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Involvement of ADAM12 in chondrocyte differentiation by regulation of TGF- β 1-induced IGF-1 and RUNX-2 expression

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Abstract

A disintegrin and metalloproteinase 12 (ADAM12) is known to be involved in chondrocyte proliferation and maturation however the mechanisms are not fully understood. In this study expression and localization of ADAM12 during chondrocyte differentiation was examined in the mouse growth plate by immunohistochemistry. *Adam12* expression during ATDC5 chondrogenic differentiation was examined by real-time PCR and compared with the expression pattern of type X collagen. The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system was used to generate *Adam12*-knockout (KO) ATDC5 cells. *Adam12*-KO and *Adam12* overexpressing cells were used for analyses of ADAM12 expression with or without TGF- β 1 stimulation. ADAM12 was identified predominantly in chondrocytes of the proliferative zone in mouse growth plates by immunohistochemistry. *Adam12* was upregulated prior to *Coll10a1* during chondrogenic differentiation in wild-type ATDC5 cells. In *Adam12*-KO ATDC5 cells, following initiation of chondrogenic differentiation, we observed a reduction in *Igf-1* expression along with an upregulation of hypertrophy associated *Runx2*, *Coll10a1*, and type X collagen protein expression. In ATDC5 wild-type cells, stimulation with TGF- β 1 upregulated the expression of *Adam12* and *Igf-1* and downregulated the expression of *Runx2*. In contrast, in *Adam12*-KO ATDC5 cells, these TGF- β 1-induced changes were suppressed. *Adam12* overexpression resulted in an upregulation of *Igf-1* and downregulation of *Runx2* expression in ATDC5 cells. The findings suggest that ADAM12 is important in the

regulation of chondrocyte differentiation, potentially by regulation of TGF- β 1 dependent signaling and that targeting of ADAM12 may have a role in management of abnormal chondrocyte differentiation.

Key words: Chondrogenic differentiation, ADAM12, IGF-1, RUNX2, Type X collagen

1. Introduction

Endochondral ossification plays important roles in skeletal growth and repair [1], peripheral osteophyte formation in osteoarthritis (OA), and the growth of osteochondromas [2, 3]. During endochondral ossification, undifferentiated chondrocytes undergo proliferation and hypertrophic differentiation. These hypertrophic chondrocytes express predominantly type X collagen, mineralize the surrounding matrix, and ultimately undergo apoptosis. This is followed by blood vessel ingrowth into the calcified cartilage, and the ultimate replacement of the cartilage with bone [4].

A disintegrin and metalloprotease 12 (ADAM12) is a member of the ADAM (a disintegrin and metalloproteases) family of proteins, comprising a prodomain, metalloproteinase, disintegrin, cysteine-rich and epidermal growth factor (EGF)-like domains, as well as a transmembrane domain and cytoplasmic tail [5]. Human ADAM12 exists in two splice variants: ADAM12-L, a transmembrane protein similar to mouse ADAM12, and ADAM12-S, a shorter secreted form that lacks the transmembrane and cytoplasmic domains [6]. ADAM12 is expressed in neonatal bone and is activated in mesenchymal condensation that leads to bone formation [7]. ADAM12-S and ADAM12-L are both expressed by human osteoblasts whereas only ADAM12-S is seen in human osteoclasts where it appears to have roles in osteoclastogenesis [8]. In genetically modified mouse models, ADAM12-S appears to stimulate bone growth by modulating chondrocyte proliferation and maturation [9]. Purified ADAM12-S cleaves insulin-like growth factor-binding protein (IGFBP)-3 and IGFBP-5 to release IGF-1 into the extracellular matrix [10], a function that can potentially lead to chondrocyte proliferation *in vivo*. Significantly, transforming growth factor- β (TGF- β), a regulator of chondrocyte proliferation and cluster

formation in OA articular cartilage, increases the expression of ADAM12-L in human OA chondrocytes [11].

Collectively, these reports stimulated the current study into the localization and expression of ADAM12 during chondrocyte differentiation in the mouse growth plate and investigation of the potential roles of this molecule in the regulation of chondrocyte differentiation *in vitro*.

