α-synuclein promotes progression of Parkinson's disease by upregulating autophagy signaling pathway to activate NLRP3 inflammasome

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Abstract. Mechanism by which α-synuclein affects the progression of Parkinson's disease through Pyrin Domain Containing Protein 3 (NLRP3) was explored. Peripheral blood plasma of 40 Parkinson's disease patients and 40 normal healthy people attending the department of neurology of the Third Affiliated Hospital of Qiqihar Medical University were collected from March 2018 to January 2019. The expression levels of oligomers, phosphorylated α-synuclein, interleukin-1β (IL-1β), interleukin-6 (IL-6) and transforming growth factor-α (TGF-α) in plasma were detected by ELISA. Astrocytes in mouse brain tissues were extracted by primary culture method, the cells were divided into drug group and the drug + inhibitor group. After adding 0, 5, 10 and 20 µg oligomerized α-synuclein or 5 mM autophagy inhibitor 3-Methyladenine (3-MA), the expression level of NLRP3, caspase-1, IL-1β and Atg5 proteins in the cells was detected. The expression level of IL-1β in peripheral blood of PD patients was significantly increased (0.604±0.136 µmol/l vs. 1.876±0.327 µmol/l, P<0.0001) while there was no significant difference between IL-6 and TGF-α. Both oligomers (0.171±0.045 µmol/l vs. 0.676±0.084 µmol/l, P<0.0001) and phosphorylated α-synuclein (0.128±0.041 µmol/l vs. 0.849±0.108 µmol/l, P<0.0001) in peripheral blood of PD patients were significantly elevated. The expression levels of NLRP3, caspase-1 and IL-1β in mouse astrocytes all increased with the increase of the concentration of oligomerized α-synuclein, and Atg5 protein expression also increased gradually with the concentration, and reached the highest level when the concentration was 10 µg/ml. The expression levels of NLRP3, caspase-1 and IL-1β were inhibited after the addition of autophagy inhibitor 3-MA. α-synuclein mediates the activation of NLRP3 inflammasome in PD patients by upregulating Atg5 protein expression.

Introduction

Parkinson's disease (PD) is a degenerative neurological disease affected by a variety of comprehensive factors. Its main manifestations include motor symptoms represented as sluggish movement, tremor and paralysis, postural gait disorder and other non-motor symptoms such as depression, which can seriously threaten the quality of life of middle-aged and elderly patients (1). Pathologically, PD is characterized by a severe loss of dopaminergic neurons in the striatum nistriatum, a change that is caused by the accumulation and spread of characteristic Lewy bodies and nerve sheath inclusions due to misfolded fibers (2). These misfolded fibers, mainly derived from a neurotoxic form of α-synuclein, can accumulate spontaneously at the junction of brain regions and cause degeneration of dopaminergic neurons (3,4). In recent years, a large number of studies have shown that α-synuclein plays an important regulatory role in non-specific immunity of the body. Among them, A53T mutant α-synuclein can significantly activate the key transcription factor of inflammatory response, NF-kB, thereby activating microglia and astrocytes (5). Apart from that, α-synuclein can also promote the expression of toll-like receptors (TLRs), such as TLR2, TLR3 and TLR7 (6). As an important component of non-specific immunity, TLR is regularly localized on the surface of cells or internal vesicles, recognizing molecules with conserved structures and stimulating the body to produce an immune response that causes an inflammatory response. Therefore, we highly hypothesized that mutations and abnormal aggregation of α-synuclein might have a certain regulatory effect on the inflammatory state of the body.

At present, emerging evidence exhibits that systemic low-level inflammation can accelerate the development of degenerative changes in the brain with increasing age (7), and most men and women aged over 65 years have increased...
β-amyloid and the secretion of the inflammatory factor IL-1β. Many experiments have proved that its expression is closely
related to the onset of age-related diseases, however, its role
in the pathogenesis of PD remains poorly understood. While
autophagy, acts as an intracellular, homeostatic mechanism,
mediates the inactivation or selective clearance of harmful
substrates or secondary material signals, such as damaged
mitochondria. There is increasing evidence that autophagy
exerts vital effect on the innate immune response. In addition,
autophagy-related pathways are implicated in the regulation of
NLRP3 inflammasome activation (10-12). It has been reported
in the literature that α-synuclein can promote immune activa-
tion in PD patients. Therefore, this study aimed to investigate
the mechanism of α-synuclein in affecting the progression of
PD through autophagy-related pathways.

