**Gadd45b is required in part for the anti-obesity effect of constitutive androstane receptor (CAR)**

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Received 14 May 2020; received in revised form 9 July 2020; accepted 21 August 2020

**KEY WORDS**

Obesity; Diabetes; CAR; Gadd45b; Glucogenogenesis; Lipogenesis; Xenobiotics; Nuclear receptor

**Abstract**  Crosstalk between xenobiotic metabolism and energy metabolism in the liver has provided a potential opportunity to target xenobiotic receptors to treat metabolic diseases. Activation of constitutive androstane receptor (CAR), a xenobiotic-sensing nuclear receptor, has been shown to inhibit obesity, suppress hepatic gluconeogenesis, and ameliorate hyperglycemia in rodent models of obesity and type 2 diabetes. However, the underlying molecular mechanism remains to be defined. The growth arrest and DNA damage-inducible gene 45b (Gadd45b), a well-known anti-apoptotic factor, has been shown to be an inducible coactivator of CAR in promoting rapid liver growth. It is unknown whether the effect of CAR on energy metabolism depends on GADD45B. In the present study and by using a high fat diet (HFD)-induced obesity model, we show that reduced body weight gain and improved insulin sensitivity by the CAR agonist 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) were markedly blunted in Gadd45b knockout mice. Mechanistically, the TCPOBOP-responsive inhibition of hepatic lipogenesis, gluconeogenesis, and adipose inflammation observed in wild type mice were largely abolished in Gadd45b knockout mice. We conclude that Gadd45b is required in part for the metabolic benefits of CAR activation.

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1. Introduction

Constitutive androstane receptor (CAR) was initially characterized as a xenobiotic receptor in response to chemical exposures. The xenobiotic function of CAR was achieved through its transcriptional activation of drug metabolizing enzymes and transporters in liver and intestinal tracks. At the functional level, CAR was subsequently appreciated, including its function in energy metabolism. For instance, CAR agonist phenobarbital has been anecdotally shown to have anti-obesity effects in humans, suggesting that CAR may play a role in energy metabolism. Several in vitro cell culture studies reported that activation of CAR decreased the mRNA expression of glucocorticoid enzyme genes G6pase and Pepck. Finally, several in vivo studies have demonstrated the role of CAR in obesity and diabetes in mice. Our group previously reported that activation of CAR improves insulin sensitivity in mice fed with high-fat diet (HFD), which was reasoned to be due to inhibition of lipogenesis, increased very low density lipoprotein (VLDL) secretion and energy expenditure. Improved insulin sensitivity by CAR activation was also demonstrated by others using the leptin-deficient (ob/ob) mice. At the mechanistic level, we recently reported that activation of CAR inhibits gluconeogenesis through accelerating degradation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-a), which is an essential co-activator in controlling glucose metabolism. Dong et al. showed that activation attenuates hyperglycaemia by suppressing glucose production and facilitating glucose uptake in the liver. A better understanding of the mechanisms underlying the metabolic benefits of CAR activation will help to harness the therapeutic potential of this “xenobiotic receptor”.

The growth arrest and DNA damage-inducible gene 45b (GADD45B) is an anti-apoptotic factor that can bind to mitogen-activated protein kinase kinase 7 (MEK7) and repress c-Jun N-terminal kinase (JNK) phosphorylation. Interestingly, GADD45B was later found to function as a coactivator for CAR. Specifically, the hepatic expression of Gadd45b was found to be induced in the livers of mice treated with the CAR agonist TCPOBOP in a CAR-dependent and TNF-independent manner. At the functional level, loss of Gadd45b impaired the early transcriptional stimulation caused by CAR activation, and Gadd45b is required to facilitate rapid liver growth. However, whether Gadd45b is required for CAR-mediated improvement of insulin sensitivity and inhibition of lipogenesis and gluconeogenesis is yet to know. In this study, we uncovered that Gadd45b is necessary for the metabolic benefits of CAR in inhibiting obesity and improving insulin sensitivity in mice.

