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Evogliptin, a Dipeptidyl Peptidase-4 Inhibitor, Attenuates Renal Fibrosis Caused by Unilateral Ureteral Obstruction in Mice

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Renal fibrosis is considered to be the final common outcome of chronic kidney disease. Dipeptidyl peptidase-4 (DPP-4) inhibitors have demonstrated protective effects against diabetic kidney disease. However, the anti-fibrotic effect of evogliptin, a DPP-4 inhibitor, has not been studied. Here, we report the beneficial effects of evogliptin on unilateral ureteral obstruction (UUO)-induced renal fibrosis in mice. Evogliptin attenuated UUO-induced renal atrophy and tubulointerstitial fibrosis. Immunohistochemistry and Western blotting demonstrated that evogliptin treatment inhibits pro-fibrotic gene expressions and extracellular matrix production. *In vitro* findings showed that the beneficial effects of evogliptin on renal fibrosis are mediated by inhibition of the transforming growth factor-β/Smad3 signaling pathway. The present study demonstrates that evogliptin is protective against UUO-induced renal fibrosis, suggesting that its clinical applications could extend to the treatment of kidney disease of non-diabetic origin.

INTRODUCTION

Diabetic kidney disease (DKD) is a leading cause of chronic kidney disease (CKD), and a considerable number of cases of CKD progress to end-stage renal disease (ESRD) [1]. Renal fibrosis is a major pathologic finding of ESRD and is characterized by extracellular matrix (ECM) accumulation induced by activation of the transforming growth factor-β (TGF-β)/Smad signaling pathway. It is generally accepted that TGF-β/Smad signaling induces the expression of pro-fibrotic genes, such as

α-smooth muscle actin (α-SMA), plasminogen activator inhibitor-1 (PAI-1), and type 1 collagen [2]. For this reason, inhibition of TGF-β/Smad signaling is considered a promising therapeutic strategy to delay the development of renal fibrosis [3].

Dipeptidyl peptidase-4 (DPP-4) inhibitors reduce blood glucose levels by decreasing the degradation of glucagon-like peptide-1 [4]. In addition to their glucose-lowing effect, previous studies have revealed the anti-fibrotic effect of DPP-4 inhibitors in various organ systems, including heart, lung, liver, and

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kidney [5-8].

Evogliptin is a novel DPP-4 inhibitor and has a low potential for interaction with other drugs [9]. However, few reports have described the effects of evogliptin on renal tubulointerstitial fibrosis. Thus, we investigated whether evogliptin has a protective effect against renal fibrosis following unilateral ureteral obstruction (UUO) in mice, a well-characterized animal model of renal fibrosis. We also evaluated whether evogliptin inhibits the TGF-β/Smad3 signaling pathway *in vitro*.

METHODS

To generate UUO-induced renal fibrosis, the left ureter of C57BL/6J mice was doubly ligated, as previously described [10]. To investigate the effect of evogliptin at early and late time points, mice were into two groups; those destined to be sacrificed on day 7 and those destined to be sacrificed on day 11 after UUO. For each group, mice were randomly divided into three subgroups: Sham operation+vehicle (*n*=6), UUO+vehicle (*n*=6), and UUO+evogliptin (*n*=6). Evogliptin at a dose of 300 mg/kg (Dong-A ST Co., Ltd., Seoul, Korea) [11] or vehicle was orally administered from 3 days before UUO and on each day until they were scarified at 7 or 11 days after UUO. All procedures were performed in accordance with the guidelines specified by the Committee on Laboratory Animal Ethics, Kyungpook National University (KNU 2017-0010, Daegu, Korea) [12]. Other experimental methods are described in Supplementary Materials.

RESULTS

Evogliptin attenuates UUO-induced renal fibrosis and inhibits pro-fibrotic gene expression