Materials and methods

Study subjects. This study was approved by the Medical
Ethics Committee of the Third Affiliated Hospital of Qiqihar
Medical University (Qiqihar, China), and the experiment was
conducted in accordance with the Guide for the Care and Use
of Experimental Animals. Peripheral blood samples of 40 PD
patients admitted to the department of neurology of the hospital
from March 2018 to January 2019 were collected. Inclusion
criteria: Patients with the characteristic progressive progres-
sion of the disease and 2 or more typical symptoms (including
static tremor, sluggish movement, rigidity, postural instability)
were included, while those with other neurological tumors were
excluded. Further 40 healthy normal people who participated
in physical examination were enrolled in the control group.
They were matched with PD patients according to age, sex and
education level, and those with traumatic brain injury, suspected
degenerative disease with Parkinson's disease, Parkinson-
related diseases (such as Lewy body dementia and Alzheimer's
disease), or severe organic or systemic diseases were excluded.
All the study subjects were informed of the purpose and the
protocol of the study before the blood was drawn, and written
informed consent was obtained.

Enzyme-linked immunosorbent assay (ELISA) for the detection of plasma oligomerized, phosphorylated α-synuclein and IL-1β.
Detection method of oligomerized α-synuclein: The detection method was carried out in accordance with the established
detection method (13). An amount of 100 µl of 3D5 monoclonal antibody (Santa Cruz Biotechnology, Inc., sc-47696) with a final
concentration of 1 µg/ml was added to each well of the
96-well plate, incubated at 37°C for 2 h for coating, and washed
with PBST after culturing overnight at 4°C. Then each well was
incubated at 37°C for 2 h with 200 µl of 10% BSA sealant and
then washed with PBST. Then recombinant human α-synuclein (rPeptide, s-1005-2) diluted by multiple ratio (concentrations
of 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0 mol/l, respectively) was
added with the sample to be tested into each well, incubated at
37°C for 2 h before rinsing with PBST. Then, 100 µl of 1 mg/l
avidin (Solarbio, A8280) was added, and the cells were incu-
bated for 2 h at 37°C and washed with PBST. Diluted human
avatin-labeled alkaline phosphatase (Solarbio, k0068r-ap)
was added, incubated at 37°C for 1 h and then washed with
PBST. Finally, 100 µl PNP was added to each well, and the
color was developed at 37°C for 30 min, and the absorbance
was measured using a 405 nm wavelength microplate reader
(Thermo Fisher Scientific, Inc., Multiskan FC). Three repli-
cate wells were set for each sample and the experiment was
performed three times.

Detection method of phosphorylated α-synuclein: α-synuclein (p-Ser129) (Abcam, ab51253) monoclonal mono-
body (100 µl) with a final concentration of 0.1 mg/l was added
to each well of the 96-well plate, and the remaining steps were
the same as stated above.

Detection method of IL-1β, IL-6 and TGF-α: The collected peripheral blood of the patient was allowed to stand at room
temperature for 40 min, and the serum was precipitated and
centrifuged at 1,000 x g for 10 min at 4°C. After centrifuga-
tion, the light yellow transparent serum from the upper layer
was collected and stored frozen at -80°C in a refrigerator for
later use. Then the expression levels were measured using
IL-1β (ProteinTech Group, Inc., KE00021), IL-6 (ProteinTech
Group, Inc., KE00007) and TGF-α (Qiaoyu Biotechnology,
QY-MB10203) ELISA kits, and the procedures were in accor-
dance with the kit instructions.