2. Materials and methods

2.1. Animals

Wild type (WT) and Gadd45b<sup>−/−</sup> mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Gadd45b<sup>−/−</sup> knockout (KO) mice were generated by crossbreeding Gadd45b<sup>+/−</sup> mice (stock number: 013101). Genotyping primers for KO mice are 10936: GCAACCCAGTAACCTTGGGA; 10937: CCTGCAGGAGAGAGGAGTGTG; o1MR7996: CTTCCTTTGTCACGCTCGT, provided by the Jackson Laboratory. Eight-week-old male WT and KO mice were subjected to 60% calories high fat diet (ENVIGO #TD.06414, containing 23.5% protein, 27.3% carbohydrate, and 34.3% fat). Harlan Laboratories, Madison, WI, USA) for 17 weeks. Mice received once per week intraperitoneal injections of 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP, 0.5 mg/kg) or vehicle (DMSO) as we have previously described. Body composition was analyzed in live animals using EchoMRI-100TM from Echo Medical Systems (Houston, TX, USA). Mice were sacrificed 24 h after the last dose of drug. The use of animals in the study was approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh (Pittsburgh, PA, USA).

2.2. Glucose tolerance test (GTT) and insulin tolerance test (ITT)

For GTT, mice were fasted for 16 h before receiving an intraperitoneal injection of d-glucose at 1.75 g/kg body weight. For ITT, mice were fasted for 6 h before receiving an intraperitoneal injection of human insulin (Novo Nordisk, Bagsvaerd, Denmark) at 1 unit/kg body weight. Blood samples were taken at different time points, and the concentrations of glucose were measured with a glucometer (OneTouch, Malvern, PA, USA). GTT and ITT were performed five days after the last TCPOBOP injection.

2.3. Liver triglyceride and cholesterol extraction and analysis

Liver lipids were extracted using chloroform–methanol method reported by Folch et al. Briefly, liver samples were homogenized in methanol first and chloroform was added to the sample. The whole mixture was agitated for 2 h at room temperature. Extra methanol was added, and the mixture was centrifuged at 1734×g for 5 min. Supernatant was collected and chloroform was added to the supernatant. After washing, the mixture was centrifuged at 771×g for 20 min and the bottom phase was collected and evaporated under nitrogen at 60 °C. The organic layer was reconstituted in a mixture of tert-butyl alcohol:Triton-114: methanol (4.5:2.1, v/v/v). Commercial assay kits from Stanbio (Boerne, TX, USA) were used to measure triglyceride and cholesterol levels.

2.4. Gene expression analysis

Total RNA was isolated using the TRIZOL (Invitrogen, Carlsbad, CA, USA) and treated with DNase I to remove genomic DNA. cDNA was synthesized using reverse-transcription kit (Thermo Fisher, Waltham, MA, USA). SYBR Green real time PCR was performed with the ABI 7500 real time PCR system (Waltham, MA, USA).
(Bethesda, MD, USA). Crown-like structures in white adipose tissue (WAT) were quantified using Image J software.

2.6. Measurements of serum chemistry

Serum levels of total triglyceride, cholesterol (Stanbio Laboratory, Boerne, TX, USA) and insulin (Crystal Chem, Downers Grove, IL, USA) were measured using commercial kits according to manufacturers’ instructions.

2.7. Statistical analysis

Results are presented as means ± standard deviation (SD). Student’s t-test was used for comparison between two groups. Analysis of variance (ANOVA) was used for the comparison among the means of three or more groups, followed by Tukey’s post-test, using GraphPad PRISM software (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant at P < 0.05.