First, we investigated whether evogliptin ameliorates UUO-induced renal tubulointerstitial fibrosis by performing hematoxylin and eosin (H&E) and Sirius red staining. On day 11 after UUO, UUO induces prominent renal tubular atrophy and tubulointerstitial fibrosis. However, evogliptin-treated kidneys showed significant attenuation of both these pathological features (Fig. 1A). To evaluate the mechanism by which evogliptin attenuates UUO-induced renal fibrosis and ECM accumulation, we examined the effects of evogliptin on TGF-β/Smad3 signaling, which is a key mediator of renal fibrosis caused by UUO. Immunohistochemistry revealed that for phosphorylated Smad3, PAI-1, fibronectin, α-SMA, and type I collagen were markedly increased in the UUO kidneys, whereas these effects were ameliorated by evogliptin (Fig. 1B). These inhibitory effects of evogliptin on pro-fibrotic gene expression and ECM production were further evaluated by Western blot analysis. Consistent with the result of immunohistochemistry, the protein levels of phosphorylated Smad3, PAI-1, fibronectin, α-SMA, and type I collagen were lower in evogliptin-treated UUO kidneys than in vehicle-treated kidneys (Fig. 1C). We have also observed weak tubulointerstitial fibrosis 7 days after UUO, which was partially but significantly reduced by evogliptin treatment (Supplementary Fig. 1). As shown in Supplementary Figs. 2 and 3, body weight and food intake decreased
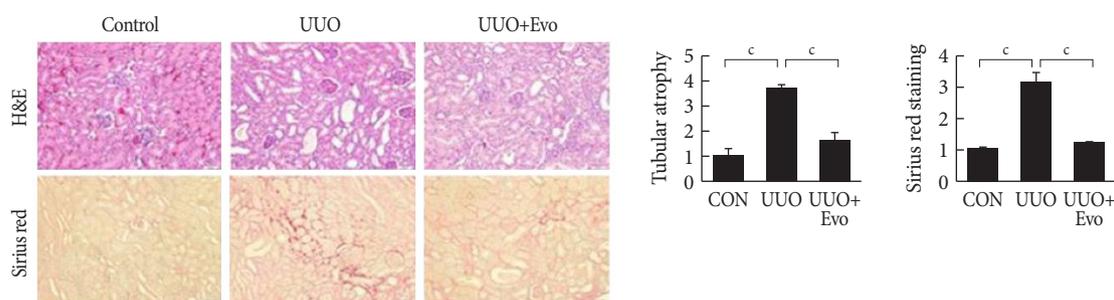
