The effect of clozapine on the AMPK-ACC-CPT1 pathway in the rat frontal cortex

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Abstract

Clozapine is an antipsychotic drug that has a greater efficacy than other medications in some contexts, especially for the treatment of treatment-resistant schizophrenia. However, clozapine induces more metabolic side-effects involving abnormality in lipid metabolism compared to other antipsychotics. AMP-activated protein kinase (AMPK) plays a central role in controlling lipid metabolism through modulating the downstream acetyl CoA carboxylase (ACC) and carnitine palmitoyl transferase 1 (CPT1) pathway. In this study, we investigated the effect of a single intraperitoneal injection of clozapine on the AMPK-ACC-CPT1 pathway in the rat frontal cortex, which has been implicated as a target site for this antipsychotic drug. At 2 h after injection, the clinically relevant dose of clozapine had activated AMPK, with increased phosphorylation of AMPKα at Thr172, and had inactivated ACC, with increased phosphorylation of ACC at Ser79. In addition, clozapine activated the brain-specific isoform of CPT1, CPT1c, whose activity is inhibited by unphosphorylated ACC, in the rat frontal cortex. Immunohistochemistry and immunofluorescence analysis showed that clozapine induced an increase in number of p-AMPKα (Thr172)- and p-ACC (Ser79)-positive cells among the neurons of the rat frontal cortex. Taken together, these results show that the antipsychotic agent clozapine affects the lipid regulatory system of neurons in the brain.

Received 19 March 2011; Reviewed 25 April 2011; Revised 25 May 2011; Accepted 25 May 2011; First published online 28 June 2011

Key words: Acetyl CoA carboxylase, AMP-activated protein kinase, antipsychotics, carnitine palmitoyltransferase 1, metabolic signal pathway.

Introduction

Clozapine is known to have a superior efficacy in the treatment of treatment-resistant schizophrenia (Kane et al. 1998; Lewis et al. 2006; McEvoy et al. 2006; Mortimer et al. 2010). However, clozapine is also known to induce a higher prevalence of metabolic syndrome (MetS) involving abnormalities in lipid metabolism such as hypertriglyceridaemia and hypercholesterolaemia, resulting in weight gain and hypertension compared to other antipsychotics (Allison et al. 1999; Casey, 2004; Henderson et al. 2004; Meyer & Koro, 2004). These findings suggest that clozapine might affect the lipid regulatory intracellular signal pathway, which requires further clarification.

AMP-activated protein kinase (AMPK) is a serine-threonine kinase that is known to be a major metabolic sensor that plays a role in maintaining metabolic homeostasis (Carling, 2004; Hardie et al. 1989). In mammals, the activation of AMPK occurs primarily through the phosphorylation of its catalytic α-subunit at Thr172 residue by liver kinase B1 (LKB1) or by...
Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase \(\beta\) (CaMKK\(\beta\)) (Hawley et al. 2003; Woods et al. 2005). A number of physiological processes leading to alterations in the intracellular AMP/ATP ratio or the calcium concentration, such as hypoxia, hypoglycaemia, and excitotoxicity, have been shown to stimulate AMPK (Kola et al. 2006). Once activated, AMPK inhibits anabolic pathways and promotes catabolic pathways. AMPK participates in various physiological processes through phosphorylating its downstream targets (Zhang et al. 2009).

AMPK plays a central role in controlling lipid metabolism through modulating the downstream acetyl CoA carboxylase (ACC) and carnitine palmitoyl transferase 1 (CPT1) pathway (Ronnell et al. 2006). The phosphorylation of ACC at Ser\(^{79}\) by AMPK leads to the inactivation of ACC (Hardie & Carling, 2002), and the level of Ser\(^{79}\) phosphorylation is commonly used as an in-vivo measure of AMPK signalling in response to various stimuli (Carling et al. 1987). ACC catalyses the production of malonyl CoA, which is both a major building block for de-novo fatty-acid synthesis and an allosteric inhibitor of CPT1 (Hardie & Carling, 1997). CPT1, which is inhibited by unphosphorylated ACC, is the rate-limiting enzyme that transfers the long-chain fatty acyl CoA to the mitochondria for beta oxidation (Ruderman et al. 2003). Of the three isoforms of CPT1, only CPT1a, localized in the mitochondria, and CPT1c, localized in the endoplasmic reticulum (ER), are expressed in the brain. CPT1a is expressed ubiquitously throughout the body, and CPT1c is only expressed in the brain (Sierra et al. 2008).