3. Results

3.1. Gadd45b is required for the anti-obesity effect of CAR activation

We have previously reported that treatment of mice with CAR agonist TCPOBOP alleviated HFD-induced obesity. To determine whether the anti-obesity effect of CAR depends on Gadd45b, 8-week-old male WT and Gadd45b KO mice were fed with HFD for 17 weeks and weekly treated with TCPOBOP or vehicle. TCPOBOP significantly inhibited the gain of body weight as early as one week after the drug treatment in WT mice (Fig. 1A), but the inhibitory effect of TCPOBOP on body weight gain was largely abolished in KO mice (Fig. 1B). Body composition analysis by MRI show that after one week of TCPOBOP treatment in WT mice, the fat mass to body weight percentage was significantly higher in the vehicle group compared to the TCPOBOP group (Fig. 1C, top). Meanwhile, the lean mass to body weight percentage was decreased in the vehicle group, but they were steady in the TCPOBOP group (Fig. 1C, bottom). In contrast, neither the fat mass to body weight percentage (Fig. 1D, top) nor the lean mass to body weight percentage (Fig. 1D, bottom) was significantly different between the vehicle- and TCPOBOP-treated KO mice. The effect of Gadd45b on obesity was independent of changes in the food intake (Fig. 1E). At the end of week 17 treatment, the body weight gain (Fig. 1F) and fat mass to body weight percentage (Fig. 1G) of WT mice were significantly lower in the TCPOBOP group compared to the vehicle group while the lean mass to body weight percentage was significantly higher in WT mice treated with TCPOBOP, but there was no difference between the vehicle and TCPOBOP groups in KO mice. The lean mass to body weight percentage was significantly higher in WT mice treated with TCPOBOP, but there was no difference between two groups in KO mice (Fig. 1H).

Taken together, these results demonstrate that the anti-obesity effect by CAR activation was abolished in the Gadd45b knockout mice.

3.2. Gadd45b is required for the insulin sensitizing effect of CAR activation

Insulin resistance, or type 2 diabetes is one of the most prevalently metabolic complications closely associated with obesity. Compared to lean subjects, the obese population is more likely to develop insulin resistance, which can be attenuated and even reversed by weight loss. Consistent with our previous report, insulin sensitivity was significantly improved in WT mice treated with TCPOBOP, as shown by both the glucose tolerance test (Fig. 2A) and insulin tolerance test (Fig. 2B). However, TCPOBOP treatment was no longer effective in improving GTT (Fig. 2C) or ITT (Fig. 2D) performances in KO mice. Areas under the curve of GTT (Fig. 2E) and ITT (Fig. 2F) were significantly lower in TCPOBOP-treated WT mice, but not in TCPOBOP-treated KO mice. These results demonstrate that the insulin-sensitizing effect of CAR activation was also Gadd45b dependent.

At the biochemical level, analysis of serum biochemistry reveals that insulin level and fasting glucose level were significantly lower in TCPOBOP-treated WT mice but there is no difference between the vehicle group and the TCPOBOP treatment group in KO mice (Table 1). Interestingly, the serum triglyceride level was not affected by TCPOBOP in either WT or KO mice (Table 1). The serum cholesterol level was comparable between WT mice treated with or without TCPOBOP, but it was deceased by TCPOBOP in KO mice (Table 1).

3.3. Gadd45b deficiency impairs TCPOBOP-responsive suppression of hepatic lipogenesis and lipogenesis, but has little effect on fatty acid β-oxidation

As expected, the CAR target gene Cyp2b10 was robustly induced by TCPOBOP in WT mice, and a similar degree of Cyp2b10 induction was observed in KO mice (Fig. 3A). The intact induction of Cyp2b10 by TCPOBOP in KO mice was consistent with the report that the attenuation of Cyp2b10 induction by TCPOBOP in KO hepatocytes was early and transient. Meanwhile, the mRNA expression of Car was reduced in TCPOBOP-treated WT mice but not in KO mice (Fig. 3B). Consistent with results published by others and us, treatment of WT mice with TCPOBOP alleviated HFD-induced hepatic steatosis as shown by oil red O staining of lipid droplets, or biochemical measurement of the liver triglyceride level (Fig. 3D). In HFD-fed KO mice, the basal oil red O staining (Fig. 3C) and liver triglyceride content (Fig. 3D) were significantly lower than their WT counterparts, and TCPOBOP was no longer effective in ameliorating hepatic steatosis. The liver cholesterol levels were not affected regardless of the Gadd45b genotype or the TCPOBOP treatment (Fig. 3E). The acetyl-coenzyme A carboxylase (ACC-1), the fatty acid synthase (FAS), and the stearoyl-CoA desaturase (SCD-1) are three key enzymes in the de novo lipogenesis in the liver. Treatment with TCPOBOP suppressed the expression of Acc-1, Fas, and Scd-1 in WT mice, but not in KO mice (Fig. 3F). We next examined whether the expression of two upstream regulators of lipogenesis insulin-induced gene 1 protein (Insig1) and sterol regulatory element-binding protein 1 (Srebp1-c) was affected or not. The results show that Insig1 mRNA expression was not changed in either WT or KO mice, whereas TCPOBOP treatment decreased Srebp1-c mRNA expression in both genotypes (Fig. 3G). Furthermore, the suppression of phosphoenolpyruvate carboxykinase (Pepck) and peroxisome proliferator-activated receptor gamma coactivator-1 alpha (Pgc1-a) observed in TCPOBOP-treated WT mice was abolished in TCPOBOP-treated KO mice, but TCPOBOP remained effective in suppressing glucose-6-phosphatase (G6Pase) gene expression in KO mice (Fig. 3H). The suppression of peroxisome proliferator-activated receptor gamma coactivator-1 alpha (Pgc1-a) observed in TCPOBOP-treated WT mice was abolished in TCPOBOP-treated KO mice, but TCPOBOP remained effective in suppressing glucose-6-phosphatase (G6Pase) gene expression in KO mice (Fig. 3H).
alpha (Ppara) and its target genes involved in β-oxidation and fatty acid influx by TCPOBOP in WT mice was similarly observed in TCPOBOP-treated KO mice (Fig. 3I), suggesting that Gadd45b was not required for the suppression of fatty acid β-oxidation by CAR activation.