2. Materials and Methods

2.1. Animal tissues

The current study was reviewed by the Animal Care and Use Committee, Okayama University. Male DBA/1 mice (Charles River Japan; Yokohama, Japan), aged 6 to 7 weeks, were used ($n = 5$) to evaluate the localization of ADAM12 during chondrocyte differentiation *in vivo*. The mice were euthanized by the systemic perfusion of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) under general anesthesia with 2% isoflurane. The limbs were dissected and fixed in the same solution for 24 h. The samples were decalcified in 0.3 M EDTA (pH 7.5) for 1 week. After decalcification, specimens were dehydrated using a graded ethanol series, and embedded in paraffin. Serial sections (5- μ m thickness) were cut and stained with hematoxylin and eosin (H&E), according to standard laboratory protocols.

2.2. Immunohistochemistry

To detect ADAM12 and type X collagen in the growth plates, paraffin-embedded sections were deparaffinized, rehydrated, and pre-incubated in 1 mg/ml testicular hyaluronidase (Wako; Osaka, Japan) for 30 min at room

temperature. Sections were immersed in 3% H₂O₂ in PBS to prevent endogenous peroxidase reactions for 10 min at room temperature. Non-specific background signals were blocked using 1% bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO, USA). For immunostaining, sections were incubated overnight at 4°C with either a rabbit polyclonal antibody against ADAM12 that recognizes both the transmembrane and secreted forms (1:500; 14139-1-AP, Proteintech, Chicago, IL, USA) or a rabbit polyclonal antibody anti-type X collagen alpha 1 (1:500; GTX37732, GeneTex, Irvine, CA, USA). Negative control sections were treated by a BSA solution without the primary antibody. After thorough rinsing, sections were finally incubated with Histofine Simple Stain MAX-PO (Nichirei; Tokyo, Japan) for 30 min. After the color reaction using diaminobenzidine (Nichirei), sections were counterstained with hematoxylin. Immunoreactivity was evaluated by light microscopy.

The growth plate shows two distinct zones—the proliferative zone and the hypertrophic zone—classified by the shape and size of the chondrocytes. Chondrocytes with a definite, diffusely stained cytoplasm were regarded as ADAM12 positive. The positive reaction of type X collagen in the extracellular matrix also helped to define this hypertrophic zone. The populations of ADAM12-positive cells in the proliferative and hypertrophic zones of the growth plate were quantified by counting the number of cells within these two zones, respectively, and averaged. Cell counting was performed on at least three fields at 40× magnification using one section from each of 5 mouse growth plates. The number of immunopositive chondrocytes was divided by the total number of chondrocytes within the two zones to calculate the positive chondrocyte ratio.

2.3. *ATDC5 cell cultures*

The ATDC5 cell line was obtained from the Riken Cell Bank (Tsukuba, Japan). The cell line serves as an in vitro system with which to model the stages of chondrogenic differentiation and maturation [12,13]. The cells were cultured in a maintenance medium consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (Invitrogen-Gibco; Carlsbad, CA, USA), and containing 5% fetal bovine serum (FBS), 50 µg/mL penicillin, and 100 µg/mL streptomycin [14,15]. ATDC5 cells were plated at 6.4×10^4 cells/well in a 6-well plate with the maintenance medium, and cells were induced to undergo chondrogenesis when they had reached 70% to 80% confluence. Chondrogenic differentiation was induced by differentiation medium consisting of DMEM and Ham's F12, 0.1% BSA (Sigma-Aldrich), 1% insulin-transferrin-selenium (ITS; I3146, Sigma-Aldrich), 50 µg/mL penicillin, 100 µg/mL streptomycin, and 20 µg/mL L-ascorbic acid to avoid unnecessary induction by FBS [16]. The medium was replaced every other day. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air.

