Cloning and Characterization of Novel Testis-Specific Diacylglycerol Kinase η Splice Variants 3 and 4

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Abstract

Diacylglycerol kinase (DGK) phosphorylates DG to generate phosphatidic acid. Recently, we found that a new alternative splicing product of the DGKη gene, DGKη3, which lacks exon 26 encoding 31 amino acid residues, was expressed only in the secondary spermatocytes and round spermatids of the testis. In this study, we cloned the full length DGKη3 gene and confirmed the endogenous expression of its protein product. During the cloning procedure, we found a new testis-specific alternative splicing product of the DGKη gene, DGKη4, which lacks half of the catalytic domain. We examined the DGK activity and subcellular localization of DGKη3 and η4. DGKη3 had almost the same activity as DGKη1, whereas the activity of DGKη4 was not detectable. In resting NEC8 cells (human testicular germ cell tumor cell line), DGKη1, η3 and η4 were broadly distributed in the cytoplasm. When osmotically shocked, DGKη1 and η4 were distributed in punctate vesicles in the cytoplasm. In contrast, DGKη3 was partly translocated to the plasma membrane and co-localized with the actin cytoskeleton. These results suggest that DGKη3 and η4 have properties different from those of DGKη1 and that they play roles in the testis in a different manner.

Introduction

Diacylglycerol kinase (DGK) phosphorylates diacylglycerol to generate phosphatidic acid [1–6]. Diacylglycerol, which is liberated from phosphatidylinositol 4,5-bisphosphate and phosphatidylcholine upon cell stimulation, regulates a wide range of cellular functions. It is well known that DGK represents a large enzyme family. Ten mammalian DGK isozymes, namely α, β, γ, δ, ε, ζ, η, Θ, ι and κ, which contain two or three characteristic cysteine-rich C1 domains and the catalytic region in common, are subdivided into five subgroups according to their structural features [1–6]. The type II DGK [7] comprises the δ [8], η [9] and κ [10] isoforms. The occurrence of alternative splicing was reported for DGKδ (δ1 and δ2) [11] and DGKη (η1 and η2 (Fig 1)) [12]. All of the type II DGK isoforms possess a pleckstrin homology domain at their N termini and a separated catalytic domain, and DGKs δ1, δ2 and η2 but not DGKs η1 or κ contain a sterile α-motif (SAM) domain at their C termini. The pleckstrin homology domain of DGKη was found to preferentially interact with phosphatidylinositol-4,5-bisphosphate [13].
Moreover, it has been reported that DGKs δ1, δ2 and η2 formed oligomers through interactions among their SAM domains and that this oligomer formation regulates the subcellular localizations of these DGK isoforms [11, 12, 14–16]. Interestingly, DGKη is a unique enzyme with high affinity for DG [17].

We recently reported that DGKη is expressed in stomach cancer and HeLa cervical cancer cells and that it is required for the Ras/B-Raf/C-Raf/MEK/ERK signaling cascade, which is activated by epidermal growth factor [18]. Moreover, DGKη was reported to be involved in lung cancer [19]. DGKη is known to be most abundantly expressed in the brain [9, 20]. Recent genome-wide association studies implicated the DGKη gene in the etiology of bipolar disorder [21–23]. Intriguingly, deficiency of DGKη indeed induced lithium-sensitive bipolar disorder (mania)-like behavior [24].

Several reports have revealed that DGKη is abundantly expressed in the reproductive organs, testis and ovary [8–12]. Therefore, we examined the expression and spatial distribution of the DGKη1 and η2 proteins and mRNAs in the mouse reproductive organs [25]. The results indicate distinct expression patterns, which were obviously different from each other. DGKη1 was distributed in the oviductal epithelium of the ovary and in the luminal epithelium of the uterus. However, DGKη2 was not detectable in reproductive organs. Moreover, we found a new splice variant of DGKη, DGKη3, which was specifically expressed in the testis. Furthermore, DGKη3 was strongly expressed in the secondary spermatocytes and round spermatids of the testis, suggesting that this isoform plays specialized roles in spermatogenesis.

In this study, we cloned the full length DGKη3 gene. Moreover, during the cloning procedure, we found a new testis-specific alternative splicing product of DGKη gene, DGKη4, which lacks the C-terminal half of DGKη3 and then examined the properties of DGKη3 and η4.

