Neutrophil extracellular trap formation and deoxyribonuclease I activity in patients with otitis media with antineutrophil cytoplasmic antibody-associated vasculitis

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Abstract

No previous studies focused on the degradation of neutrophil extracellular traps (NETs) or deoxyribonuclease (DNase) I activity in the pathogenesis of otitis media with antineutrophil cytoplasmic antibody-associated vasculitis (OMAAV). The aim of this study was to explore the formation and degradation of NETs in the middle ear of patients with OMAAV during the onset and remission phases of the disease, with a particular focus on the relationships between the quantifiable NETs levels and DNase I activity. OMAAV patients were eligible for inclusion. Patients with otitis media with effusion (OME) were examined as controls. The levels of cell-free deoxyribonucleic acid (DNA), citrullinated-histone H3 (cit-H3)-DNA complex and myeloperoxidase (MPO)-DNA complex were quantified using an enzyme-linked immunosorbent assay. DNase I activity was measured using a fluorometric method. The quantifiable levels of cell-free DNA, cit-H3-DNA complex and MPO-DNA complex in the middle ear lavage of patients with OMAAV at onset were significantly higher than those in patients with OMAAV at remission and in patients with OME. DNase I activity in the patients with OMAAV at onset was significantly lower than those in patients with OMAAV at remission and OME, and was negatively correlated with the level of MPO-DNA complex. This study suggests that excessive NET formation and impaired DNase I activity are involved in the pathogenesis of OMAAV. NETs and DNase I activity may be useful biomarkers for the diagnosis and disease activity of OMAAV.

Introduction

Otitis media with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (OMAAV) is a relatively rare disease, which occasionally progresses to complete deafness and the systemic form of ANCA-associated vasculitis (AAV) [1]. AAV is comprised of granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA), which commonly involves various organs and is a life-threatening disorder [2]. In 2004, Brinkmann et al. demonstrated that neutrophil extracellular traps (NETs) were released as a result of neutrophil extracellular trap cell death (NETosis) [3]. NETs are composed of extracellular deoxyribonucleic acid (DNA) fibers and histones decorated with various enzymes, and lead to the formation of a physical net in which pathogens are entrapped and killed by elastase, defensin, and reactive oxygen species [3–5]. Despite their beneficial effects in host defense, the excessive formation of NETs induces vessel wall inflammation, thrombosis, and pathogenic ANCA [6, 7]. Recent studies have suggested that the vicious cycle of NETs-ANCA induction could be critical to the pathogenesis of OMAAV [8].

Although the formation and degradation of NETs are regulated in a strict manner, a failure in regulation leads to unfavorable consequences. It has been generally recognized that members of the deoxyribonuclease (DNase) families play vital roles in the degradation of extracellular DNA, including NETs, by targeting and cleaving DNA sequences [9]. The DNase I and DNase II families are the major DNase families, and multiple enzymes in each family play diverse roles in the development of various diseases [10]. The DNase I family consists of a number of distinct DNases, including DNase I, DNase X, DNase γ, DNAS1L2 and DNAS1L3 [9–11]. DNase I, a major nuclease present in the blood and other body
fluids, is the enzyme that is secreted outside of cells and cleaves extracellular DNA [9, 12]. The physiological increases and decreases in extracellular DNA are regulated to some extent by DNase I [13], which may be crucial for the prevention of autoimmune reactions [14]. Thus, reduced levels of DNase I lead to impaired fragmentation and elimination of chromatin, resulting in the accumulation of chromatin fragments in complex with chromatin-specific autoantibodies [9, 10]. Previous studies have reported that DNase I activity is related to the occurrence of systemic autoimmune diseases including MPA and systemic lupus erythematosus (SLE), suggesting that serum DNase I activity might be an indicator of the ongoing NETosis and a useful biomarker for the diagnosis and disease activity of autoimmune diseases [15, 16].