Primary cell isolation and culture. Neonatal 3-4d C57BL/6
mice were sacrificed by spinal dislocation, and the heads of
the mice were placed on ice to isolate the cerebral cortex. The
primary cell separation method was established as previously
described (13). After the cerebral cortex was removed, 0.25%
trypsin (Beyotime, C0201) was used for digestion and cell
suspension. Then, 90% Dulbecco's modified Eagle's medium:
Nutrient Mixture F-12 (DMEM/F-12) (HyClone, sh30023.01b)
+10% fetal bovine serum (FBS) (HyClone, Sv30087.01)
+100 µ/ml penicillin +100 µ/ml streptomycin (Beyotime,
C0222) was cultured in a polylsine-coated culture flask for
16 h. According to the different adhesion ability between glial
cells, the culture flask was put into a thermostatic oscillator,
oscillated at 37°C, 180 rpm, for 5 h, and the bottom layer
cells were collected as astrocytes with higher purity. Finally,
the cells were digested with 0.25% trypsin, subcultured in a
plurality of flasks at 1:2, and cultured to a density of 90%.

Drug treatment of cells. The number of astrocytes was
adjusted to 1x10^7/ml, inoculated in the 6-well plate, with 3 ml
per well. The astrocytes were divided into control group and
drug treatment group when they grew to 80% density. The
drug treatment group was divided into low, medium and high
concentration groups according to different concentrations of
the added drugs, which were 5, 10 and 20 µg/ml of oligomer-
ized α-synuclein. Judged by whether autophagy inhibitors
were added or not, the cells were divided into a drug treatment
group and an autophagy inhibition group. The drug treatment
group was treated with 10 µg/ml oligomerized α-synuclein,
while the autophagy inhibition group was added with 10 µg/ml
oligomerized α-synuclein and 5 mM 3-MA (MCE, hy-19312),
and the culture was continued for 24 h.

Western blot for protein detection. RIPA lysate (Beyotime,
P0013C) was applied for cell lysis and centrifugation at
15,000 x g for 15 min at 4°C to extract cell protein. The protein
concentration was detected by BCA assay (Beyotime, P0012S) and gel electrophoresis was performed in 15% SDS-PAGE. Then, the protein was transferred to PVDF membrane by semi-dry transfer method, and 5% skim milk powder was adopted to seal the membrane for 1 h. Then, NLRP3 (ProteinTech Group, Inc., 19771-1-AP, 1:1000), caspase-1 (ProteinTech Group, Inc., 22915-1-AP, 1:500), IL-1β (ProteinTech Group, Inc., 16806-1-AP, 1:1000) and Atg5 (ProteinTech Group, Inc., 10181-2-AP, 1:1000) were added, respectively, with GAPDH (ProteinTech Group, Inc., 60004-1-Ig, 1:5000) as the internal reference, triple rinsed with 0.1% PBST after incubation overnight at 4˚C, adding corresponding HRP-labeled goat anti-rabbit (or mouse) immunological secondary antibody (ProteinTech Group, Inc., SA00001-1/2, 1:3000), incubated for 1 h at room temperature, and washed 3 times with 0.1% PBST. Finally, ECL chemiluminescence reagent (Beyotime, P0018FS) was employed to develop, fix and photograph (Odyeesy, LI-COR) in the dark, and then it was analyzed using the Corning Axygen gel imaging system.

Statistical analysis. All the collected data were statistically analyzed by SPSS 23.0. The measurement data were expressed as mean ± standard deviation. Independent t-test was applied for comparison between the two groups, and one-way ANOVA was used for comparison among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Elevated plasma inflammation-related factor IL-1β in PD patients. The levels of inflammatory factors in the plasma of PD group and control group were detected by ELISA. It was found that IL-1β was significantly increased in the patient group (0.604±0.136 µmol/l vs. 1.876±0.327 µmol/l, P=0.002) (Fig. 1A), while the expression levels of IL-6 (321.4±45.7 pmol/l vs. 403.8±43.19 pmol/l, P=0.206) (Fig. 1B) and TGF-α (264.3±38.2 pmol/l vs. 278.8±53.3 pmol/l, P=0.827) (Fig. 1C) did not identify any significant increase.

Increased plasma α-synuclein expression in PD patients. Plasma α-synuclein levels in the PD group and the control group were detected by ELISA, and it was observed that both the expression levels of oligomerized α-synuclein (0.171±0.044 µmol/l vs. 0.676±0.083 µmol/l, P<0.0001) (Fig. 2A) and phosphorylated α-synuclein...
Oligomerized α-synuclein induction of NLRP3 inflammasome and expression of related molecules in astrocytes. Oligomerized α-synuclein with different concentrations of 0, 5, 10, and 20 µg/ml were added to the astrocytes of primary mice, respectively, and the expression level of NLRP3 was detected by western blot. It was found that NLRP3 expression was elevated with the increase of drug concentration in the cells (Fig. 3A and B). Further detection of other inflammasome related molecular proteins revealed a similar trend in caspase-1 and IL-1β (Fig. 3C and D).