2.4. Creation of Adam12-knockout (KO) ATDC5 cells using the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system

To create the single-guide RNA (sgRNA) expression vectors, the annealed oligonucleotides for the target site of ADAM12 (5'-GCATCATGAACCCGTCCACG-3' and 5'-TATTCTGACATCGACGATTG-3') were cloned into the MLM3636 vector (#43860; Addgene, Cambridge, MA). These plasmids and the Cas9 expression plasmid (#51142; Addgene) were co-transfected into ATDC5 cells for 2 days. The adherent cells were trypsinized and resuspended in 2% FBS in PBS. Single-cell suspensions were sorted into 96-well plates using a FACS Aria II cell

sorter (BD Biosciences; San Jose, CA, USA). To confirm the KO, two-dimensional gel electrophoresis of the genomic PCR product and Sanger sequencing were performed (Fig. 1A and B). The cells were cultured and chondrogenesis was induced in the same way as that for wild-type ATDC5 cells (as above).

2.5. RNA extraction and real-time quantitative PCR analysis

To examine the expression of *Adam12*, *Coll0a1*, *Igf-1* and runt-related transcription factor2 (*Runx2*) in ATDC5 cells, total RNA was prepared using an RNeasy Lipid Tissue Mini Kit (Qiagen; Venlo, The Netherlands) according to the manufacturer's instructions. Complementary DNA of ATDC5 cells after 0, 7, 14, 21, 28, and 35 days of culture were reverse transcribed from 1 µg of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR analysis was performed using an Mx3000P QPCR System (Agilent Technologies; Santa Clara, CA, USA) with Taqman Gene Expression Assays for mouse *Adam12* (Mm00475719_m1), *Coll0a1* (Mm00487041_m1), *Igf-1* (Mm00439560_m1), and *Runx2* (Mm00501584_m1) (Applied Biosystems; Foster City, CA, USA). Amplification of the housekeeping gene *Gapdh* was used to normalize the efficiency of cDNA synthesis and the amount of RNA. We calculated the final expression levels by dividing the expression levels of *Adam12*, *Coll0a1*, *Igf-1*, and *Runx2* by the expression level of *Gapdh*.

2.6. Western blotting

ATDC5 cells were induced to undergo chondrogenic differentiation for up to 5 weeks. At 3, 4 and 5 weeks, cells were scraped on ice with 1× Laemmli buffer (Bio-Rad; Hercules, CA, USA) containing 5% β-mercaptoethanol.

Total protein samples were homogenized in SDS buffer (4% 180 SDS, 125 mM Tris–glycine, 10% 2-mercaptoethanol, 2% bromophenol blue in 30% glycerol) and centrifuged at 9,000 ×g for 10 min at 4°C to remove debris. Aliquots were subjected to polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) followed by electrotransfer onto PVDF membranes (Hybond-P; GE Healthcare, Tokyo, Japan). Membranes were blocked overnight with Block Ace (UKB80; Dainippon Sumitomo Pharma, Osaka, Japan) and then probed overnight at 4°C with a rabbit polyclonal antibody against IGF-1 (1:1000 dilution; ab9572, Abcam, Cambridge, UK), a rabbit polyclonal antibody against RUNX2 (1:1000 dilution; ab23981, Abcam), a goat polyclonal antibody against type X collagen alpha 1 (1:500 dilution; sc-323750, Santa Cruz Biotechnology, Dallas, TX, USA) and a mouse monoclonal antibody against GAPDH (1:1000 dilution; sc-32233, Santa Cruz Biotechnology). Signals were detected with goat anti-rabbit IgG horseradish peroxidase (HRP) (1:50,000 dilution; sc-2004, Santa Cruz Biotechnology), donkey anti-goat IgG HRP (1:50,000 dilution; sc-2020, Santa Cruz Biotechnology) and goat anti-mouse IgG HRP (1:50,000 dilution; sc-2005, Santa Cruz Biotechnology) and Immunostar LD (290-69904, Wako) detection reagents. The lumino-labeled membranes were analyzed on an Amersham Imager 600 CCD-based 190 chemiluminescent analyzer (GE Healthcare). Relative band intensities were quantified using ImageQuant TL software (GE Healthcare).