Fig. 1. Effects of evogliptin (Evo) on renal fibrosis and pro-fibrotic gene expression in kidneys of unilateral ureteral obstruction (UUO) mice. A UUO kidney from a mouse at day 11. (A) Representative images of hematoxylin and eosin (H&E) and Sirius red staining of kidney tissue sections from control (CON) mice and UUO mice treated with (300 mg/kg) or without Evo. The number of atrophic tubules was determined by measuring the amount of abnormal irregular and dilated tubular basement membranes in H&E-stained sections under high power magnification (×200). Areas of positive staining with Sirius red were quantitated by computer-based morphometric analysis. All morphometric data were normalized against the corresponding val-ues in CON animals. Data in all bar graphs are expressed as fold increases relative to the CON (*n*=6 in each group).   (*Continued to the next page*)
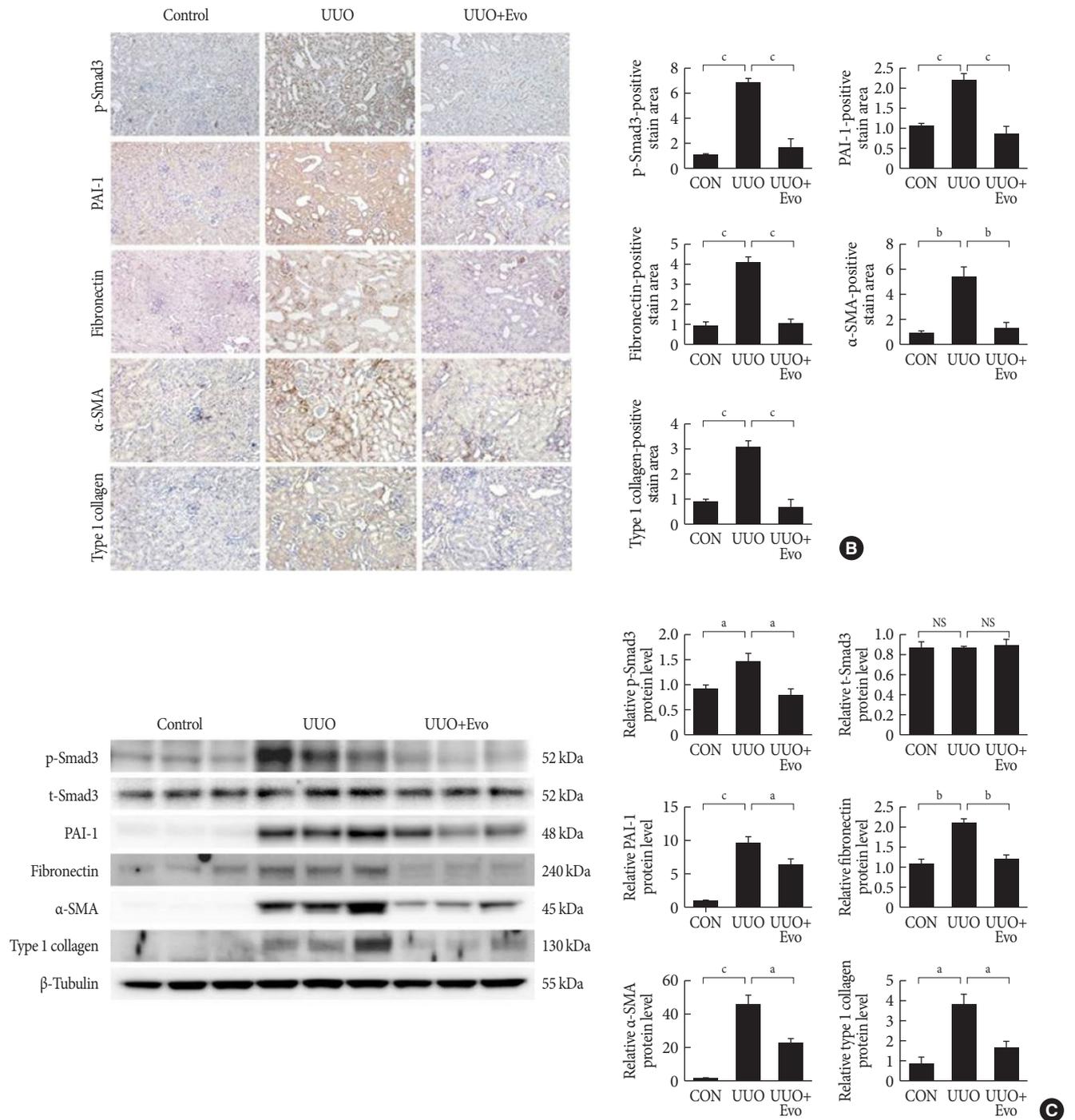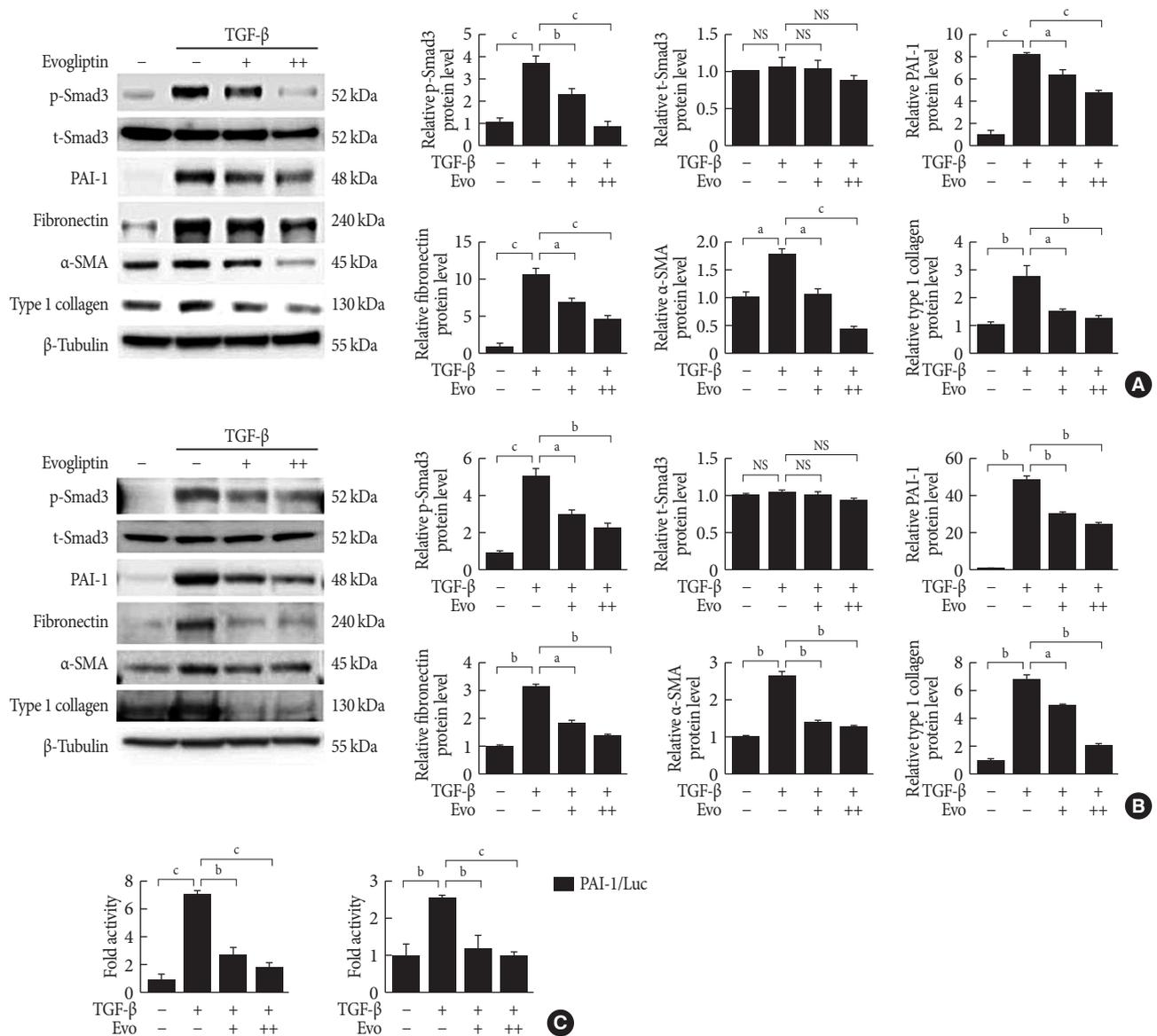
Fig. 1. Continued. (B) Representative images of immunohistochemical staining for phosphorylated-Smad3 (p-Smad3), plasmino-gen activator inhibitor 1 (PAI-1), fibronectin, α-smooth muscle actin (α-SMA), and type I collagen in kidney tissue sections from CON mice or UUO mice treated with (300 mg/kg) or without Evo (n=6 in each group). Areas of positive staining with p-Smad3, PAI-1, fibronectin, α-SMA, and type I collagen antibodies were quantitated by computer-based morphometric analysis. Data are the mean±standard error of the mean (SEM) of five random fields from each kidney. (C) Representative western blot analysis of p-Smad3, total-Smad3 (t-Smad3), PAI-1, fibronectin, α-SMA, and type I collagen protein level in UUO kidneys from mice treated with (300 mg/kg) or without Evo (n=6 in each group). Data in the bar graphs are the mean±SEM. NS, not significant. [a]P<0.05, [b]P<0.01, [c]P<0.001.