NETosis-derived products, such as cell-free DNA, citrullinated-histone H3 (cit-H3)-DNA complex, and myeloperoxidase (MPO)-DNA complex, may be potentially useful as markers for assessing the development and prognosis of autoimmune diseases [6, 15, 17–20]. A recent study indicated a high level of NET induction in patients with OMAAV [8]. However, no previous studies focused on the degradation of NETs or evaluated the importance of DNase I activity in the pathogenesis of OMAAV. Long-lasting exposure to NETs that are digested poorly due to low DNase I activity may result in severe, life-threatening complications [14]. Therefore, the aim of this study was to explore the formation and degradation of NETs in the middle ear of patients with OMAAV during the onset and remission phases of the disease, with a particular focus on the relationships between the quantifiable levels of NETosis-derived products and DNase I activity. This is the first report to validate the importance of DNase I activity as well as NETs formation as a new biomarker for the diagnosis and disease activity of OMAAV.

**Materials and methods**

**Ethics**

This prospective study included patients diagnosed with OMAAV in the Department of Otolaryngology, Head and Neck Surgery, between April 2018 and March 2021. All patients were instructed on the potential risks and benefits of the management program, and written informed consent for the use of their samples and clinical data was obtained after a full explanation. This research adhered to the tenets of the Declaration of Helsinki and was approved by our Institutional Review Board (No. 021–0230).

**Patients and controls**

OMAAV patients were eligible for inclusion in this analysis. OMAAV was diagnosed using the criteria proposed by the OMAAV study group of the Japan Otological Society as follows: 1) intractable otitis media with effusion or granulation, which was resistant to antibiotics and insertion of tympanic ventilation tubes, accompanied by progressive hearing loss; 2) at least one of the following four findings: (a) diagnosis of GPA, MPA and EGPA before the occurrence of ear symptoms; (b) positivity for serum MPO- or PR3-ANCA; (c) histopathologically consistent with AAV; and (d) at least one accompanying AAV-related symptom involving organs other than the ear (eye, nose, pharynx/larynx, lung, kidney, facial palsy,
hypertrophic pachymeningitis, mononeuropathy and others); and 3) exclusion of other types of intractable otitis media such as bacterial otitis media, cholesterol granuloma, cholesteatoma, malignant osteomyelitis, tuberculosis, neoplasms and eosinophilic otitis media, as well as exclusion of other autoimmune diseases and vasculitis other than AAV, such as Cogan's syndrome and polyarteritis nodosa among others [1].

Patients with otitis media with effusion (OME) were examined as controls. OME was defined as the presence of fluid in the middle ear without signs or symptoms of acute ear infection based on the clinical practice guidelines on OME published by the American Academy of Otolaryngology Head and Neck Surgery Foundation [21].

The exclusion criteria for subjects and controls were as follows: 1) fluid samples of less than 0.1 ml that cannot provide a quantifiable level of NETosis-derived products; 2) a history of definitive ear disease such as familial hearing loss, chronic noise exposure, ototoxic drug intake, head trauma, radiation therapy, acoustic neuroma or inner ear malformation; 3) a history of cancer, diabetes, deep vein thrombosis, acute coronary syndrome, ischemic stroke or other systemic autoimmune diseases such as Cogan's syndrome, SLE, rheumatoid arthritis, IgG4-related disease, sarcoidosis or aortitis syndrome, in which NETs may be involved [5, 16, 17, 19, 22, 23]; 4) pregnancy; and 5) less than 20 years of age.

### Treatment Procedure

The treatment protocol was adjusted based on the severity of disease, age, comorbidities, patient wishes and/or the attending physician's discretion in each case. Patients with OMAAV were essentially treated with intravenous methylprednisolone pulse therapy (500–1000 mg/body/day) for 3 days followed by high-dose oral prednisolone (1 mg/kg/day) for 4 or more weeks at active-stage, which was then tapered gradually and maintained (5–10 mg/body/day) while serum ANCA titer and C-reactive protein levels were monitored. An immunosuppressant, such as cyclophosphamide, rituximab, methotrexate or azathioprine, was added to the steroid therapy.

### Evaluation of the disease activity

The disease activity of vasculitis was measured using the Birmingham Vasculitis Activity Score (BVAS) version 3.0, which was previously validated and has been generally used for clinical trials for vasculitis [24]. The BVAS form is divided into 9 organ-based systems, with each section including symptoms that are typical of that particular organ involvement in systemic vasculitis. The scores range from 0 to 63, and higher scores indicate more active disease. Remission of OMAAV was defined as a state in which the BVAS was 0 (or ≤ 1, if all items were persistent) and the maintenance dose of oral prednisolone was ≤ 10 mg/body/day [24].