2.7. *TGF- β stimulation in ATDC5 cells*

Both wild-type and *Adam12*-KO ATDC5 cells were cultured in a maintenance medium consisting of a 1:1 mixture of DMEM and Ham's F12 medium (Invitrogen-Gibco), and containing 5% FBS, 50 µg/mL penicillin, and 100

µg/mL streptomycin. Cells were plated at 2.0×10^4 cells /well into the wells of a 24-well plate. The cells were transferred to serum free medium for 12h before treatment of TGF-β. Cells were stimulated with 10 ng/mL recombinant TGF-β1 (Cell Signaling Technology) for 24 h. After treatment, the gene expression of *Adam12*, *Igf-1* and *Runx2* was examined by real-time PCR to compare expression changes between wild-type and *Adam12*-KO cells.

2.8. *Adam12* overexpression in ATDC5 cells

To construct the *Adam12* expression vector, the mouse *Adam12* coding region was obtained from the mouse *Adam12* open reading frame clone (FLH481306.01X) with Platinum Super Fi DNA Polymerase (Invitrogen), and subcloned into the pcDNA3.1 vector (pcDNA3.1 Mm ADAM12). ATDC5 cells were seeded into the wells of a 24-well plate at 2.0×10^4 cells/cm² and transiently transfected with the pcDNA3.1 vector using Lipofectamine 2000 reagent (Invitrogen). We used empty pcDNA3.1 vector as a control. The expression of *Adam12* in the *Adam12*-overexpressed cells was elevated 100,000-fold above that of the empty vector-transfected controls (data not shown). At 24 h after transfection, the gene expression of *Igf-1* and *Runx2* was examined by real-time quantitative PCR.

2.9. Statistical Analysis

The results are expressed as the mean ± standard deviation. All in vitro work was performed in triplicate and repeated on three (or more) independent occasions. Statistical analyses were performed using R for Windows

(www.r-project.org). The student's *t*-test was used to compare two groups of data. Differences among more than two groups were compared using ANOVA with Bonferroni post hoc test. Significance was set at *p* values < 0.05.

3. Results

3.1. Expression of ADAM12 in the mouse growth plate

ADAM12 expression was observed in the chondrocytes of the proliferative and hypertrophic zones of the mouse growth plate (Fig. 2A-C). The positive cell ratio for ADAM12 in the chondrocytes of the proliferative zone (49.4% $\pm$ 8.8%) was significantly higher (*p* < 0.01) than that of the hypertrophic zone (28.2% $\pm$ 9.6%) (Fig. 2D).

3.2. Gene expression pattern during insulin-induced chondrogenic differentiation of ATDC5 cells

No significant differences were seen in *Adam12* gene expression at 1, 2, 3, 4, and 5 weeks compared to basal levels (week 0). A significant increase in *Adam12* expression was however seen after 3 weeks differentiation when compared to levels at 1 week. (Fig. 3A). *Col10a1* expression became evident after 3 weeks of insulin-induced chondrogenic differentiation before peaking at 4 weeks (Fig. 3B).

3.3. Effect of Adam12 gene knock-out during insulin-induced chondrogenic differentiation in ATDC5 cells

During insulin-induced chondrogenic differentiation of wild-type ATDC5 cells *Igf-1* expression increased significantly to a maximum level at 4 weeks. However in *Adam12*-KO ATDC5 cells this *Igf-1* response was completely absent (Fig. 4A). In contrast to *Igf-1*, *Runx2* expression initially increased significantly after 1 week

of insulin-induced chondrogenic differentiation and thereafter decreased to basal levels in wild-type ATDC5 cells. In *Adam12*-KO ATDC5 cells *Runx2* expression increases following the initiation of chondrogenic differentiation, peaks at 2 weeks and thereafter decreases to basal levels at 4 and 5 weeks (Fig. 4B). At 3, 4, and 5 weeks after the initiation of chondrogenic differentiation, *Coll10a1* expression was significantly higher in the *Adam12*-KO ATDC5 cells than in the wild-type ATDC5 cells (Fig. 4C). The results of western blot analysis showed that Igf-1 protein expression is decreased in *Adam12* KO ATDC5 cells compared to wild-type cells at 3-5 weeks of chondrogenic differentiation. Runx2 protein expression peaks at 4 weeks in *Adam12* KO ATDC5 cells. Type X collagen protein expression showed a time-dependent upregulation in *Adam12*-KO ATDC5 cells during 3 to 5 weeks. (Fig. 4D).