at 3 days after UUO but fully recovered at 7 days after UUO. Blood glucose levels did not change after UUO or after evogliptin treatment in either the early or late time groups after UUO, suggesting that the anti-fibrotic effect of evogliptin was independent of its glucose-lowering effect. The kidney weights

did not differ between groups.

Evogliptin attenuates pro-fibrotic molecules via suppression of TGF-β/Smad3 signaling
To determine whether the anti-fibrotic effect of evogliptin is

Fig. 2. Effects of evogliptin (Evo) on transforming growth factor-β (TGF-β)-induced pro-fibrotic gene expression in cultured kidney cell lines. Representative Western blot analysis of phosphorylated-Smad3 (p-Smad3), total-Smad3 (t-Smad3), plasminogen activator inhibitor 1 (PAI-1), fibronectin, α-smooth muscle actin (α-SMA), and type I collagen protein level in TGF-β-stimulated (A) human proximal renal tubular epithelial (HK-2) cells and (B) normal rat kidney fibroblasts (NRK-49F) cells. Cells were incubated with TGF-β (5 ng/mL) treated with (100 or 200 μg/mL) or without Evo co-treatment for 24 hours. Data are the mean±standard error of the mean (SEM) of three independent measurements. (C) Effects of Evo on PAI-1 promoter activity in NRK-49F and NRK-52E cells. Cells were treated with TGF-β (5 ng/mL) treated with (100 μg/mL) or without Evo co-treatment for 24 hours. Data are the mean±SEM of three independent measurements. NS, not significant; Luc, luciferase. [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$.