### Sample collection

The middle ear lavage samples were obtained at onset (initial presentation) and remission of disease. Tympanic membrane anesthesia using iontophoresis was applied to the external auditory canal with 4% lidocaine (AstraZeneca Co., Ltd., London, UK). The samples were aspirated from the middle ear through
the anterior-inferior portion of the tympanic membrane using a 1-ml tuberculin syringe with a 24- or 26-gauge needle under a microscope by lavaging the middle ear cavity with a 0.3-ml saline bolus. In cases of OMAAV with tympanic membrane perforation or tympanic ventilation tube insertion during remission, the middle ear lavage samples were collected through these routes in similar manner. The supematants were centrifuged at 1500 rpm for 5 minutes and stored at -80°C until analysis. The levels of NETosis-derived products were quantified by detecting the major components consisting of DNA fibers and histones decorated with neutrophil-specific, cytoplasmic granule proteins, such as cell-free DNA, cit-H3-DNA complex, and MPO-DNA complex in the fluid samples, which is consistent with the method used in most previous studies [6, 8, 18–20].

Blood was collected into serum separator tubes containing clot activator and serum separator gel by a trained hospital phlebotomist. After completion of biochemical testing ordered by the clinician, the remaining serum was released to the research laboratory. Serum samples were immediately divided into small aliquots and stored at -80°C until analysis. DNase I activity in the serum at onset (initial presentation) and remission of disease was measured using a fluorometric method.

**Immunofluorescent staining**

For histological analysis, each middle ear lavage sample was immediately smeared on a glass slide. To identify NETs, DNA and cit-H3 as the main components and MPO as neutrophil-specific granules were visualized simultaneously by immunofluorescence, as previously described [15, 25]. The samples on the glass slides were fixed with 4% paraformaldehyde for 30 min, washed with phosphate-buffered saline (PBS), and then incubated for 60 min with a mouse anti-histone H3 (citrulline R2 + R8 + R17) antibody (Abcam, ab5103) and a mouse anti-human MPO antibody (4A4; Bio-Rad Laboratories, Tokyo, Japan) as the primary antibody. After washing in PBS, each primary antibody was visualized using secondary antibodies coupled to Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen, A27039) and Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, A11001). After incubation for 60 min with the secondary antibodies, the specimens were washed with PBS, and the DNA was stained with 4', 6-diamidino-2-phenylindole (DAPI; Invitrogen, P36962) in PBS for 5 min. All procedures were performed at room temperature. The specimens were analyzed using a confocal laser-scanning microscope (LSM 710; ZEISS, Jena, Germany). This analysis considered string- or web-like extracellular DNA and cit-H3 extending from the cell body colocalized with MPO to be NETs-positive cells. Samples were considered negative for the presence of NETs if no cells harboring NETs were identified in 300 neutrophils by immunostaining.

**Evaluation of the cell-free DNA level**

The cell-free DNA level in the middle ear lavage was determined by enzyme-linked immunosorbent assay (ELISA) using Cell Death Detection ELISA PLUS (Roche, Cat. No: 1177442500) according to the manufacturer's protocol [6, 8, 18–20]. The determination was based on quantitative sandwich ELISA using anti-DNA antibody and anti-histones antibody, specifically binding mono- and oligonucleosomes derived from the nuclei of eukaryotic cells. The optical absorbance was measured at 405 nm using an ELISA reader (Bio-Rad 680; Bio-Rad Laboratories, Tokyo, Japan).
Evaluation of the cit-H3-DNA complex level

The cit-H3-DNA complex level in the middle ear lavage was quantified using ELISA, as previously described [6, 8, 18–20]. An anti-histone H3 (citrulline R2 + R8 + R17) antibody (Abcam, ab5103) was coated on 96-well microtiter plates, with 1% bovine serum albumin used for blocking. The fluid sample, together with a peroxidase-labeled anti-DNA monoclonal antibody (Cell Death Detection ELISA kit; Roche, Cat. No.: 11774425001), was then added. The optical absorbance was measured at 405 nm using an ELISA reader (Bio-Rad 680; Bio-Rad Laboratories, Tokyo, Japan).