3.4. Gadd45b deficiency abolishes the alleviation of inflammation in white adipose tissue (WAT) by TCPOBOP, but has little effect on the adipose lipogenesis and lipolysis

Obesity is commonly associated with a state of chronic and low-grade inflammation that contributes to insulin resistance and type 2 diabetes25,26. Treatment of WT mice with TCPOBOP reduced adiposity in epididymal WAT, and this effect was abolished in TCPOBOP-treated KO mice, as shown by H&E staining (Fig. 4A) and quantification of adipocyte size (Fig. 4B). Adipose macrophage accumulation is higher in obesity, which is tightly related to insulin resistance27,28. Additionally, macrophage infiltration in the adipose tissue and liver also plays an important role in the pathogenesis of nonalcoholic fatty liver disease (NAFLD)29. TCPOBOP-treated WT mice showed a decreased number of macrophage-enriched crown-like structures (CLS) in their WAT, compared with vehicle-treated WT mice. In vehicle-treated KO mice, the basal density of CLS was decreased, and TCPOBOP was no longer effective in reducing the CLS density (Fig. 4A and C). The same pattern of Gadd45b knockout effect was observed when the adipose expression of macrophage marker genes Cdb8 and F4/80 was measured (Fig. 4D). These results suggest that Gadd45b was essential for the effects of CAR activation on HFD-induced adipose tissue inflammation. The adipose expression of genes involved in lipogenesis (Fig. 4E) and lipolysis (Fig. 4F) was not affected by Gadd45b ablation.

Figure 1 Gadd45b is required for anti-obesity effect of CAR activation. Growth curve of male WT C57BL/6 mice (A) or Gadd45b KO mice (B) fed with HFD for 8 weeks, in the absence or presence of TCPOBOP treatment (0.5 mg/kg, intraperitoneal, once per week). Fat mass to body weight percentage (top) and lean mass to body weight percentage (bottom) in male WT C57BL/6 mice (C) or Gadd45b KO mice (D) fed with HFD for 8 weeks, in the absence or presence of TCPOBOP treatment (0.5 mg/kg, intraperitoneal, once per week). Fat mass and lean mass were determined by MRI, n = 6 for each group. (E) Food consumption of WT and KO mice fed with HFD treated with vehicle or TCPOBOP for 8 weeks. Body weight change (F), fat mass to body weight percentage (G), and lean mass to body weight (H) in male WT and KO C57BL/6 mice fed with HFD for 17 weeks. Results are presented as mean ± SD, n = 6 for each group. *P < 0.05; **P < 0.01 compared with the vehicle group.
3.5. Gadd45b ablation has little effect on TCPOBOP-responsive attenuation of HFD-induced brown adipose tissue (BAT) whitening

Brown adipose tissue contains a large number of mitochondria, where fatty acid oxidation and heat emission take place. The whitening of brown adipose tissue was evident in obese animals30. We show that TCPOBOP treatment decreased the whitening of BAT in both WT and KO mice at the histological level (Fig. 5A), which indicated that Gadd45b may not be required for the effects of CAR activation on BAT. The Gadd45b genotype and HFD had little effect on the BAT expression of genes involved in lipogenesis (Fig. 5B) and β-oxidation (Fig. 5C).