Oligomerized α-synuclein affects NLRP3 inflammation by affecting autophagy. After the addition of 0, 5, 10, and 20 µg/ml oligomerized α-synuclein, the autophagy-related molecule Atg5 was upregulated in astrocytes with increasing drug concentration (Fig. 4A and B). To further explore whether oligomerized α-synuclein causes NLRP3-related inflammatory responses by affecting autophagy, we added oligomerized α-synuclein/oligomerized α-synuclein + autophagy inhibitors (3-MA) to cells. Western blot results showed that the expression levels of NLRP3, caspase-1 and IL-1β expression were inhibited by 3-MA (Fig. 4C and D), indicating that the activation of NLRP3 inflammatory pathway by oligomerized α-synuclein was mediated by autophagy.

Discussion
As a neurodegenerative disease, Parkinson's disease has been extensively confirmed to be significantly associated with age, but its specific pathogenesis remains a subject of investigation. In recent years, studies have found a positive correlation between age and inflammatory status (7), as serum levels of IL-6, TNF, and IL-18 are elevated in older people over the age of 65 (8,9). A growing number of studies have found that with the increase of age, a systemic mild inflammatory state can lead to the progression of degenerative changes in the brain, thus forming the 'hypothalamic microinflammation' theory, which mainly believes that under the condition of aging and metabolic syndrome, the abnormal stimulation of the hypothalamus activates the inflammatory signal pathway, leading to the formation of chronic and stable inflammatory background (14,15). The NLRP3 inflammasome acts as a multimeric complex in the cytoplasm and serves as a platform for caspase-1 activation and promotion of IL-1β maturation (16). It has been found that overexpression is associated with many age-related inflammatory disorders (10). The role of NLRP3 inflammasome in degenerative diseases may be related to their responses to various danger-associated molecule patterns (DAMP), including α-synuclein, extracellular adenosine triphosphate (ATP), excessive glucose, ceramide, amyloid, urate and cholesterol crystals (17). In this study, the plasma levels of oligomerized and phosphorylated α-synuclein in peripheral blood of patients with PD were both higher than

(0.128±0.041 μmol/l vs. 0.849±0.108 μmol/l, P<0.0001) (Fig. 2B) in the PD group were markedly increased, with statistically significant differences.
those in normal subjects. ELISA method applied to analyze the expression level of inflammatory factors in plasma of PD patients, revealed that the expression level of IL-1β was significantly increased, while there was no significant difference in IL-6 and TGF-α. These results suggest that α-synuclein in PD patients may activate NLRP3 inflammatory factors to release excess IL-1β, thus leaving the patient in a state of chronic mild inflammation.

The characteristic pathological changes of PD are Lewy body formation and dopaminergic neuropathy, of which the latter often occurs in the late stage of the disease, when the neurodegeneration rate can be as much as 70% (18). The formation of Lewy body is usually much earlier than that of dopaminergic neuropathy, so the early Lewy body formation is the best target for disease occurrence and intervention. The main component of Lewy body is abnormally aggregated α-synuclein. Studies have shown that α-synuclein can be released from neurons into extracellular fluids, including cerebrospinal fluid and plasma. In the current study, ELISA was used to find a significant increase in plasma α-synuclein expression in PD patients. α-synuclein normally exists in presynaptic nerve terminals and mediates normal physiological functions (19). In special cases, its neurotoxic abnormal metabolites are released into the extracellular matrix, which can activate glial cells and innate immune responses, leading to neuronal damage (20-22). While in turn, neurons can be