3.4. Effect of TGF- β stimulation on *Adam12*-KO and *Adam12*-overexpressing ATDC5 cells

Stimulation with 10ng/ml TGF- β 1 for 24 hours induced expression of *Adam12* and *Igf-1* in wild-type ATDC5 cells whilst decreasing expression of *Runx2*. In contrast, in the *Adam12*-KO ATDC5 cells there was no effect of TGF- β 1 on expression of *Igf-1* and *Runx2* (Fig. 5A-C). Overexpression of *Adam12* in ATDC5 cells caused increased expression of *Igf-1* whilst decreasing *Runx2* expression (Fig. 5D and E).

4. Discussion

In the current study we have demonstrated for the first time that ADAM12 is expressed in the mouse growth plate, predominantly in the chondrocyte proliferative zone but also to a lesser extent in the hypertrophic zone. Furthermore, through our *in vitro* studies, we show that *Adam12* expression is increased during chondrogenic

differentiation and begins to decline after 3 weeks during chondrocyte hypertrophy. The human ADAM12 gene is alternatively spliced, resulting in two major protein isoforms: a long, transmembrane form called ADAM12-L, and a short, secreted form designated ADAM12-S, which lacks the transmembrane and cytoplasmic domains. Both forms are active metalloproteinases [5,17]. As ADAM12 in mice is most similar to the human ADAM12-L splice variant [5], we examined the function of the transmembrane form during chondrogenic differentiation in ATDC5 cells.

Several molecules, including IGF-1, RUNX-2, and type X collagen, have roles in endochondral ossification [18,19], with RUNX2 known to regulate endochondral ossification through the control of chondrocyte proliferation and differentiation [20]. RUNX2 is a crucial transcription factor for type X collagen expression and chondrocyte hypertrophy. Interestingly, *Adam12*-KO ATDC5 cells showed marked upregulation of both *Runx2* and *Coll10a1* expression. Type X collagen protein expression levels were also upregulated in the *Adam12*-KO ATDC5 cells as compared with wild-type ATDC5 cells. As overexpression of *Adam12* downregulated *Runx2* gene expression, the results suggest that ADAM12 may have a role in regulating type X collagen gene expression through RUNX2.

TGF- β signaling is recognized as having a critical role in regulation of chondrocyte homeostasis and articular cartilage degradation [21]. A prior study has shown that TGF- β -induced ADAM12-L expression enhances the bioavailability of IGF-1 from the IGF-1-IGFBP-5 complex by selective digestion of IGFBP-5, indicating possible involvement of ADAM12-L in pathways that lead to chondrocyte proliferation and cloning in human osteoarthritic articular cartilage [11]. TGF- β 1 also arrests the downstream differentiation of chondrocytes at an early stage of

hypertrophy [22]. In the current study, treatment of ATDC5 cells with TGF- β 1 upregulated *Igf-1* and downregulated *Runx2*, with these effects suppressed in *Adam12*-KO cells. Therefore we hypothesize that the effect of TGF- β 1 on gene expression of IGF-1 and RUNX2 might be, at least in part, modulated by ADAM12. The mechanisms are not yet clear but as BMP signaling directly accelerates the expression of *Col10a1* in concert with RUNX2 [23,24], ADAM12 may have a negative influence on BMP signaling pathways potentially by enhancing TGF- β 1 signalling. TGF- β promotes the early stage of chondrogenesis by enabling Smad3 to form an active transcriptional complex with CEBP/p300 and Sox9 [25]. However, at the later stages of maturation TGF- β signalling promotes the maturation and hypertrophy of chondrocytes [26]. As Smad3-deficient chondrocytes show enhanced BMP signalling and accelerated hypertrophic differentiation *in vitro*, endogenous TGF- β signalling is likely to have a role in suppressing BMP signalling during chondrocyte maturation [27]. Recently SnoN has been shown to mediate a negative feedback mechanism by which TGF- β can inhibit BMP signalling and allow hypertrophic maturation of chondrocytes [28]. SnoN acts as a negative regulator of ADAM12 expression in a Smad2/3-dependent manner, binding to nuclear Smad complexes and repressing their transcriptional activities [29,30]. In response to TGF- β stimulation, SnoN undergoes ubiquitination and rapid proteasomal degradation [31] with subsequent de-repression of ADAM12 expression.