Evaluation of the MPO-DNA complex level

The MPO-DNA complex level in the middle ear lavage was quantified using ELISA, as previously described [6, 8, 15, 25]. A mouse anti-human MPO antibody (4A4; Bio-Rad Laboratories, Tokyo, Japan) was coated on 96-well microtiter plates. After blocking with 1% bovine serum albumin, the fluid sample was then added together with a peroxidase-labeled anti-DNA monoclonal antibody (Cell Death Detection ELISA kit; Roche, Cat. No: 11774425001). After incubation, the peroxidase substrate was added according to the manufacturer's instructions. The optical absorbance was measured at 405 nm using an ELISA reader (Bio-Rad 680; Bio-Rad Laboratories, Tokyo, Japan).

Measurement of DNase I activity

DNase I activity in serum was measured using a DNase I Activity Assay Kit (Abcam, ab234056) according to the manufacturer's protocol. Samples, a positive control, and a background control were added together with DNA probe to each well. Enzyme activity was detected upon cleavage of a DNA probe, which yields a fluorescent DNA product measured at Ex/Em = 651/681 nm in kinetic mode every 30 seconds for 90 minutes at 37°C, using a SpectraMax Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 6.0; GraphPad Software Inc.; La Jolla, CA, U.S.A.). Statistical differences were analyzed using the Wilcoxon matched-pairs signed-ranks test, Mann-Whitney U-test for two independent groups and Kruskal-Wallis test for three or more independent groups, with a p value of less than 0.05 considered statistically significant. Differences in quantitative data were analyzed using one-way ANOVA among more than three groups, followed by a post-hoc Steel-Dwass test. Potential associations between the levels of NETosis-derived products and DNase I activity for patients with OMAAV were determined using Spearman’s correlation analysis.

Results

Clinical profiles of patients and controls
Characteristics of the patients with OMAAV and controls are summarized in the Table. The study population consisted of 10 males and 27 females, ranging in age from 48 to 84 years (median, 67 years). Nine patients were diagnosed with GPA, 8 with MPA, 11 with EGPA and 9 patients with localized forms of OMAAV. Twenty-three patients were MPO-ANCA positive and 5 patients were PR3-ANCA positive, whereas 9 patients were ANCA negative at the initial time of sampling. As a systemic treatment, 32 patients were treated with steroid pulse therapy followed by high-dose prednisolone combined with immunosuppressive agents (cyclophosphamide, rituximab, methotrexate or azathioprine), 3 patients with steroid pulse therapy followed by high-dose prednisolone, and 2 patients with high-dose prednisolone monotherapy. The dose of oral prednisolone was tapered and maintained ≤ 10 mg/body/day while the BVAS was ≤ 1, and serum ANCA titer and C-reactive protein levels were normalized. The resolution of middle ear effusion or granulation was confirmed using otoscopic examination or CT scanning during remission of disease.

The control group comprised 37 subjects, consisting of 18 males and 19 females, ranging in age from 44 to 83 years (median, 67 years). There were no differences in background characteristics, such as age or gender distribution, between the patient and control groups.

**Presence of NETs in the middle ear lavage samples for patients and controls**

Hematoxylin-eosin staining detected high numbers of neutrophils in the middle ear lavage samples in patients with OMAAV at onset (Fig. 1A), while lower numbers of neutrophils were detected in patients with OME (Fig. 1B). Triple staining with DNA, cit-H3, and neutrophil-specific proteins was then performed using the middle ear lavage samples, and NET formation was identified in all patients with OMAAV at onset (Fig. 1C). Meanwhile, no NET formation was observed in the samples from patients with OME (Fig. 1D).