Figure 4. Expression levels of Atg5 and NLRP3 related proteins in mouse astrocytes at different drug concentrations. (A) Western blot of expression of Atg5 protein in mouse astrocytes after adding 0, 5, 10, and 20 µg/ml oligomerized α-synuclein. (B) Expression and statistical representation of NLRP3, caspase-1 and IL-1β in cells after administration of 10 µg/ml oligomerized α-synuclein and/or 3-MA in mouse astrocytes. (C and D) The western blot of expression levels and quantification of NLRP3, caspase-1 and IL-1β expression were inhibited by 3-MA. NLRP3, Pyrin Domain Containing Protein 3; 3-MA, 3-methyladenine; IL-1β, interleukin-1β. *P<0.05; **P<0.01; ***P<0.001.
internalized by neurotoxic α-synuclein through endocytosis to avoid extensive damage, but long-term pathological accumulation of α-synuclein will result in apoptosis or degeneration of neuronal cells (21). A study demonstrated that the overexpression of α-synuclein in rodent models (23) without obvious toxicity could impair the release of neurotransmitters glutamate and dopamine. Through direct imaging of the synaptic vesicle circulation process, it was found that the synaptic vesicle circulation pool was significantly reduced, and ultrastructural analysis exhibited that the density of synaptic vesicles in the active area was decreased. Imaging further revealed the defect of reaggregation of synaptic vesicles after endocytosis. Therefore, the increased level of α-synuclein in PD patients will lead to a special physiological defect in the process of synaptic vesicle regeneration, which precedes the detectable neuro-pathology and provides a solid theoretical basis for further exploration of the influence of α-synuclein on the progress of PD disease. In the present study, mouse astrocytes were extracted in the primary generation, and different concentrations of oligomerized α-synuclein were added to the culture medium. The activation of NLRP3-related signaling pathway was detected by western blot, and significant activation of NLRP3, caspase-1 and IL-1β was observed. Combined with a large number of literature and the results of this study, it can be concluded that α-synuclein plays an important regulatory role in non-specific immune responses and is of great significance in the pathogenesis of neurodegenerative diseases.

Some studies have analyzed the autopsy of brain tissue of PD patients, and the results showed that there was obvious microglial cell activation and glial cell invasion in the regions with degeneration of dopaminergic neurons (24). The reason is that abnormal lipids, proteins and metabolic stress factors accumulated in tissues and neurons of PD patients (such as α-synuclein or mitochondrial dysfunction) could send stress signals to small glial cells and astrocytes in the environment (23). Others (25) have reported that when severe metabolic and lipid disorders occur in neurons, fatty acids and other molecules can directly participate in the activation of neuronal inflammation in brain tissue. In recent years, researchers have found that α-synuclein activates microglia activation and produces neuroinflammation in neurodegenerative diseases such as PD and other related diseases. A study (26) displayed that the combined application of wild-type α-synuclein and toll-like receptor stimulation (TLR) in the microglia of primary mice could significantly increase the secretion levels of IL-6, McP-1 and CXCL10. In addition, it showed that microglia stimulated by α-synuclein could simultaneously differentiate into M1/M2 intermediate phenotype. According to some other studies, α-synuclein upregulates the expression of immunosome-regulating receptors TLR2, TLR3, and TLR7 (5,6), and activates NF-κB, a key transcription factor for inflammation. Previous studies mainly focused in microglia and neuronal cells. In this study, astrocytes from mouse brain tissue were extracted for testing, and we found that α-synuclein could activate NLRP3 inflammasome through autophagy signaling pathway and promote the occurrence of chronic inflammatory state, which was also consistent with the previous results. Moreover, a study on traumatic brain injury also revealed that trauma could cause severe brain inflammation and significantly increase the level of α-synuclein in the brain (27), increasing the risk of PD, further validating the relationship between inflammation and Parkinson's disease. Most of the studies were designed to investigate the promotion of normal or abnormal structural α-synuclein levels by different stimuli. However, there is still scarce research on the influence of abnormally high expression of oligomerized α-synuclein on brain tissue. In this study, combined with previous literature reports, we found that there was a mutual regulatory relationship between inflammation and α-synuclein.