As yet, there is limited information on the roles of ADAM12 in endochondral ossification. A recent study using zebrafish showed that ADAM12 expressed in both cartilage and bone might regulate bone growth [32]. *Adam12*-deficient mice have been reportedly constructed by homologous recombination but 30% of the homozygotes died before weaning within 1 week of birth [33], albeit, the cause of death is unknown. A previous study reported that

both the width of the hypertrophic zone in the growth plate and the degree of longitudinal bone growth were increased in *Adam12*-S transgenic mice. However, these *Adam12*-deficient mice showed normal femur length [9], possibly due to compensation by other ADAM proteins [34].

In conclusion, we have demonstrated that ADAM12 is expressed in the proliferative and hypertrophic zones of the mouse growth plate. Our *in vitro* data using ATDC5 cells supports a role for ADAM12 being involved in TGF- β /BMP signaling pathway cross talk as a negative regulator of BMP signaling in the regulation of chondrocyte differentiation. It will be interesting to explore the potential involvement of microRNAs in future studies, some of which have been reported to regulate ADAM12 expression [35].

Conflicts of interest: The authors declare that they have no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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Figure Legends

Fig. 1 Creation of *Adam12*-knockout (KO) ATDC5 cells using the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system. (A) Schematic illustration of the knockout strategy for targeting the ADAM12 gene. Two gRNAs were designed in the exons of the *Adam12* gene. The gRNA targeting sequence is marked by red, and the protospacer adjacent motif (PAM) is marked by green. (B) Chromatogram of the Sanger sequencing result for homozygote mutant

Fig. 2 Expression of ADAM12 in the mouse growth plate. Immuno-staining for ADAM12 (A) and type X collagen (B). Negative control (C). (D) The expression of ADAM12 in the hypertrophic zone was significantly higher than that in the proliferative zone ($n = 5$. $*p < 0.01$ between proliferative and hypertrophic zones). Scale bar: 50 μm .

Fig. 3 Results of real-time PCR showing (A) *Adam12* and (B) *Coll10a1* gene expression during insulin-induced chondrogenic differentiation in ATDC5 cells. ($n = 3$. $*p < 0.05$, relative to 1 week)

Fig. 4 Results of real-time PCR showing the effect of *Adam12* knockout (KO) on the expression of *Igf-1*, *Runx2*, and *Coll10a1* during insulin-induced chondrogenic differentiation in ATDC5 cells. *Adam12*-KO ATDC5 cells showed significant reduction in (A) *Igf-1* and significant upregulation of both (B) *Runx2* and (C) *Coll10a1* when compared with wild-type (wt) ATDC5 cells ($n = 3$. $*p < 0.05$ relative to 0w (wt), $^{\#}p < 0.05$ relative to 0w (*Adam12* KO), $^{\S}p < 0.05$ between wt and *Adam12* KO). Results of western blot analysis (D) showing protein levels of Igf-

1, Runx2 and Col10a1 in the *Adam12*-KO and wild-type (wt) ATDC5 cells at 3 to 5 weeks of chondrocyte differentiation.

Fig. 5 Results of real-time PCR showing the effect of TGF- β 1 stimulation for 24 h on the expression of (A) *Adam12*, (B) *Igf-1*, and (C) *Runx2* in wild-type (wt) and *Adam12*-knockout (KO) ATDC5 cells. ($n = 4$. $*p < 0.05$).

Results of real-time PCR showing the effect of *Adam12* overexpression on (D) *Igf-1* and (E) *Runx2* gene expression in ATDC5 cells. ($n = 3$. $*p < 0.05$ between wt and *Adam12* overexpressed cells)

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- Masahiro Horita: Study design, Data collection, Data analysis and interpretation, Manuscript preparation
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All authors revised the paper critically for intellectual content and approved the final version. All authors agree to be accountable for the work and to ensure that any questions relating to the accuracy and integrity of the paper are investigated and properly resolved.

Fig. 1

A

B

Fig. 2

Fig. 3

A

B

Fig. 4

A

B

C

D

Fig. 5