mediated by effects on TGF-β-induced pro-fibrotic gene expression, we investigated whether evogliptin suppresses TGF-β-induced Smad3 phosphorylation, PAI-1, fibronectin, α-SMA, and type I collagen abundance in cultured renal cells (human proximal renal tubular epithelial [HK-2] and normal rat kidney fibroblast [NRK-49F] cells). In accordance with the *in vivo* findings, evogliptin inhibited TGF-β-stimulated Smad3 phosphorylation and upregulation of PAI-1, fibronectin, α-SMA, and type I collagen in renal tubular and fibroblast cells (Fig. 2A and B). We further examined whether evogliptin inhibits TGF-β/Smad3 signaling at the transcriptional levels by measuring PAI-1 luciferase activity, and, indeed, evogliptin treatment inhibited TGF-β-stimulated PAI-1 promoter activity in both NRK-49F and NRK-52E cells (Fig. 2C). These findings suggested that renoprotective effect of evogliptin is mediated by downregulation of Smad3 phosphorylation. Finally, we investigated the effect of evogliptin on plasma and renal DPP-4 activity after UUO. We observed that renal DPP-4 activity was significantly increased by UUO and that treatment with evogliptin markedly suppressed DPP-4 activity, in agreement with our previous finding (Supplementary Fig. 4A) [10]. Plasma DPP-4 activity was not significantly different between control and UUO mice; however, evogliptin treatment markedly reduced DPP-4 activity in UUO mice (Supplementary Fig. 4B).

DISCUSSION

This study was undertaken to address whether evogliptin directly ameliorates renal fibrosis induced by UUO in mice and to elucidate the potential mechanism. Here, we show that evogliptin protects against renal fibrosis in this mouse model and that it inhibits TGF-β-stimulated Smad3 phosphorylation and ECM protein production in cultured renal cells.

TGF-β/Smad3 signaling is a crucial pathway in the pathogenesis of renal fibrosis [2,3]. Among Smad family, Smad3 is considered to be the principal regulator of the transcription of genes associated with renal fibrosis [13]. Upon its phosphorylation and activation by TGF-β receptor, Smad3 transactivates collagen genes to induce synthesis of ECM components and inhibit matrix degradation [14]. Recent study has shown that sitagliptin improves renal fibrosis by suppressing TGF-β/Smad3 signaling [15]. Alogliptin treatment of UUO mice had renoprotective effects through downregulation of the expression of TGF-β mRNA and α-SMA [16]. Previously, we also found that gemigliptin improved renal fibrosis in streptozoto-

cin-induced diabetic mice by reducing TGF-β-stimulated Smad3 phosphorylation, which lowers the expression of ECM proteins, including type 1 collagen and fibronectin [17]. In addition, it was previously demonstrated that DPP-4 plays a role in TGF-β-induced receptor hetero-dimerization and that TGF-β-induced formation of the TGFR1/2 heterodimer is suppressed by small interfering RNA (siRNA)-mediated inhibition of DPP-4 [18]. Moreover, vildagliptin and linagliptin were successful in lowering the renal TGF-β level in a streptozotocin-induced diabetic rat [19,20]. Although we did not investigate the TGF-β level, the mechanisms described above could be, at least in part, responsible for evogliptin-mediated inhibition of TGF-β signaling. Therefore, further studies using Smad3-null transgenic mouse model are warranted to clarify the molecular mechanism responsible for evogliptin's suppressive effect on TGF-β/Smad3 signaling. The present study provides evidence that evogliptin has a protective effect on renal fibrosis by inhibiting TGF-β-stimulated Smad3 phosphorylation and its downstream signaling.

In conclusion, we have shown that evogliptin can prevent renal fibrosis by inhibiting the TGF-β/Smad3 signaling pathway. Our data suggest that evogliptin could be applied to prevent the progression of CKD or other etiologies, as well as DKD. Therefore, the present study provides the rationale for further clinical trials to evaluate the therapeutic efficacy of evogliptin in patient with DKD.

SUPPLEMENTARY MATERIALS

Supplementary materials related to this article can be found online at https://doi.org/10.4093/dmj.2018.0271.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

AUTHOR CONTRIBUTIONS

Conception or design: M.J.K., N.Y.K., Y.K.C., K.G.P.
Acquisition, analysis, or interpretation of data: M.J.K., Y.A.J., S.H.L., G.S.J., J.G.K., I.K.L., S.L.
Drafting the work or revising: N.Y.K., Y.K.C., K.G.P.
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