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Original Article

γ-aminobutyric acid inhibits the growth of cholangiocarcinoma via cAMP/PKA signal pathway

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Abstract: Background: To explore the molecular mechanism of γ-aminobutyric acid (GABA) inhibiting the growth of cholangiocarcinoma via cAMP/PKA signal pathway. Methods: QBC939 cells were cultured in different groups and treated with GABA, GABA + 8Br (cAMP agonists), GABA + H89 (PKA antagonist) for 48 hours. MTT assay was used to determine the proliferation of QBC939 cells. Annexin V-FITC/PI binding assay was used to detect apoptosis in the QBC939 cells. Western blot was applied to detect the expression of PKAI and PKAII and ERK proteins in different groups of QBC939 cells. Animal models of cholangiocarcinoma bearing nude mice were established by subcutaneous injection of QBC939 cells and randomized into 2 groups: control and GABA-treated groups. The effect of GABA was evaluated after 5 weeks, including tumor volume. The expression of PKAI and PKAII and ERK was detected by Western blot in xenograft tumors. Results: MTT and FCM assays all showed that the inhibitory effect of GABA on the proliferation and the induced apoptosis of QBC939 cells could be antagonized by H89, but not 8Br. Western blot analysis showed that GABA significantly down-regulated the expression of PAK I protein (0.0878±0.003 vs. 0.1521±0.003, t=29.687, P<0.05), up-regulated the expression of PKA II (0.2042±0.012 vs. 0.1461±0.072, t=8.152, P<0.05), and also decreased the expression of ERK protein (0.3683±0.007 vs. 0.4687±0.01, t=13.647, P<0.05) and xenograft tumor volume [(0.50±0.02 vs. 0.32±0.03) cm^3, *t*=15.354, P<0.05]. The expression of PKAI and ERK was significantly decreased, and PKAII was increased in GABA-treated group as compared with control group. Conclusions: GABA may inhibit the growth of cholangiocarcinoma cells QBC939 through cAMP/PKA signal pathway, which down-regulated PAK I and up-regulated PKAII expression, and then decreased the expression of ERK.

Keywords: Bile duct neoplasms, gamma-aminobutyric acid, camp, pka, erk

Introduction

Cholangiocarcinomas (CCA) encompass all tumors originating in the epithelium of the bile duct. More than 90% of CCA are adenocarcinomas. CCA are diagnosed throughout the biliary tree and are typically classified as either intrahepatic or extrahepatic CCA. CCA accounts for approximately 3% of gastrointestinal tumors and 10%-25% of all hepatobiliary malignancies. The incidence is increasing, especially the incidence of intrahepatic CCA [1, 2]. However, the mechanisms of CCA are still unclear. Increasing evidence suggests that the growth of tumor cells is not only a consequence of genetic alterations, but also due to chemokines, neuropeptides, and neurotransmitters such as gamma aminobutyric acid (GABA) [3]. GABA is originally identified as a principal inhibitory neurotransmitter in the adult mammalian brain. Outside the brain, GABA have also been found in non-neuronal peripheral tissues such as gastrointestinal system [4], lung [5], and liver [6], including biliary tract system [7]. They have been implicated they play an important role not only in synaptic inhibition, convulsion, pain, depression and cognition [8], but also in inhibiting cancer growth and tumor cell migration, such as colorectal carcinoma [9], and breast cancer [10]. In our previous study, we showed that GABA may inhibit the growth of cholangiocarcinoma cell QBC939 through $GABA_B$ receptor, and the anticancer effects may be partly mediated via JAK/STAT3 pathway [11]. However, the development of tumor is not regulated by a single signaling pathway, but a multiple cell signal-

ing pathways. Recently, investigators considered that cAMP/PKA signal pathway may play an important role in the CCA growth and migration; nevertheless the mechanisms of inhibition effect were not fully understood.

So, in this study, we aim to investigate whether GABA can mediate its antiproliferative and proapoptotic effects in QBC939 cells through the suppression of the cAMP/PKA pathway.