The relationship between inflammation and PD has long been elucidated. The 1918 influenza pandemic caused encephalitis, and some patients further developed PD, which was then called Von Ikonomo disease (28). Since then, a significant link between inflammation and Parkinson’s disease has been found by studying other viral types, in which mice with H5N1 infection showed that the virus caused microglia activation from peripheral infection to the central nervous system, and α-synuclein polymerization and death of dopaminergic neurons in the substantia nigra. Whereas, previous studies believed that inflammatory response and activation of immune cells are secondary processes in PD. In recent years, studies have clarified that inflammation can directly participate in the pathogenesis of PD. First, the expression levels of pro-inflammatory factors, including TNF-α, IL-1β, IL-6 and TLR, were increased in PD brain tissue. Second, injection of PD-associated toxins (MPTP and 6-hydroxydopamine) in a mouse experimental model could cause significant inflammation in the brain tissue after injection and lead to degeneration of dopaminergic neurons (29,30). However, intracerebral injection of lipopolysaccharide (LPS) can induce intracellular accumulation of insoluble polymeric α-synuclein (31), reducing the resistance of dopamine neurons to MPTP and 6-hydroxydopamine (32), which is mainly related to the activation of related proinflammatory factors, especially IL-1β (33). Accorded with previous results, the addition of IL-1β receptor antagonists significantly reduced TNF-α and IFN-γ expression levels and abolished LPS-mediated dopamine neuronal death below toxic doses (34). In another model of inflammation, injection of the TLR3 agonator polyglucoside:polycysteine [poly(IC)] into the substantia nigra of mice led to persistent inflammation, increased sensitivity to low doses of 6-hydroxydopamine, and interfered with proteins involved in synaptic transmission and axonal transport (35). Compared with the normal control group, the expression of TLR2 and TLR9 in the brain of PD patients was increased, and the expression of TLR2 was positively correlated with the accumulation of α-synuclein (36) in the body.

Furthermore, it has been reported that α-synuclein stimulation can significantly activate inflammatory monocytes and microglia in mice, and promote PD disease progression by producing excessive inflammatory responses (5,6). Moreover, studies have indicated that autophagy is closely related to immune cells and may be involved in the pathogenesis of many diseases, including neurodegeneration, metabolic disorders and aging-related diseases. For example, autophagy is reported to participate in the negative regulation of NLRP3 related signaling pathways, and the product of mitochondrial reactive oxygen species (37) and autophagosome dysfunction,
cathepsin B (38), can be used as a ‘second signal’ to stimulate the activation of NLRP3 inflammasome. Previously, it was exhibited that the exposure of methamphetamine (METH) to α-synuclein significantly reduced chaperon-mediated autophagy activity, and the decreased lam-p2a expression could alleviate neurotoxic reactions induced by α-synuclein (39). On the contrary, overexpression of the heat shock protein 70 (HSP70) reduced abnormally aggregated α-synuclein and inhibited apoptosis induced by METH exposure. A study on PD (40) suggested that the addition of autophagy small molecule inhibitors in cell experiments and animal models could inhibit the formation of NLRP3-PYCARD-CASP1 complex, thereby delaying the progression of PD. In line with previous studies, we found that the expression of autophagy-associated molecule Atg5 was significantly increased when stimulated by α-synuclein, and autophagy inhibitor 3-MA inhibited α-synuclein-mediated activation of NLRP3-related molecules. α-synuclein, as an immunogenic substance, is usually an abnormal metabolite of nerve cells, which activates the NLRP3 inflammasome pathway through the autophagy-related molecule Atg5.

We did not yet carry out in vivo experiments in mice, nor did we explore the activation of α-synuclein and autophagy on the inflammatory response in vivo, or the dynamic process of the changes of Lewy body and dopaminergic neurons in the substantia nigra. Thus, further studies are anticipated. By examining α-synuclein and inflammation-related factors in peripheral blood of PD patients, this study not only verified the activation effect of α-synuclein on NLRP3-related molecules and autophagy in cells, but also found that the application of autophagy inhibitor 3-MA could significantly inhibit the inflammatory pathway, providing a solid basis for autophagy inhibitors to be potential targets for PD treatment.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

XW, JC, DH and LD led the conception and design of this study. XW, JC, XZ, LJ, YY and FG were responsible for the data collection and analysis. DH, LD and YY were in charge of interpreting the data and drafting the manuscript. XW and DH made revision from critical perspective for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Third Affiliated Hospital of Qiqihar Medical University (Qiqihar, China). Signed informed consents were obtained from the patients and/or the guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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