**The levels of cell-free DNA, cit-H3-DNA complex and MPO-DNA complex in patients with OMAAV at onset and remission**

Figure 2A-F shows the levels of NETosis-derived products based on cell-free DNA, cit-H3-DNA complex and MPO-DNA complex ELISA results in the patients with OMAAV and OME. The mean quantifiable levels of cell-free DNA, cit-H3-DNA complex and MPO-DNA complex expressed as arbitrary units (a.u.) were 14.2 a.u. (ranging from 0.01 to 48.2 a.u.), 12.6 a.u. (ranging from 0.01 to 41.9 a.u.), and 11.2 a.u. (ranging from 0.11 to 32.6 a.u.) in the patients with OMAAV at onset, 1.33 a.u. (ranging from 0.01 to 3.77 a.u.), 1.11 a.u. (ranging from 0.01 to 3.87 a.u.), and 0.89 a.u. (ranging from 0.02 to 2.93 a.u.) in the patients with OMAAV at remission, and 0.73 a.u. (ranging from 0.02 to 2.93 a.u.), 0.73 a.u. (ranging from 0.01 to 3.50 a.u.), and 0.73 a.u. (ranging from 0.01 to 3.50 a.u.) in the patients with OME, respectively. Patients with OMAAV at onset showed significantly higher levels of cell-free DNA (p < 0.001 and p < 0.001, respectively), cit-H3-DNA complex (p < 0.001 and p < 0.001, respectively) and MPO-DNA complex (p < 0.001 and p < 0.001, respectively) compared with the patients with OMAAV at remission and those with OME, respectively. Meanwhile, there was no significant difference in the levels of NETosis-derived products between patients with OMAAV at remission and those with OME.
DNase I activity in patients with OMAAV

Figure 3A and 3B shows the serum DNase I activity in the patients with OMAAV and OME. The mean quantifiable serum DNase I level was 160 nU/ml (ranging from 53 to 432 nU/ml) in the patients with OMAAV at onset, 600 nU/ml (ranging from 200 to 1173 nU/ml) in the patients with OMAAV at remission, and 651 nU/ml (ranging from 344 to 1026 nU/ml) in the patients with OME, respectively. The DNase I activity in the patients with OMAAV at onset was significantly lower in comparison with that in the patients with OMAAV at remission ($p < 0.001$) and OME ($p < 0.001$), respectively. Meanwhile, no significant difference in the DNase I activity was found between patients with OMAAV at remission and those with OME. It is important to stress that DNase I activity in the patients with OMAAV at remission reached an equivalent level to that in patients with OME.

The correlation between DNase I activity and cell-free DNA, cit-H3-DNA complex or MPO-DNA complex

The correlations between DNase I activity and each NETosis-derived product were examined (Fig. 4A-C). DNase I activity was negatively correlated with MPO-DNA complex ($r = -0.40$, $p = 0.015$), while there was no significant correlation between DNase I activity and cell-free DNA or cit-H3-DNA complex.

Discussion

The excessive formation of NETs has been suggested to be involved in the pathogenesis of OMAAV [8, 26, 27]. Novel methods for the evaluation of NETs are essential to providing a definite diagnosis as well as to predicting the activity and severity of OMAAV [26]. To date, immunofluorescent staining has been widely used to visualize and directly evaluate NETs themselves released as a result of NETosis [28]. The presence of NETs is defined by the identification of web-like extracellular DNA colocalized with citrullinated histones and neutrophil-derived proteins extending from neutrophils [15, 24]. This study demonstrated that NET formation in middle ear lavage of patients with OMAAV at onset could be identified by immunofluorescent staining. It has been generally recognized that the accumulation of NETs in localized regions, such as the skin, kidneys, lungs and peripheral nerves, is a characteristic of AAV and results in tissue damage [6, 7]. The deposition of NETs in the middle ear of patients with OMAAV may play a central role in disease pathogenesis through complement activation, neutrophilic infiltration and the release of destructive enzymes which, in turn, lead to damage to the stria vascularis and hair cells in the cochlea. However, the lack of objectivity and quantitativity remain critical methodological drawbacks to immunofluorescent staining [28].

In the case of fluid samples, the soluble NET remnants could be quantified using ELISA. This methodology seems to be the most specific, objective, and quantitative for the monitoring NETosis at present [28]. This study demonstrated that the quantifiable levels of cell-free DNA, cit-H3-DNA complex and MPO-DNA complex were higher in the middle ear lavage of patients with OMAAV at onset in comparison with those in patients with OME. In particular, even ANCA-negative cases indicated high
quantifiable levels of cell-free DNA, cit-H3-DNA complex and MPO-DNA complex. These findings suggest that NETosis-derived products may be useful as a biomarker for the diagnosis of OMAAV. However, cell-free DNA might be derived from dead cells other than NETosis, such as apoptosis and necrosis [28], and cit-H3-DNA complex might also be generated by cells other than neutrophils, such as macrophages, mast cells and eosinophils [29–32]. Thus, it is important to measure the complex of DNA and neutrophil-specific enzymes including MPO as indicators for the more appropriate monitoring of NETosis [26].