Materials and methods

Cell culture and drugs preparation

Human cholangiocarcinoma cell line QBC939 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). It was cultured in PRMI 1640 (Sigma) medium supplemented with 10% fetal bovine serum (FBS) and penicillin and streptomycin (Sigma) at 37°C in humidified 5% CO_2 atmosphere. GABA (Sigma), 8Br (cAMP agonist, Sigma) and H89 (PKA inhibitor, Sigma) were dissolved in culture medium, and then all were diluted for experiments.

Cell proliferation assay

QBC939 cells were seeded into 96-well plates (3,000 per well) in a final volume of 200 ml medium. After a 24-hour attachment, cells were incubated with GABA (100 μmol/L), GABA (100 μmol/L) + 8Br (20 μmol/L), GABA (100 μmol/L) + H89 (10 μmol/L) for 48 hours. During this time, the culture medium was replaced with fresh medium every 24 h. After treatment, cell proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium (MTT, Sigma) assay. The OD values were measured at 490 nm with a microplate spectrophotometer (Molecular Devices). Proliferation index was calculated as the ratio of the absorbance of cells with different treatments compared with controls.

Annexin V-FITC/PI assay

After cells were incubated with GABA (100 μmol/L), GABA (100 μmol/L) + 8Br (20 μmol/L), GABA (100 μmol/L) + H89 (10 μmol/L) for 48 hours. The Annexin V-FITC/PI assays were performed according to the manufacture's protocol (Roche). Briefly, the cultured cells were collected, washed with binding buffer, and incubated in 200 μl of a binding buffer containing 5 μl of Annexin-V-FITC. The nuclei were counterstained with PI. The percentage of apoptotic cells was determined using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Western blot

QBC939 cells were cultured and treated with GABA, GABA (100 μmol/L) + 8Br (20 μmol/L), GABA (100 μmol/L) + H89 (10 μmol/L) for 48 hours. Cells were collected and lysed in radioimmunoprecipitation assay (RIPA) buffer solution [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM ethylenediamine tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)]. After measurement of protein concentrations with a BCA protein assay kit (Pierce, USA), equal amounts of total protein per lane were subjected to SDS gel electrophoresis, followed by semidry transfer of proteins to polyvinylidene fluoride (PVDF) membrane (Sigma, USA). Nonspecific binding of proteins to the membrane was blocked by incubation in TBS-T buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween-20) containing 5% skimmed milk. After blocking of nonspecific sites, filters were reacted with primary antibodies and corresponding horseradish peroxidase-conjugated secondary antibodies. Primary rabbit polyclonal antibodies were as follows: anti-PKAI (1:200 dilution), anti-PKAII (1:200 dilution), anti-ERK (1:200 dilution), and anti-b-actin (1:500 dilution), all from Cell Signal Technology. Western blot images were captured using an Epi Chemi II Darkroom and Sensicam imager with Labworks 4 software (UVP).

Xenograft tumors

Female Balb/c nude mice (18-22 g at 4 weeks of age) were from the Vital River Laboratories (Beijing, China). Cells (5×10^6 cells in 200 ml of serum-free medium) were subcutaneously injected into the flank of the Balb/c nude mice (n=8 per group). When tumors had reached a volume of about 0.1 cm^3, the mice were given a tumor injection of GABA (1000 mg/kg/d, dissolved in 0.9% Nacl) or 0.9% Nacl every other day. Tumor growth was monitored every 5 days by measuring two perpendicular diameters with calipers. The tumor volumes were calculated using the equation $V = (D\times d^2)/2$, where V (cm^3) is the tumor volume, D is the longest diameter,