**Experimental Procedures**

**Cell culture, transfection and osmotic shock**

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Wako Pure Chemicals, Osaka, Japan) containing 10% fetal bovine serum (Corning, Corning, NY) at 37°C in an atmosphere of 5% CO2. cDNA was transfected into COS-7 cells by electroporation with a Genecel Xcell™ Electroporation System (Bio-Rad Laboratories, Tokyo, Japan) according to the manufacturer’s instructions.

NEC8 (a human embryonal carcinoma cells derived from testis) were obtained from Japanese Collection of Research Bioresources (Tokyo, Japan). The cells were maintained in RPMI-1640 medium (Wako Pure Chemicals) containing 10% fetal bovine serum at 37°C in an atmosphere of 5% CO2. The cells were transiently transfected with cDNA using FuGENE HD Transfection Reagent (Promega, Tokyo, Japan) according to the manufacturer’s instructions. Twenty-four hours after transfection, the cells were serum starved for 3 h and incubated in RPMI-1640 with 500 mM sorbitol for 30 min.

**Plasmid constructs**

cDNAs encoding for mouse DGKη1 (1–1156 aa), DGKη3 (1–1125 aa) and DGKη4 (1–558 aa) were amplified by PCR using KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The products were inserted into the Sall/SacII site of pAcGFP-C1 vector (Clontech-Takara Bio, Kusatsu, Japan).

**Western blot analysis**

The testes from 10-week-old male mice were homogenized in lysis buffer (50 mM HEPES, pH7.2, 150 mM NaCl, and 5 mM MgCl2) containing 1 mM phenylmethylsulfonyl fluoride,
20 μg/ml each of leupeptin, pepstatin, aprotinin and soybean trypsin inhibitor and Complete EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics), and centrifuged at 1,000 x g for 5 min. The protein concentration in the supernatants was determined using a bicinchoninic acid protein assay kit (Thermo Scientific, Hudson, NH, USA). The tissue lysates (50 μg of protein) were separated on SDS-PAGE, and the separated proteins were transferred to a polyvinylidene difluoride membrane (Pall Life Sciences, Port Washington, NY, USA). The membrane was blocked with 5% skim milk and incubated with an anti-DGKη polyclonal antibody [24] overnight at 4°C. The immunoreactive bands were visualized using a peroxidase-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and the ECL Western Blotting Detection System (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

COS-7 cell lysates were separated on SDS-PAGE. The separated proteins were transferred to polyvinylidene difluoride membrane and blocked with 5% skim milk. The membrane was incubated with anti-GFP antibody (sc-9996, Santa Cruz Biotech, Santa Cruz, CA) for 1 h. The immunoreactive bands were visualized using peroxidase-conjugated anti-mouse IgG antibody and the Enhanced Chemiluminescence Western Blotting Detection System.

Reverse transcription (RT)-PCR

Total RNA was isolated from each tissue of the 10 to 12-week-old male and female mice using a Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA). cDNA synthesis was performed with the Transcription First-Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany). PCR amplification was performed using rTaq polymerase (Toyobo) and the following mouse-DGKη specific primers: primer A (nucleotide positions 2416–2436, 5'-GGGAATTCCGGGAGCTACTACAGAGATC-3') and primer B (nucleotide positions 3451–3471, 5'-CTTCCTCGGAGCTACTACAGAGATC-3'). The PCR conditions for these two primers were as follows:
94°C for 3 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 2 min, and 72°C for 5 min. PCR was also performed with primer C (nucleotide positions 1411–1422, 5'- CCTGAA CCGTGCAGCAACTG-3') and primer D (nucleotide positions 1681–1702, 5'- GGACTC GACTGGGCTGAGAGTC-3'). The PCR conditions used for these primers were as follows: 94°C for 3 min, 35 cycles of 94°C for 30 sec, 48°C for 30 sec, 72°C for 1 min 30 sec and 72°C for 5 min.