The effect of clozapine on the AMPK-ACC-CPT1 signalling pathway remains unclear. There are a few reports about the effect of clozapine on the AMPK-ACC pathway. The studies have demonstrated that there are increased p-AMPK levels in the mouse hypothalamus after clozapine injection (Kim et al. 2007), increased p-AMPK and p-ACC levels in C2C12 cells after a single clozapine treatment (Kim et al. 2010a), and no effect on AMPK activity in PC12 cells (Takami et al. 2010). There has been little research on the activity of CPT1 after clozapine treatment.

The AMPK-ACC-CPT1 pathway is found throughout the body, including brain regions (Coccurello & Moles, 2010; Dwyer et al. 2005; Kim et al. 2007). Previous studies have suggested organ- and tissue-dependent roles for the AMPK-ACC-CPT1 pathway: controlling gluconeogenesis in the liver (Yamauch et al. 2002); regulating insulin secretion in pancreatic \(\beta\) cells (da Silva Xavier et al. 2003); adjusting lipolysis in adipocytes (Gaidhu et al. 2006); and regulating food intake in the hypothalamus (López et al. 2008). These results suggest the possibility of a tissue-specific role for the AMPK-ACC-CPT1 pathway.

The frontal cortex has been suggested as a target site for the action of antipsychotics (Artigas, 2010; MacDonald et al. 2005). In addition, alteration of energy metabolism in the frontal cortex of schizophrenia patients has been reported (Hazlett et al. 2000; Martins-de-Souza et al. 2009; Middleton et al. 2002). As mentioned above, clozapine has been reported to affect AMPK activity in the hypothalamus and muscle cells, which have been suggested to be involved in MetS induced by clozapine. Outside of these possible implications, we investigated whether the systemic administration of clozapine affected AMPK and its downstream signalling pathway related to the lipid regulatory system in the frontal cortex, a possible target region of antipsychotics.

In this study, we investigated the changes in activity of the AMPK-ACC-CPT1 pathway in the rat frontal cortex following a single intraperitoneal (i.p.) injection of clozapine. We found that clozapine activated AMPK and inactivated its downstream target, ACC. In addition, clozapine activated CPT1c, whose activity is inhibited by ACC, in the rat frontal cortex.

Methods

Animals and drugs

Male Sprague–Dawley rats (200–250 g) were housed under a 12-h light/dark cycle (lights on 08:00 hours) with food and water available ad libitum. Animals were treated in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Clozapine (Tocris, USA), dissolved in vehicle (0.3% tartaric acid in distilled water), was injected i.p. into each rat in a volume of 1 ml. Control animals received the equivalent volume of vehicle. The rats were randomly assigned to either the clozapine group or the control group. Clozapine doses of 5, 10, or 20 mg/kg were used, and all drugs were adjusted to pH 6.0–6.5 with NaOH. The dose of 5–20 mg/kg was selected based on our previous study (Roh et al. 2007) and on clinical relevance determined by the relationship among drug dose, plasma levels, and in-vivo dopamine D\(_{2}\) receptor occupancy (Kapur & Mamo, 2003).

Immunoblot analysis

The rats were decapitated at the designated time after (i.p.) injection of clozapine or vehicle, and the frontal cortex was dissected and homogenized as described previously (Kim et al. 2008). After centrifugation at
20,000 g for 20 min at 4 °C, the supernatants were boiled with Laemmli sample buffer. The immunoblot analysis was performed as described previously (Kim et al. 2008). The membranes were incubated with primary antibodies overnight at 4 °C, followed by a second incubation with anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Jackson Immuno Research Laboratories Inc., USA). Primary antibodies specific for p-AMPKa (Thr172), AMPKa, p-ACC (Ser79), ACC (Cell Signaling Technology, USA), p-CaMK4 (Thr172), CaMK4, p-LKB1 (Ser416), LKB1 (Santa Cruz Biotechnology, USA), α-tubulin, β-actin (Sigma-Aldrich, USA), and SOD2 (Abcam, USA) were used. The membranes were developed using the enhanced chemiluminescence system (Pierce Biotechnology, USA).