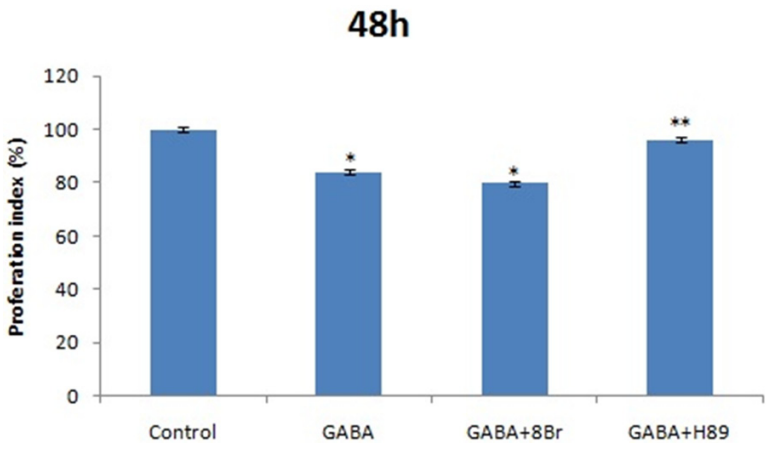

Figure 1. The effect of GABA on QBC939 cell proliferation in the presence of the selected antagonists. MTT assay shows that GABA induced (100 mmol/L) decrease of cholangiocaricinoma proliferation at 48 hours is blocked by pre-incubation with H89 (10 mmol/L), but not 8Br (10 mmol/L). *P<0.05, versus control group; **P<0.05, versus GABA group.

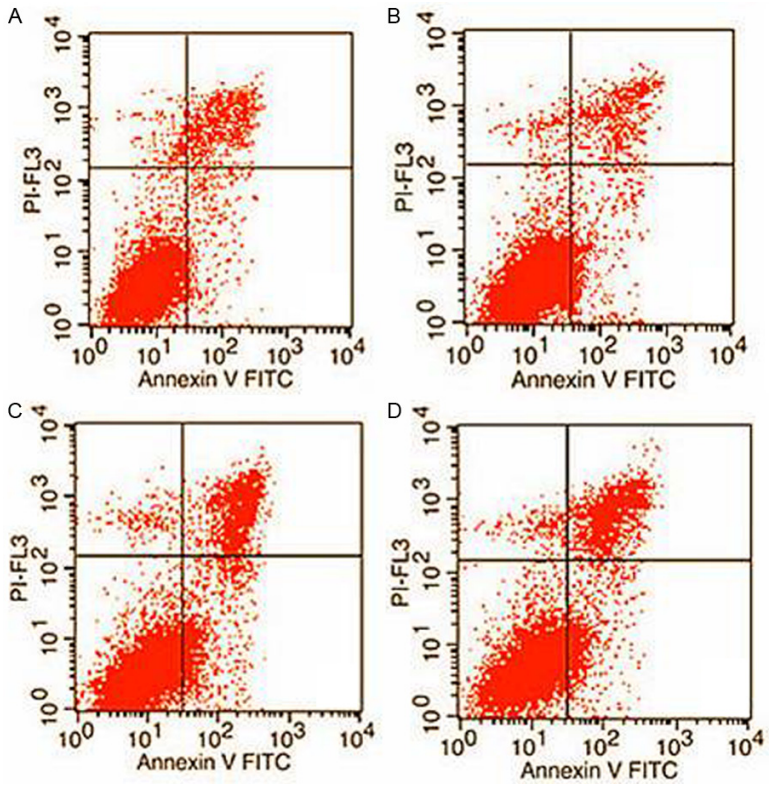

Figure 2. The Annexin V-FITC/PI staining of GABA on QBC939 cell apoptosis was in the presence of the selected antagonists and inhibitor (A. Control; B. GABA; C. GABA + 8Br; D. GABA + H89).

and d is the shortest diameter. Anti-tumor efficacy was measured as tumor growth inhibition rate, defined as [1-(T/C) ×100], where T and C represent the mean tumor volumes in the treated and untreated control groups, respectively. After treatment for 5 weeks, mice were sacrificed. The study was carried out in accordance with Chinese government guidelines.

Western blot in xenograft tumors

Total protein was collected from fresh tumor tissue samples and lysed in radioimmunoprecipitation assay (RIPA) buffer solution. The subsequent steps as above-mentioned in Western blot.

Statistics

All statistical analyses were performed using SPSS13.0 software. The Pearson's chi square (x^2) test or Fisher's exact test was used to analyze the differences in the comparison of rates. The experiments results were presented as mean ± SD and differences between various groups were assessed using the ANOVA or Dunnett t-test. *P* value of <0.05 was considered to indicate statistical significance.