Confocal laser scanning microscopy

NEC8 cells grown on poly-L-lysine (Sigma-Aldrich, St. Louis, MO)-coated glass coverslips were transfected with pAcGFP-DGKη1, η3, η4 or pAcGFP vector alone. After 24 h, the cells were serum starved for 3 h and then incubated in 500 mM sorbitol for 30 min. The cells were then fixed in 3.7% formaldehyde. The filamentous actin (F-actin) was stained with Alexa 594-conjugated phalloidin (Thermo Fisher Scientific, Waltham, MA), and the nuclei were stained with 4',6-diamino-2-phenylindole (DAPI). The coverslips were mounted using Vectashield (Vector Laboratories, Peterborough, UK). The cells were examined using inverted confocal laser microscopy (FV1000-D, Olympus, Tokyo, Japan).

DGK activity assay

pAcGFP-DGKη1, η3, η4 or pAcGFP vector were transfected into COS-7 cells. After 48 h, the cells were harvested and suspended in ice-cold lysis buffer (50 mM HEPES (pH 7.2), 150 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, complete™ EDTA-free protease inhibitor (Roche Diagnostics)) and were then sonicated. The octylglucoside mixed micellar assay of DGK activity was performed as described previously [8]. In brief, the assay mixture (50 μL) contained 50 mM MOPS (pH 7.2), 50 mM n-octyl-β-D-glucoside, 1 mM dithiothreitol, 20 mM NaF, 10 mM MgCl₂, 1 μM CaCl₂, 27 mol% PS, 5.0 mol% 1,2-dioleoyl-sn-glycerol (18:1/18:1-diacylglycerol) and 1 mM [γ-³²P]ATP (100000 cpm/nmol). The reaction was initiated by adding the cell lysates, and it continued for 5 min at 30°C. Lipids were extracted from the mixture, and phosphatidic acid was separated by thin layer chromatography. The phosphatidic acid spot was scraped and counted by a liquid scintillation spectrophotometer.

Statistical analysis

Statistical comparisons were performed using one-way ANOVA followed by a Tukey’s test.

Results

Cloning of full length DGKη3

We previously found a new alternative splicing product of DGK gene, DGKη3, which lacks exon 26 encoding 31 amino acid residues [25] (Fig 1). However, it is still unknown whether the DGKη3 mRNA is a derivative of the DGKη1 mRNA or the DGKη2 mRNA. To clarify this, it is needed to determine whether DGKη3 gene contains exon 29, which produces DGKη2 (Figs 1 and 2A), or not. Thus, we performed RT-PCR using primer A (in exon 20) and B (in exon 30) indicated in Fig 2A. If DGKη3 contains exon 29, a 1094 bp product will be amplified (Fig 2A). If not, a 963 bp band will be detected (Fig 2A). As shown in Fig 2B, only the 963 bp product was amplified, indicating that the DGKη3 mRNA does not contain exon 29 and is derived from the DGKη1 mRNA. We next cloned and completely sequenced the full-length DGKη3 mRNA, and consequently confirmed that the DGKη3 mRNA is a derivative of the DGKη1 mRNA (Fig 1). Therefore, the protein product of DGKη3 does not contain a SAM domain (Fig 1).
**A**

Primer A → 963 bp → Primer B
Exon 20 → Exon 21 → Exon 22 → Exon 23 → Exon 24 → Exon 25
Exon 27 → Exon 28 → Exon 30

Primer A → 1094 bp → Primer B
Exon 20 → Exon 21 → Exon 22 → Exon 23 → Exon 24 → Exon 25
Exon 27 → Exon 28 → Exon 29 → Exon 30

**B**

Size marker → Testis

- 1500 bp
- 1000 bp
- 900 bp
- 963 bp

**C**

WT

- 126 kDa
- 118 kDa

DGK\(\eta\)-KO

- 130 kDa

Anti-DGK\(\eta\)

- 100 kDa

Anti-\(\beta\)-actin

- 35 kDa
Although the DGK\(\eta\)3 mRNA was strongly expressed in the testis, its protein band has not been detected in our previous report [25]. It is possible that the DGK\(\eta\)3 protein is unstable and quickly degraded. Therefore, in the present study, we added various protease inhibitors (see Experimental Procedures) into testis samples to prevent proteolysis, and performed Western blotting. As shown in Fig 2C, we robustly detected anti-DGK\(\eta\) antibody-reactive bands of 126 kDa, which approximately correspond to a calculated molecular mass of 124 kDa of DGK\(\eta\)3, and 118 kDa. Moreover, we confirmed that the 126 and 118 kDa bands were disappeared in testes of DGK\(\eta\)-knockout mice [24]. These results strongly suggest that the DGK\(\eta\)3 mRNA is translated in the testis. The lower band (118 kDa) may be a product of proteolytic degradation from the upper band (126 kDa).