**Immunohistochemistry**

Different animals from those used in the immunoblotting experiments were used. Rats were anaesthetized with an i.p. injection of 1.5 g/kg urethane (Sigma-Aldrich) 10 min before laparotomy, which was performed 2 h after clozapine injection. Each rat was transcardially perfused with 0.1 M PBS (pH 7.4) and then fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS. Brains were immediately removed and cryoprotected in 30% sucrose (Sigma-Aldrich) in PBS. The sections were incubated in DAB substrate and subsequently mounted with DPX mountant (Biomeda, USA), sections on slides were analysed using a Meta confocal microscope (Model LSM 510; Carl Zeiss Micro Imaging Inc., Germany).

**CPT1 activity assay**

The homogenized brain tissues were fractionated into mitochondrial and non-mitochondrial cytoplasmic proteins using a Mitochondria Isolation kit (Pierce Biotechnology) according to the manufacturer’s instructions. The mitochondrial and cytoplasmic fractions were used for CPT1a and CPT1c activity assays, respectively. Fractionated proteins were quantified using the BCA protein assay method (Pierce Biotechnology). CPT1 activity was measured by the method described by Shin et al. (2006a). CPT1 activity was determined spectrophotometrically by following the release of CoA-SH from palmitoyl CoA using DTNB [5,5′-dithio-bis-(2-nitrobenzoic acid)]. Fractionated proteins (20 μl) were incubated with 200 μl reaction buffer containing 2 mM (indicating final concentration) DTNB, 116 mM Tris–HCl (pH 8.0), 2.5 mM EDTA, and 0.2% Triton-X 100 at room temperature in 96-well plates (SPL Life Science, Korea) for 20 min to eliminate all of the reactive thiol groups pre-existing in the fractionated proteins. After incubation, the absorbance was measured at 412 nm using a Versa Max microplate reader ( Molecular Devices, USA). To start the reaction, 100 μM of freshly mixed palmitoyl CoA (Sigma-Aldrich) in distilled water and 1 mM carnitine (Sigma-Aldrich) in 1 M Tris (pH 8.0) were added to the reaction mixtures. The reaction mixtures were incubated for 10 min at 37 °C. After incubation, the absorbance at 412 nm was measured again. The difference between the absorbances with and without substrates was used as a measure of the release of CoA-SH. The activity was defined as the absorbance at 412 nm/mg protein.

**Statistical analysis**

Immunoblot signals on developed X-ray films were quantified with TINA 2.10 G (Raytest, Germany), and the results were translated into optical densities (OD). The mean of OD values of the clozapine-treated group was compared to that of the control group using...
one-way analysis of variance (ANOVA) followed by LSD post-hoc test. To compare the mean values of the control group with those of the clozapine-treated group for the immunohistochemistry and CPT1 activity assay data, an independent t test was performed. p values < 0.05 were considered statistically significant. All tests were performed using SPSS 17.0 for Windows (SPSS Inc., USA).

Results

To investigate the effect of clozapine on AMPK activity in rat frontal cortex, immunoblot analysis was performed. AMPK activity was determined by measuring the phosphorylation level of the AMPKα subunit at Thr172, which reflects the activation of AMPK (Hawley et al. 1996). A single injection of clozapine induced dose- and time-dependent changes in the phosphorylation level of AMPKα (Thr172). After 30 min, no significant changes were found in the phosphorylation of AMPKα (Thr172) (F=0.224, d.f.=3, n.s.). The phosphorylation level of AMPKα (Thr172) was significantly different at 1 h (F=6.900, d.f.=3, p<0.01); it was significantly higher after injection of 5, 10, or 20 mg/kg clozapine (p<0.01, p<0.01, p<0.05, respectively). At 2 h after clozapine injection, the phosphorylation level of AMPKα (Thr172) was also significantly altered (F=10.481, d.f.=3, p<0.001); again, 5, 10, and 20 mg/kg clozapine significantly increased the phosphorylation level (p<0.05, p<0.001, p<0.001, respectively). At 4 h (F=4.219, d.f.=3, p<0.05), the phosphorylation of AMPKα (Thr172) was significantly increased by 20 mg/kg clozapine (p<0.05) (Fig. 1).