Results

GABA inhibits QBC939 cells proliferation through c AMP/PKA signal pathway

The effects of GABA and its other drugs stimulation on the proliferation of cholangiocarcinoma QBC939 were determined by MTT assay. As illustrated in **Figure 1**. GABA significantly suppressed cells growth, the effect could be antagonized by H89 (P<0.05), but not 8Br (P> 0.05); the proliferation index of 100 μmol/L GABA, 100 μmol/L GABA + 20 μmol/L 8Br and 100 μmol/LGABA + 10 μmol/L H89 were (83.89±

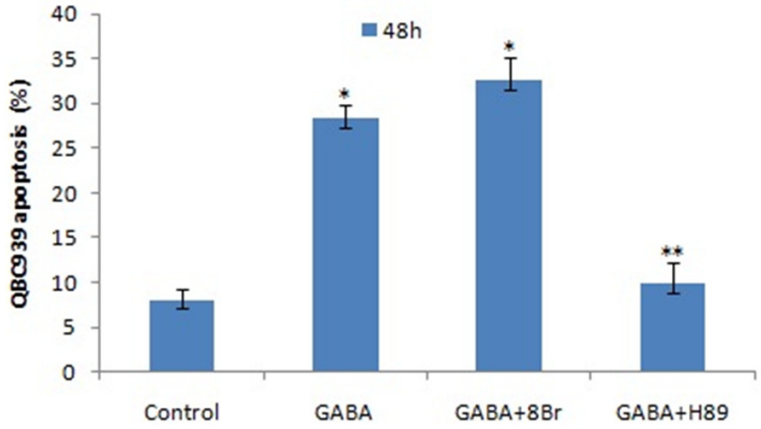

Figure 3. The effect of GABA on QBC939 cell apoptosis was in the presence of the selected antagonists. Annexin V-FITC/PI assay shows that GABA-induced (100 mmol/L) increase of cholangiocaricinoma apoptosis at 48 hours is blocked by preincubation with H89 (10 mmol/L), but not 8Br (20 mmol/L). *P<0.05, versus control group; **P<0.05, versus GABA group.

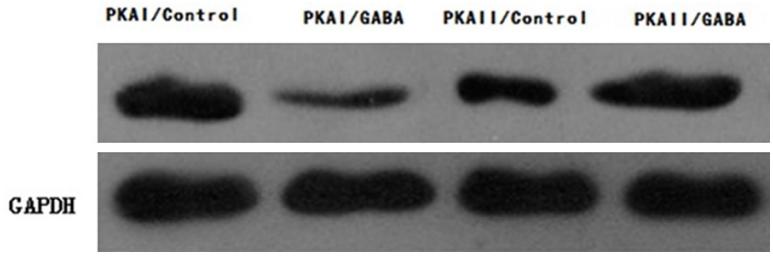

Figure 4. Western blot analysis for protein expression of PKAI and PKAII in QBC939 cells with the presence of the GABA.

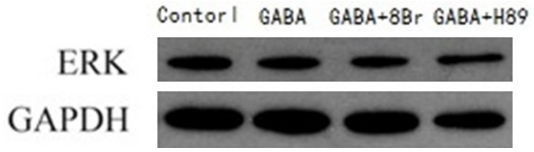

Figure 5. Western blot analysis for protein expression of ERK in QBC939 cells with the presence of the selected antagonists and inhibitor.

0.73)%, (79.7±0.57)%, (95.94±0.29)% respectively. Significant difference was observed among groups (P<0.05).

GABA promotes QBC939 cells apoptosis through c AMP/PKA signal pathway

The influence of GABA and other drugs stimulation on the apoptosis of cholangiocarcinoma QBC939 was determined by Annexin V-FITC/PI assay. The apoptosis rates of control, 100 μmol/LGABA, 100 μmol/L GABA + 20 μmol/L 8Br and 100 μmol/L GABA + 10 μmol/L H89 were (8.07±1.24)%, (28.38±1.53)%, (32.62±2.45)%, (9.90±2.42)%, respectively (**Figure 2A-D**). As showed in **Figure 3**, GABA significantly promoted cholangiocarcinoma QBC939 cells apoptosis, and the action also could be antagonized by H89, but not 8Br. Significant difference was observed among groups (P< 0.05).