Identification of DGK\(\eta\)4

When we performed RT-PCR for sequencing, we detected a longer DGK\(\eta\) mRNA (327 bp) that includes 35 bp of additional nucleotides derived from intron 14 (Fig 3A and 3B). This insertion leads to a frame shift, resulting in an in-frame stop codon in exon 15 and generating the extra 15 aa hydrophobic tail, IFPSFMSFLMSAQS (Fig 3A and 3C). Therefore, we designated it DGK\(\eta\)4 as a new splice variant of the DGK\(\eta\) gene (Fig 1). RT-PCR using primers C in exon 12 and primer D in exon 15 (Fig 3A and 3B) showed that the product derived from DGK\(\eta\)4 was approximately 46% and that derived from DGK\(\eta\)3 was approximately 54% (Fig 3B). The encoding protein of the DGK\(\eta\)4 gene stops at the middle of the intermediate region between separated catalytic subdomains (Fig 1). Therefore, the DGK\(\eta\)4 protein lacks the C-terminal half of the catalytic domain.

Expression of DGK\(\eta\)3 and DGK\(\eta\)4 mRNAs

In addition to the testis, expression of DGK\(\eta\)3 and \(\eta\)4 mRNAs in other organs such as brain, liver, kidney, lung, skeletal muscle, vesicula seminalis, prostate gland, epididymis, ovary and uterus were analyzed using primers A and B, which amplify 828 bp (DGK\(\eta\)1 and \(\eta\)2) and 735 bp (DGK\(\eta\)3) products, respectively, and were analyzed using primers C and D, which amplify 327 bp (DGK\(\eta\)1, \(\eta\)2 and \(\eta\)3) and 292 bp (DGK\(\eta\)4) products, respectively. Shionoya et al. reported that DGK\(\eta\)3 was not expressed in ovary or uterus whereas DGK\(\eta\)1 and \(\eta\)2 were expressed there. Thus, the expression of DGK\(\eta\)3 in brain, liver, kidney, lung, skeletal muscle, vesicula seminalis, prostate gland and epididymis was checked. We confirmed that the 735 bp band derived from DGK\(\eta\)3 mRNA was expressed in testis alone (Fig 4). On the other hand, the 828 bp product (DGK\(\eta\)1 and \(\eta\)2) was detected in brain, liver, kidney, lung, skeletal muscle, vesicula seminalis, prostate gland and epididymis.

The 327 bp product amplified from DGK\(\eta\)4 mRNA was also detected only in the testis (Fig 4). The 292 bp product (DGK\(\eta\)1, \(\eta\)2 and \(\eta\)3) was detected in testis, brain, liver, kidney, lung, skeletal muscle, prostate gland, ovary and uterus. In vesicula seminalis, epididymis, liver and skeletal muscle, other products having different lengths were detected, suggesting that other alternative splicing variants of the DGK\(\eta\) gene may exist. These results strongly suggest that DGK\(\eta\)3 and \(\eta\)4 mRNAs are testis-specifically expressed.
A

DGKη1, 2, 3
Exon 12 Exon 13 Exon 14 Exon 15

DGKη4
Exon 12 Exon 13 Exon 14 Intron 14 Exon 15

B

327 bp
292 bp

400
300

C

ATACCTCTGTGAAAACCTGAAAGGCTCTCTGTGCCCAAGTGAGAAGCCAGAGGCACAGAACAA
I L C S T V K D F V A K V E K A Q D R T

CTGGAAAAATACAGATTGAAGGCGAGCCGGTGGCCAGTAAA
LENTVVABAASK

ATTTTTCCCTCCCTTTATGACTTTTCTGATGCTAAG
IFPSFMSFLMLSL

TGCTCAGTCCTAAAGGCAGGCTTGGACAGCTCTGAAGCCCTTGCACAGGACTCTCAG
CSVLEKLEQLQALHADSQ

DGKη1, 2, 3, 4

DGKη1, 2, 3, 4

Exon 14

Exon 14-2 (Intron14)