We next examined the effect of clozapine on the change in the activity of ACC and CPT1 2 h after clozapine injection. We observed the effect of clozapine 2 h after injection because as this time-point showed, the p-AMPKα (Thr172) level of the clozapine-treated group was more than 200% higher than that of the vehicle-treated group under our experimental conditions. ACC activity was determined by measuring the phosphorylation level of ACC at Ser79 where AMPK phosphorylates (Boone et al. 1999). As shown by the immunoblot analysis, the phosphorylation level of ACC (Ser79) was significantly increased by 20 mg/kg clozapine (p<0.05) (Fig. 1).

![Fig. 1. Time- and dose-dependent changes in the phosphorylation level of AMPKα (Thr172) in the rat frontal cortex after clozapine injection. (a) Representative immunoblots of the rat frontal cortex at 30 min, 1, 2, and 4 h after injection of clozapine at the indicated doses. (b) Quantification of the immunoblot data using densitometric analysis of the intensity of the p-AMPKα (Thr172) bands. Data are expressed as relative optical densities (OD) that are percentages of the OD of each vehicle-treated control group. The bars express the p-AMPKα (Thr172) level of the clozapine-treated group relative to that of the vehicle-treated group. The data are shown as the mean ± S.E.M. (* p<0.05, one way ANOVA, n=3–5/group).](https://academic.oup.com/ijnp/article-abstract/15/7/907/638793/data/638793.html)
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Fig. 2. Effect of clozapine injection on the phosphorylation level of ACC (Ser79) and the activity of CPT1 in the rat frontal cortex. (a) Representative immunoblots of the rat frontal cortex at 2 h after injection of clozapine at the indicated doses. (b) The bars express p-ACC (Ser79) levels of vehicle- or clozapine-treated groups expressed as relative optical densities (OD) that are percentages of the OD of each vehicle-treated control group. The data represent the mean ± S.E.M. Data were processed as described in the legend of Fig. 1 (*p < 0.05, one way ANOVA, n = 3–5/group). (c) Bars express CPT1α or CPT1c activity 2 h after injection of clozapine (10 mg/kg) or vehicle expressed as a percentage of the activity of each vehicle-treated control group. Values are expressed as mean ± S.E.M. Subcellular fractionation was confirmed by immunoblot analysis with cytoplasmic marker, α-tubulin, and mitochondrial marker, SOD2 (*p < 0.05, t test, n = 7/group).

fraction was not affected by the clozapine injection (t = 0.555, d.f. = 12, n.s.) (Fig. 2c). Subcellular fractionation was confirmed by immunoblot analysis with antibodies specific for α-tubulin and superoxide dismutase 2 (SOD2), as a non-mitochondrial cytoplasmic marker and a mitochondrial marker, respectively (Fig. 2c, bottom panels).

To further specify the location at which clozapine treatment affects the AMPK-ACC-CPT1 signalling pathway in the rat frontal cortex, immunohistochemistry and double-label immunofluorescence analysis were performed. At 2 h after injection of 10 mg/kg clozapine, immunohistochemistry showed that the density of p-AMPKα (Thr172)-positive cells was significantly increased in clozapine-treated rats compared to vehicle-treated controls in the prefrontal (t = 5.670, d.f. = 8, p < 0.001), orbital (t = 3.968, d.f. = 8, p < 0.01), insular (t = 4.077, d.f. = 8, p < 0.01), and cingulate (t = 3.278, d.f. = 8, p < 0.05) cortices (Paxinos & Watson, 1998) (Fig. 3a). The number of p-ACC (Ser79)-positive cells was also significantly increased with clozapine injection in the prefrontal (t = 3.318, d.f. = 8, p < 0.05), orbital (t = 5.627, d.f. = 8, p < 0.01), insular (t = 5.909, d.f. = 8, p < 0.001), and cingulate (t = 3.462, d.f. = 8, p < 0.01) cortices (Fig. 3b). In addition, double-label immunofluorescence analysis revealed that the immunofluorescence of p-AMPKα (Thr172) or p-ACC (Ser79) 2 h after injection of 10 mg/kg clozapine was co-localized with that of Neu-N, a marker of neuronal cells, but not with that of GFAP, a marker of glial cells (Fig. 3c, d).