cAMP/PKAI/PKAII/ERK may regulate the inhibition effect of GABA on QBC939 growth

Western blot analysis showed that the expressions of PKAI, PKAII and ERK were observed in QBC939. As illustrated in **Figure 4**, GABA exposure significantly decreased PKAI level (0.0878±0.003 vs. 0.1521±0.003, t=29.687, P< 0.05), increased PKAII level (0.2042±0.012 vs. 0.1461± 0.072, t=8.152, P<0.05), and also decreased ERK level (0.3683±0.007 vs. 0.4687± 0.01, t=13.647, P<0.05) in **Figure 5**. Similarly, this effect also could be partly antagonized by H89 (0.3959±0.166), but not 8Br (0.3191±0.009).

GABA suppressed QBC939 xenograft tumor growth in vivo

To further explore the mechanisms of GABA in CAA development, we examined the effect of GABA on QBC939 xenograft growth in nude mice. We observed that the administration of GABA significantly suppressed tumor growth (**Figure 6A**, **6B**). The mean tumor volume of treated group was smaller than that of the control group [(0.32±0.03) cm^3 vs. (0.50±0.02)) cm^3, P<0.05]. The anti-tumor efficacy of 15 d, 20 d, 25 d, 30 d was 20.45%, 26.42%, 31.85%, 34.87%, respectively (P<0.05). During the 35-day treatment, no abnormal deaths were observed.

Expression of PKAI, PKAII, and ERK was examined in GABA-treated xenograft tumors

Furthermore, we analyzed the tumor sections from control and GABA groups for PKAI, PKAII, and ERK using western blot. Significant difference was found in **Figures 7** and **8**, the expres-

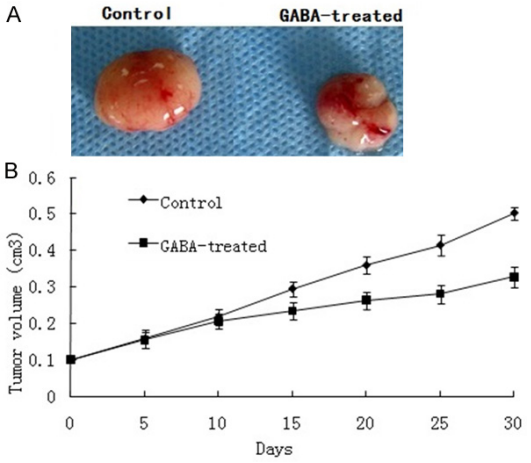

Figure 6. GABA suppressed QBC939 xenograft growth. Establishment of Balb/c nude mice bearing QBC939 cells as described in Materials and methods. A: The representative photograph of GABA suppressed QBC939 xenograft growth. B: Mice in GABA group were given tumor injections of GABA (1000 mg/kg/d) for 35 days. The Control group received same volume of NaCl (0.9%). Tumor growth was evaluated by measuring the relative tumor volume. *Represented P<0.05.

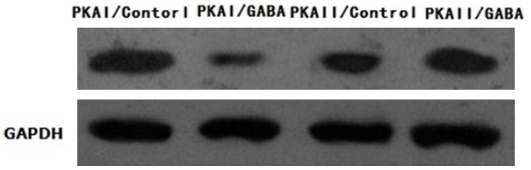

Figure 7. Western blot analysis for detecting the protein expression of PKAI and PKAII in the QBC939 xenograft tumor tissue.

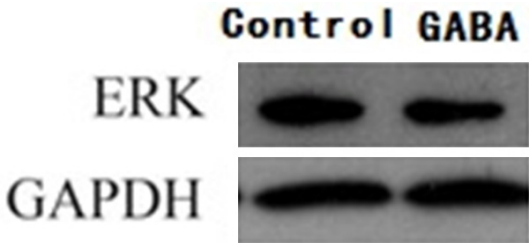

Figure 8. Western blot analysis for protein expression of ERK in the QBC939 xenograft tumor tissue.

sion levels of PKAI (0.130±0.001 vs. 0.080±0.002, t=27.19, P<0.05) and ERK (0.6378±0.007 vs. 0.9441±0.022, t=23.199, P<0.05) were significantly decreased in the tumor sections of mice treated with GABA, however, the PKAII was increased (0.010±0.001 vs. 0.015±0.002, t=28.14, P<0.05).

