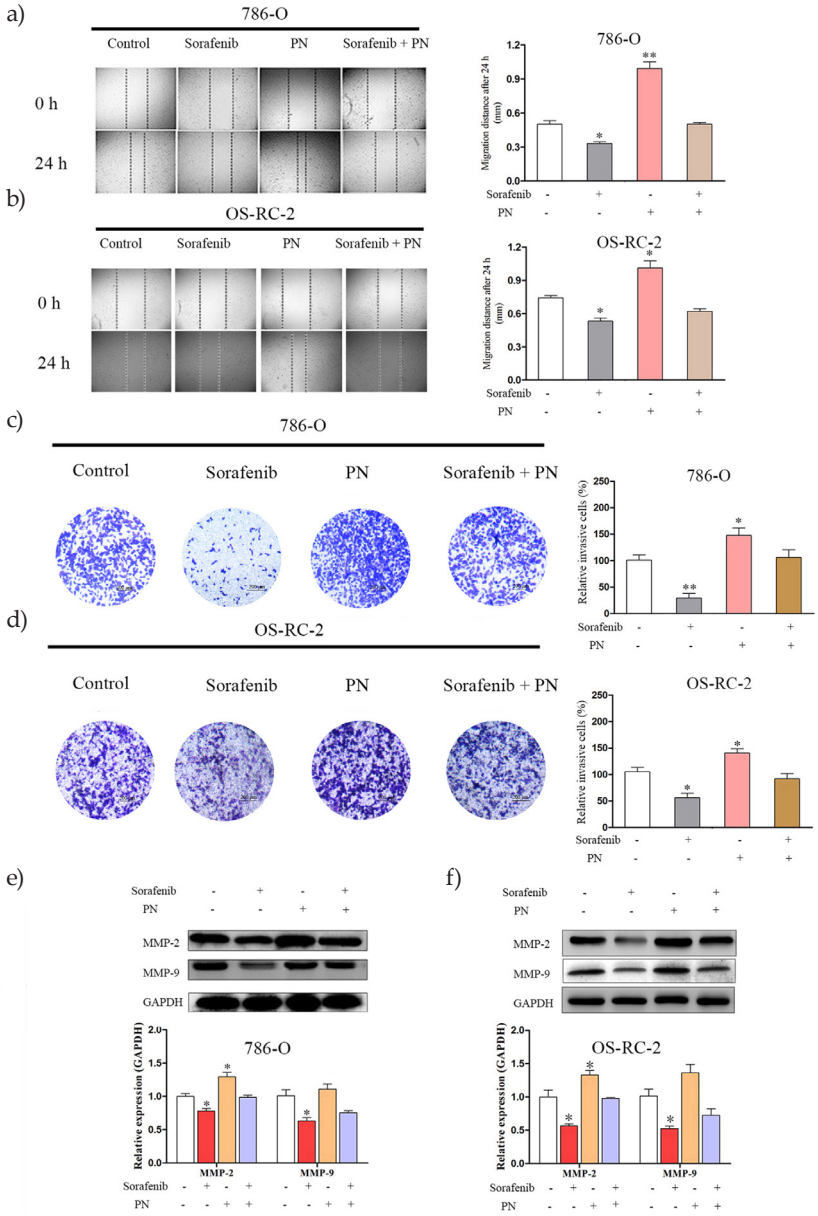

Fig. 5. Sorafenib regulated cell migration and invasion through CREB signaling. Migration and invasion experiments were performed in 786-O and OS-RC-2 cells after treatment with PN for 24 h. Cell migration distances were evaluated by a wound healing assay (a, b), cell invasion was measured by the transwell assay (c, d) and the expression levels of MMP-2 and MMP-9 were assayed by western blotting (f, g). Data are presented as means ± SD, all experiment were repeated three times. *$p < 0.05$, **$p < 0.01$ *vs.* the control group.

of N-cadherin, fibronectin, E-cadherin and MMP-2/9, but cannot affect the expression and distribution of vimentin because of the lack of CER sequence in the promoter of vimentin (24). Pradines *et al.* showed that CREB could regulate the transcriptional expression of MMP-9 and its inhibitor TIMP-1 (30). Park *et al.* revealed that CREB functioned in the signaling between Akt and MMP-2/MMP-9 and mediated the ICAM-3-induced cell migration and invasion in human non-small cell lung cancer cells (31). Similar results were also shown in human cholangiocarcinoma cells and human osteosarcomas (32–33). It, therefore, seemed that CREB signaling functioned as a regulator in the process of cell locomotion. To explore whether CREB played pivotal roles in the sorafenib-treated RCC cell migration and invasion, we treated cells with sorafenib, PN and their combinations. As expected, PN markedly boosted 786-O and OS-RC-2 cell migration distances. In comparison with sorafenib-treated cells, cells treated with the combination of PN and sorafenib had greater increases in their migration distances (Fig. 5a and b). Similarly, the sorafenib-reduced cell invasion was abrogated by addition of PN and sorafenib (Fig. 5c and d). In addition, we found that MMP-2 and MMP-9 expression levels were increased when pCREB was induced by PN. Compared to the sorafenib group, higher increases of MMP-2/MMP-9 levels were observed with the combination of PN and sorafenib (Fig. 5e, f), indicating that CREB was an upstream regulator of MMP-2/MMP-9. These data verified the role of CREB-induced MMP-2/9 expression in the progression of tumor metastasis.

CONCLUSIONS

This study has demonstrated that CREB signaling is involved in the sorafenib-inhibited cell proliferation, migration and invasion, accompanied by up-regulation of MMP-2 and MMP-9. A search for more exact mechanisms will be conducted in our future study.

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