DGKη4

Exon 15

DGKη1, 2, 3

DGKη4

Exon 15

DGKη1, 2, 3

DGKη1, 2, 3

Exon 15

DGKη1, 2, 3

DGKη1, 2, 3
DGK activities of DGKη3 and η4

We next characterized properties of the newly identified splice variants, DGKη3 and η4. To measure DGK activities of protein products encoded by DGKη3 and η4 genes, these proteins were overexpressed in COS-7 cells. We confirmed expression of DGKη3, η4 and η1, which is a positive control (Fig 5A). Compared to DGK activity of DGKη1, DGKη3 exhibited almost the same (approximately 80%) activity (Fig 5B), indicating that DGKη3 is catalytically active. On the other hand, DGK activity of DGKη4 was not detectable (Fig 5B), indicating that this isoform is kinase negative.

Subcellular localization of DGKη3 and η4 in NEC8 cells

We next tested whether DGKη3 and η4 respond to stress stimulation. To address this, we determined subcellular localization of DGKη3 and η4 in NEC8 cells (human testicular germ cell tumor cell line) in the presence and absence of 0.5 M sorbitol (osmotic stress). AcGFP alone were broadly distributed in the cytoplasm and nucleus in the presence and absence of 0.5 M sorbitol (Fig 6A and 6B). In the absence of sorbitol, DGKη1, η3 and η4 were broadly distributed in the cytoplasm, and localization of either DGKη1, η3 or η4 at the plasma membrane was not detectable (Fig 6A). When osmotically shocked, DGKη1 and η4 were distributed in
Fig 5. Catalytic activities of DGK n3 and n4. (A) Expression of AcGFP alone, AcGFP-DGK n1, n3 and n4 in COS-7 cells. COS-7 cells were transfected with pAcGFP vector alone, pAcGFP-DGK n1, n3 or n4. The cell lysates (12 μg of protein) were analyzed by Western blotting using anti-GFP antibody. (B) The relative activities of DGK n3 and n4 compared to DGK n1. The cell lysates (5 μg of protein) were analyzed by Western blotting using anti-DGK antibody.
punctate vesicles in the cytoplasm (Fig 6B). Unexpectedly, DGK3 was partly translocated to the plasma membrane and co-localized with actin cytoskeleton in an osmotic stress-dependent manner (Fig 6B). As shown in Fig 6C, DGK3 was translocated to the plasma membrane in approximately 45% of the cells in response to osmotic stress, whereas DGK1 and 4 were located at the plasma membrane in only 10–15% of the cells. These results strongly suggest that DGK3 was specifically translocated to the plasma membrane in NEC8 cells in response to osmotic stress.

Discussion

In this study, we cloned cDNA of the testis specific full length DGK3 mRNA (Figs 1 and 2). Moreover, we found a new testis-specific alternative splicing product of the DGK gene, DGK4, during the cloning procedure (Figs 1 and 3). DGK4 lacks half of the catalytic domain (Fig 1).

The DGK3 mRNA is a splice variant derived from the DGK1 mRNA but not from the DGK2 mRNA (Figs 1 and 2). Therefore, DGK3 does not contain the SAM domain (Fig 1). DGK4 also lacks the SAM domain (Fig 1). In addition to DGKδ1 and δ2 [11, 14], DGKδ2 formed an oligomer through its SAM domain [12]. However, it is unlikely that DGK3 and 4 are able to form oligomers.

The biochemical and cell biological properties of DGK3 and 4 were different from those of DGK1. First, DGK activity was not detectable for DGK4 (Fig 5). The loss of activity is reasonable because Rittiner et al. [26] revealed that a DGK1 mutant lacking the C-terminal half of the catalytic domain did not show DGK activity. On the other hand, DGK3 showed almost the same activity as DGK1 (Fig 5), indicating that 31 amino acid deletion between two coiled-coil structures (Fig 1) does not significantly affect its catalytic activity.