Discussion

Cholangiocarcinoma is one of the highly malignant gastrointestinal tumors and has poor prognosis. It is difficult to diagnose owing to its anatomic location, growth patterns and lacking of definite diagnostic criteria. The prognosis of this malignancy is dismal owing to its silent clinical character, difficulties in early diagnosis and limited therapeutic approaches; the median survival is less than 24 months [12, 13]. So it is important for us to understand its pathogenesis and find effective treatments. With the development of molecular biology, neuropsychological factors for the disease have been taken seriously and proved. What's more, many neurotransmitters in cancer development and metastasis have been revealed. These have become the new hot spots to explore the prevention and treatment of cancer. GABA, as a neurotransmitter, which inhibited the growth of cholangiocarcinoma cell line QBC939, and cAMP/PKA signaling pathway may be involved in its regulatory process, but the mechanism was not understood.

cAMP, as the component of signaling system, was the first discovered intracellular messenger, which involved in the regulation of life processes and function in cells [14]. As a major regulator of cAMP, PKA involved in proliferation and differentiation in many tumors. The study showed that the content of cAMP and PKA could be increased significantly after GABA action. 8Br (cAMP agonist) significantly enhanced the effect of GABA to inhibit proliferation and induce apoptosis in cholangiocarcinoma cell line QBC939, but H89 (PKA inhibitor) can significantly antagonize the effect of GABA. It suggested that cAMP/PKA signaling pathway may play an important role in inhibiting the growth of QBC939 cells by GABA. However the numerous PKA subunits played different roles, they were mainly divided into PKAI and PKAII [15] according to the differences of subunits. What was the specific role of the subunit was not determined.

Therefore, we further found that the expression of PKA I protein in GABA action after 48 h was significantly lower than that of control group, while the expression of PKA II was significantly higher, the difference was statistically significant. In animal experiments, we also observed the expression of PKAI protein decreased, and

the expression of PKAII protein increased in GABA-treated group. These suggested that GABA may inhibit the growth of cholangiocarcinoma cell line QBC939 by regulation the expression of PKAI and PKAII in this process. At present, the functional studies of PKAI and PKAII were extensive. For example, a study [16] found that PKAI was overexpression in breast cancer; antisense PKAI oligonucleotides could inhibit cells proliferation and tumor growth. In addition, 8-cl-cAMP analogue, which had site selectivity, could identify the cAMP binding sites on PKA and selectively bound with PKA II specifically to increase the expression, which had the effect of inhibiting growth, promoting differentiation and reversing malignant phenotype in most cancers, sarcoma and leukemia cells.

GABA could regulate the growth of cholangiocarcinoma cells through cAMP/PKA signaling pathway, the target genes were not clear. A research [17] showed that ERK was involved in the proliferation and differentiation of cholangiocarcinoma. In our study, we found that the expression of ERK protein was significantly down regulated in the experiment after GABA treated, and 8Br (cAMP agonist) could significantly enhanced the effect, H89 (PKA inhibitor) inhibited that. In the animal experiments, the expression of ERK protein was also decreased in xenograft tumors tissue, it indicated that ERK protein may play an important role in the regulation of cAMP/PKA signaling pathway. It had been reported that ERK was closely related to tumor growth, such as the activation of it (p-ERK) could avoid apoptosis and promote the progress of cell cycle in pancreatic cancer [18]. Chen et al. also found that it was closely related to the invasion and metastasis in renal cell carcinoma [19].

In conclusion, our present results indicated that GABA had the effect of inhibiting the growth of cholangiocarcinoma cell line QBC939, and its inhibition may be mediated by cAMP/PKA signaling pathway, through down-regulated PAK I and up-regulated PKAII expression, and the decrease in ERK was an underlying mechanism of functioning action. We considered the effect of GABA may constitute a cell response and an immune defense mechanism against tumor development. Therefore, activation of the GABA-mediated pathway, possibly combined with cAMP/PKA/ERK agonist, may have potential therapeutic value to prevent cancer progression.

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Disclosure of conflict of interest

None.

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