4. Discussion

Gadd45b was originally known to be involved in cell death and proliferation31. Gadd45b was later found to be a gene inducible by CAR activation and was established as a co-activator of CAR. Activation of CAR is known to promote hepatocyte proliferation and cause hepatomegaly. It was found that loss of Gadd45b impairs the early transcriptional stimulation caused by CAR activation after partial hepatectomy, and Gadd45b is required to facilitate rapid liver growth11,12. CAR and its agonists such as TCPOBOP and phenobarbital are known to be potent tumor promoters in rodents and upon the diethylnitrosamine initiation32,33. Besides being a tumor promoter in rodents, TCPOBOP has also been shown to be a non-genotoxic hepatocarcinogen34. However, it has not been reported whether Gadd45b is required for the tumor promoting effect of CAR and its agonists. It is recognized that phenobarbital was not shown as a tumor promoter in humanized CAR mice35. It appeared that CAR-mediated liver carcinogenesis is rodent specific and upon diethylnitrosamine initiation, but it does not seem to be relevant for humans36. Phenobarbital was even reported to suppresses some liver tumors in certain context37,38.

CAR is more recently known for its endobiotic functions, including its activity in attenuating obesity and type 2 diabetes. Several in vitro studies suggested that the inhibitory activities of CAR on lipogenesis and gluconeogenesis may have been due to the competitive binding of CAR to several transcription factors such as FOXO1, HNF4a, and PGC1a onto the promoter regions of gluconeogenic or lipogenic genes. However, the in vivo significance of these transcriptional factors in mediating the metabolic benefits of CAR remains unclear. In this study, we demonstrated that as a CAR coactivator, Gadd45b is required for the anti-obesity and anti-diabetic effects of CAR in vivo. The improved systemic insulin sensitivity by CAR activation was abolished in KO mice. The serum insulin lowering effect of TCPOBOP was also abolished in Gadd45b KO mice. Loss of CAR-responsive suppression of hepatic lipogenesis and gluconeogenesis may have accounted for the loss of metabolic benefits of CAR activation in Gadd45b KO mice. Another interesting

Figure 2  Gadd45b is required for the insulin sensitizing effect of CAR activation. Mice were fed with HFD for 8 weeks in the presence or absence of TCPOBOP (0.5 mg/kg, once per week) before glucose tolerance test (GTT, A) and insulin tolerance test (ITT). GTT (A) and ITT (B) in male WT C57BL/6J mice. GTT (C) and ITT (D) in male KO mice. Area under curve (AUC) of GTT (E) and ITT (F) in male WT and KO mice before GTT or ITT. Results are presented as mean ± SD, n = 6 for each group. *P < 0.05, **P < 0.01 compared with the vehicle group.

| Index          | WT Vehicle | TCPOBOP   | KO Vehicle | TCPOBOP   |
|----------------|------------|-----------|------------|-----------|
| Insulin (ng/mL) | 4.088 ± 2.613 | 0.1454 ± 0.0528** | 3.46 ± 2.031 | 2.075 ± 1.37 |
| Triglyceride (mg/dL) | 119.7 ± 22.06 | 111.6 ± 17.93 | 132.2 ± 35.88 | 99.85 ± 18.1 |
| Cholesterol (mg/dL) | 131.2 ± 16.09 | 117.4 ± 10.81 | 172.7 ± 43.11 | 118.9 ± 23.52* |
| Fasting glucose (mg/dL) | 153.8 ± 17.63 | 126.0 ± 12.88** | 114.5 ± 21.24 | 107.4 ± 21.88 |

Data are mean ± SD, n = 6. *P < 0.05, **P < 0.01 vs. Vehicle.
Figure 3  
Gadd45b deficiency impairs TCPOBOP-responsive suppression of hepatic lipogenesis and lipogenesis, but has little effect on fatty acid β-oxidation. Mice were the same as described in Fig. 1. (A) and (B) The hepatic mRNA expression of Cyp2b10 (A) and Car (B). (C) Hepatic steatosis was evaluated by oil red O staining. Scale bar, 50 μm. (D) (E) Liver triglyceride (D) and cholesterol (E) levels. (F)–(I) The hepatic mRNA expression of genes involved in lipogenesis (F) and (G), gluconeogenesis (H), and β-oxidation (I) was measured by real-time PCR. Results are presented as mean ± SD, n = 6 for each group. *P < 0.05; **P < 0.01 compared with the vehicle group.