To investigate the upstream regulators involved in clozapine-induced AMPK-ACC-CPT1 signalling, the activity of LKB1 and CaMKKβ was examined. The activity of LKB1 was determined by measuring the phosphorylation of LKB1 at Ser431 (Sapkota et al. 2001), and the activity of CaMKKβ was determined by measuring the phosphorylation level of CaMK4 at Thr194, which reflects CaMKKβ activity (Swulius & Waxham, 2008). The phosphorylation levels of LKB1 (Ser431) (F = 7.239, d.f. = 3, p < 0.01) and CaMK4 (Thr194) (F = 7.581, d.f. = 3, p < 0.01) were significantly affected 2 h after clozapine injection. The phosphorylation levels of LKB1 (Ser431) was significantly increased after injection of 10 and 20 mg/kg clozapine (p < 0.05, p < 0.01, respectively), and that of CaMK4 (Thr194) was also significantly increased after injection of 10 and 20 mg/kg clozapine (p < 0.01 for both) (Fig. 4).
Discussion

In this study, at 2 h after injection, a clinically relevant dose of clozapine activated AMPK, as demonstrated by the increased phosphorylation of AMPKα at Thr\textsuperscript{172}, and inactivated its downstream target, ACC, as demonstrated by the increased phosphorylation of ACC at Ser\textsuperscript{79}. In addition, clozapine activated CPT1, whose activity is inhibited by ACC in the rat frontal cortex. Clozapine induced an increase in the number of p-AMPKα (Thr\textsuperscript{172})- and p-ACC (Ser\textsuperscript{79})-positive cells in the neurons of the rat frontal cortex, including prefrontal, orbital, insular, and cingulate cortices. Clozapine also activated two kinases upstream of AMPK, LKB1 and CaMKKβ as demonstrated by the increased phosphorylation levels of LKB1 (Ser\textsuperscript{431}) and CaMK4 (Thr\textsuperscript{196}).

The activation of AMPK by clozapine has been reported in the mouse hypothalamus (Kim et al. 2007) and C2C12 myotube cells (Kim et al. 2010a). These studies suggested that the role of clozapine induced-activation of AMPK might control weight gain in the
hypothalamus or regulate glucose uptake in skeletal muscle. In this study, we demonstrated the activation of AMPK by clozapine in the rat frontal cortex, along with inhibition of ACC and the activation of CPT1 (Figs 1 and 2). AMPK plays a central role in lipid metabolism via inhibiting ACC and activating CPT1 through release of its inhibition by ACC (Ronnett et al. 2006). The frontal cortex has been suggested as a target site for the action of antipsychotics (Artigas, 2010; MacDonald et al. 2005). In addition to the implications regarding the possible role of clozapine on the AMPK pathway in the hypothalamus and muscle cells in weight gain and glucose metabolism, this finding in the frontal cortex could suggest that clozapine controls the lipid-related regulatory system in the frontal cortex.

Of the isoforms of CPT1 present within the brain, clozapine only increased the activity of the brain-specific isoform of CPT1, CPT1c; clozapine had little effect on CPT1a in the rat frontal cortex (Fig. 2c). The traditional role of CPT1, i.e. the roles of CPT1a and CPT1b, is to increase the capacity of fatty acid flux into the mitochondria and to improve insulin sensitivity and glucose utilization by eliminating unoxidized fatty acids (Bruce et al. 2009; Folmes & Lopaschuk, 2007). Although the role of CPT1c is far from fully elucidated, it has been suggested that CPT1c might alter the synthesis of ceramide and sphingolipids in neurons through facilitating the entry of palmitoyl-CoA to the ER lumen (Sierra et al. 2008; Washington et al. 2003). The synthesis of ceramide and sphingolipids affects the lipid composition of the cell membrane, changing the environment and the activity of the membrane receptors (Chattopadhyay & Paila, 2007). Therefore, this finding suggests that clozapine might affect the lipid compositions of the neuronal membranes in the frontal cortex through increasing the activity of CPT1c.