Second, the subcellular localization of DGK3 was different from that of DGK1 (Fig 6). Environmental stress causes testicular dysfunction [27, 28]. Thus, we tested whether DGK3 and 4 respond to osmotic stress stimulation. DGK1 and 2 were translocated to punctate vesicles in the cytoplasm in response to osmotic stress, as previously reported [12, 13, 26, 29]. In NEC8 cells DGK1 or 4 were also osmotic stress-dependently translocated to punctate vesicles in the cytoplasm (Fig 5). Interestingly, DGK3, but not DGK1 or 4, was partly translocated to the plasma membrane and co-localized with F-actin in NEC8 cells in response to osmotic stress. Therefore, a 31 amino acid deletion between two coiled-coil structures in DGK3 (Fig 1) would affect its osmotic stress-dependent localization. Thus, the length between two coiled-coil structures may be important for subcellular localization. The strong plasma membrane localization of DGK3 was not observed in COS-7 cells (data not shown), suggesting that its translocation is cell line dependent.

It is possible that the splice variants DGK3 and DGK4 might be artifacts of cDNA preparation. However, this possibility is unlikely because the splice variants were detected only in the testis (Fig 4), although the artifacts would arise in every tissues. Moreover, we obtained the same RT-PCR products amplified from DGK3 and DGK4 mRNAs in three independent experiments. Furthermore, alternative splicing products of the DGKs gene (accession number XP_011243403, XP_011243404 and XP_006519232), which lack exon 26, are predicted and deposited into the NCBI database. These records are derived from a genomic sequence annotated using gene prediction method Gnomon, which is supported by mRNA and EST evidence.
A

| AcGFP alone | AcGFP-DGK11 | AcGFP-DGK13 | AcGFP-DGK14 |
|-------------|-------------|-------------|-------------|
| DAPI        | F-actin     | GFP         | Merge       |

Scale bar: 10 μm

B

| AcGFP alone | AcGFP-DGK11 | AcGFP-DGK13 | AcGFP-DGK14 |
|-------------|-------------|-------------|-------------|
| DAPI        | F-actin     | GFP         | Merge       |

Scale bar: 10 μm

C

Percentage of cells exhibiting plasma membrane transition (%)
Finally, we robustly detected anti-DGK\(\eta\) antibody-reactive bands of 126 kDa, which approximately corresponds to a calculated molecular mass of 124 kDa of DGK\(\eta\)\(3\) and 118 kDa (Fig 2C). Moreover, we confirmed that the 126 and 118 kDa bands were disappeared in testes of DGK\(\eta\)-knockout mice [24]. Because available anti-DGK\(\eta\) antibodies detect the C-terminal half of DGK\(\eta\)\(1–3\), we are not able to detect endogenous DGK\(\eta\)\(4\) protein at present. Thus, it is also possible that the DGK\(\eta\)\(4\) mRNA is a non-coding RNA. However, this possibility is unlikely because the splice variant contains initiation methionine and stop codons. Even if the DGK\(\eta\)\(4\) mRNA is a non-coding RNA, it is predicted to play specialized roles in the testis.

DGK\(\eta\)\(3\) and \(\eta\)\(4\) are expressed in the testis (Fig 4), especially in the secondary spermatocytes and round spermatids [25]. The round spermatids are generated from the secondary spermatocytes through the second meiotic division [30, 31], implying that these isoforms play specialized roles in meiosis during spermatogenesis. DGK\(\eta\)\(3\) is catalytically active and stress-dependently localizes at the plasma membrane but not the cytoplasm where DGK\(\eta\)\(1\) exists. DGK\(\eta\)\(4\) exhibited the same localization with DGK\(\eta\)\(1\) but is catalytically inactive. DGK\(\eta\)\(1\) is known to enhance proliferation [18]. Thus, these isoforms may attenuate mitosis/proliferation and promote meiosis through their inactivity and different localization. Therefore, our present work would provide useful information to the study of spermatogenesis. However, to explore their specialized physiological functions, further study is required.

**Author Contributions**

**Funding acquisition:** FS.

**Investigation:** EM TS SK YS.

**Project administration:** FS.

**Supervision:** FS.

**Writing – original draft:** EM FS.

**Writing – review & editing:** FS.

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