Figure 4  
Gadd45b deficiency abolishes the alleviation of inflammation in white adipose tissue (WAT) by TCPOBOP, but has little effect on the adipose lipogenesis and lipolysis. Mice were the same as described in Fig. 1. (A) H&E staining of white adipose tissue (WAT). Scale bar = 100 μm. (B) Quantification of adipocyte size, and (C) quantification of crown-like structures (CLS). (D) The mRNA expression of macrophage marker genes and genes involved in (E) gluconeogenesis and (F) lipolysis in WAT was measured by real-time PCR. Results are presented as means ± SD, n = 6 for each group. *P < 0.05; **P < 0.01 compared with the vehicle group.
finding is that Gadd45b ablation abolished the alleviation of inflammation in WAT by TCPOBOP, which may have also contributed to the loss of metabolic benefits in KO mice. We have previously reported that WAT does not have appreciable expression of CAR10. As such, the WAT phenotype in TCPOBOP-treated KO mice was probably secondary to the loss of metabolic benefits in tissues outside of the adipose tissues.

Besides Gadd45b’s role in mediating the metabolic benefits TCPOBOP, we found that the vehicle-treated KO mice exhibited reduced liver triglyceride levels and decreased expression of lipogenic genes compared to their WT counterparts. The density of the CLS structure and the expression of adipose macrophage marker gene were also significantly decreased in vehicle-treated KO mice. These results suggested that Gadd45b may have CAR independent effects on energy metabolism. Since intestinal microbiota has been shown to play a vital role in HFD-induced obesity41, we cannot exclude the possibility that changes in intestinal microbiota may have also contributed to the phenotypic exhibition.

Besides GADD45B, several other CAR coactivators of CAR, such as SRC1, SRC2, and PGC1a42,43, have also been identified based on mammalian two-hybrid experiment and reporter gene assays. However, whether these coactivators contribute to the anti-obesity effects of CAR in vivo is not known. Meanwhile, we recently reported that Gadd45b can also function as a coactivator for another xenobiotic receptor aryl hydrocarbon receptor (AHR). Interestingly, Gadd45b was not required for the promotion of liver carcinogenesis by AHR activation44.

There are several limitations of our study: 1) Mice of the C57BL background were used. It remains unclear whether the phenotype was strain dependent; 2) Only male mice were used. Although we have previously reported that the anti-obesity effects of TCPOBOP was not sex-specific10, we cannot exclude the possibility that the phenotype of Gadd45b ablation was sex specific; and 3) The mechanism by which Gadd45b contributes to the metabolic benefits of CAR activation is yet to be defined. Previous reports have shown that CAR could compete with HNF4a for binding to the promoters of gluconeogenic genes, or facilitate the degradation of PGC1a. It will be interesting to investigate whether Gadd45b affects the interaction between CAR and HNF4a or PGC1a, and thus conveying metabolic benefits.

In summary, we demonstrated that Gadd45b as a coactivator of CAR is required in part for the metabolic benefits of CAR activation in vivo.

Acknowledgment

Wen Xie was supported in part by the Joseph Koslow Endowed Professorship from the University of Pittsburgh School of Pharmacy (Pittsburgh, PA, USA).

Author contributions

Xinran Cai, Ye Feng, and Wen Xie designed the study, analyzed the data, and drafted the original manuscript. Xinran Cai, Ye Feng, and Meishu Xu conducted the experiments. Xinran Cai, Ye Feng, Chaohui Yu, and Wen Xie reviewed and finalized the manuscript.

Conflicts of interest

Ye Feng was a visiting scholar at the University of Pittsburgh in the laboratory of Wen Xie from April 2017 to March 2019, during which Ye Feng was a graduate student of Zhejiang University First Affiliated Hospital (Hangzhou, China) under the supervision of Chaohui Yu. The authors declare no conflict of interest.

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