The activation of the AMPK pathway by clozapine was mainly observed in neurons of the rat frontal cortex (Fig. 3). This seems to be because AMPK is expressed at a much higher level in neurons than in glia (Ramamurthy & Ronnett, 2006). Within neurons, AMPK maintains metabolic homeostasis via phosphorylation of its downstream targets (Zhang et al. 2009). In addition to its metabolic function, emerging
evidence has demonstrated novel roles of AMPK in neurons. For instance, AMPK has been reported to protect neurons after ischaemia (Kuramoto et al. 2007), to regulate brain development (Dasgupta & Milbrandt, 2009), and to modulate neuronal plasticity (Potter et al. 2010). Thus, although we focused on the AMPK-ACC-CPT1 pathway, it is necessary to further investigate the effect of clozapine on other downstream targets of AMPK.

Although there has been a report that clozapine activates AMPK only by CaMKKβ not by LKB1 in C2C12 myotube cells (Kim et al. 2010a), the clozapine-induced activation of AMPK signalling pathway in the in-vivo rat frontal cortex seems to be influenced not by a single, but by both upstream pathways of AMPK under our experimental conditions because clozapine activated both LKB1 and CaMKKβ similarly with the pattern of AMPK (Figs 1 and 4). p-LKB1 (Ser431) is regulated by PKA and p90 ribosomal s6 kinase (Sapkota et al. 2001), and p-CaMK4 (Thr286) is regulated by CaMKKβ, which is activated by calcium influx (Swulius & Waxham, 2008). Since clozapine has been reported to act on upstream pathways of LKB1 and CaMKKβ, such as the cAMP-dependent pathways (Bateup et al. 2008; Ma et al. 2006; Pozzi et al. 2003) and the Ca²⁺/CaM-dependent pathways (Rushlow et al. 2009; Shin et al. 2006b), activation of AMPK by clozapine from both pathways might be possible. In order to prove this hypothesis, the direct causal relationship among these molecules should be further examined.

In this study, we demonstrated that clozapine changes the activity of the AMPK-ACC-CPT1 pathway in the rat frontal cortex, this pathway is known to play a central role in lipid metabolism. This result suggests that clozapine might affect the lipid regulatory system, not only in peripheral tissues but also in the brain. Although the role of the AMPK-ACC-CPT1 pathway in the frontal cortex is still poorly understood, we hypothesize that clozapine might affect the lipid compositions of the neuronal membrane in the frontal cortex by increasing the activity of CPT1c. Interestingly, abnormal membrane lipid composition has been reported in the frontal cortex in individuals with schizophrenia (Horrobin et al. 1991; Narayan et al. 2009; Pearce et al. 2009). The activation of AMPK-ACC-CPT1c signalling by clozapine in the frontal cortex might contribute to the understanding of its therapeutic efficacy against schizophrenia.

As noted above, previous studies on AMPK activation by clozapine have suggested its roles related to MetS: controlling weight gain in the hypothalamus (Kim et al. 2007) and regulating glucose uptake in skeletal muscle (Kim et al. 2010a). Further investigations are necessary to elucidate whether clozapine’s effects on the AMPK-ACC-CPT1c signalling pathway in the frontal cortex might be involved in the therapeutic action of antipsychotics other than the previously suggested involvement of clozapine-induced MetS. To examine the exact role of the AMPK pathway in clozapine’s effects on peripheral organs and the brain, chronic experiments reflecting long-term changes are required. Moreover, extended investigations using other antipsychotic drugs such as haloperidol, with a lower liability of inducing MetS, and olanzapine, with a higher risk of MetS, are necessary to determine whether these findings are related only to MetS liable antipsychotics or common signalling effects for various antipsychotics.

In conclusion, we found that a single i.p. injection of clozapine activated CPT1c through the AMPK-ACC signalling pathway in neurons of the rat frontal cortex, and this activation of the AMPK-ACC signalling pathway seemed to be mediated by both LKB1 and CaMKKβ pathways. These findings indicate that the antipsychotic agent clozapine affects the lipid metabolic regulatory system in the brain. These results provide further insight into the working mechanism of clozapine.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0012160), and by a grant (A080534) of the Korea Healthcare technology R&D Project, Ministry for Health, Republic of Korea.

The English in this manuscript has been checked by at least two professional editors, both native speakers of English [for a certificate, see: www.journalexperts.com/certificate (2D58-D2B6-C6F7-44F3-A24A)].

Statement of Interest

None.

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