It is noteworthy that no NET formation was identified by immunofluorescent staining in the middle ear lavage of patients with OMAAV at remission, and the quantifiable ELISA levels of NETosis-derived products were equivalent to those in patients with OME. These findings suggest that the formation and accumulation of NETs may be useful as an indicator of disease activity in patients with OMAAV. The level of NETosis-derived products could allow early assessment for deciding the appropriate dose of medication as maintenance immunosuppressive therapy for patients with OMAAV during the remission stage of the disease. In addition, such assessment could allow more aggressive treatments to target OMAAV with a high level of NETs, particularly in relation to subjects with a poor prognosis.

The clearance of NETs also has a pivotal role in the induction of autoimmune disease [33]. Previous studies have shown that the decreased clearance of NETs is correlated with DNase I activity, suggesting DNase I is responsible for NETs degradation [15, 24, 33]. Low DNase I activity could lead to the persistence of NETs and an immunological imbalance [15, 24, 33]. DNase I is inhibited by complement over-activation as well as excessive deposition of the complement protein, C1q, which results in an inability to degrade NETs [35]. This study showed that DNase I activity in the patients with OMAAV at onset was significantly lower than that in the patients with OME, which is consistent with the previous published findings regarding SLE and MPA [15, 16]. Additionally, DNase I activity was negatively correlated with the levels of MPO-DNA complex. The accumulation of NETs due to disordered DNase I activity may induce ANCA production and activate complement [6, 7, 10]. ANCA stimulates neutrophils and induces NETosis, the production of oxygen radicals and proinflammatory cytokines, which then exaggerate the autoimmune response [6, 7]. Thus, DNase I may play a key role in the vicious cycle of NETs-ANCA induction involved in the pathogenesis of OMAAV. It is noteworthy that DNase I activity in the patients with OMAAV at remission improved to the equivalent level as that in the patients with OME. Patients with OMAAV undergoing steroid and immunosuppressant therapy could normalize the ability to degrade NETs, suggesting that DNase I activity may be useful as an indicator of disease activity.

**Limitations**

This study had several limitations. The results of the analyses might have been affected by the small number of samples, as well as by the detection and quantification methods employed for NETs and DNase I activity. To date, various methods and markers have been utilized to demonstrate NETosis in vitro and in vivo [28]. It should be noted that cell-free DNA and cit-H3-DNA complex are derived from dead cells, aside from neutrophils, that undergo NETosis. Methods for the quantification of DNase I activity, such as colorimetry, precipitation, fluorometry, and viscometry, are limited by technical and biological
factors. The technical factors include sample processing, choice of collection tube, and hemolysis [36]. The biological factors that could affect DNase I activity are physical activity, circadian rhythm, and hormones [13]. Additionally, it remains unclear how other enzymes in the DNase I family, such as DNase X, DNase γ, DNAS1L2 and DNAS1L3, affect the degradation of NETs [9–11]. The relationship between NET accumulation, DNase I activity, and/or the activity and severity of OMAAV is not fully elucidated. Therefore, further studies are required to understand the role of NETs and DNases I in the pathogenesis of OMAAV.

**Conclusion**

This analysis demonstrated that immunofluorescent staining could be used to identify NET formation in the middle ear lavage of patients with OMAAV at onset and the quantifiable ELISA levels of NETosis-derived products were significantly higher compared with those in patients with OMAAV at remission and those with OME. Furthermore, DNase I activity in the patients with OMAAV at onset was significantly lower than those in the patients with OMAAV at remission and OME, and was negatively correlated with the level of MPO-DNA complex. It should be noted that the levels of NETosis-derived products and DNase I activity in the patients with OMAAV after remission induction therapy improved to the equivalent levels as those in the patients with OME. These findings would suggest that excessive formation of NETs and impaired DNase I activity are involved in the pathogenesis of OMAAV. NETs and DNase I activity may be useful as biomarkers for the diagnosis and disease activity of OMAAV.

**Abbreviations**

AAV: antineutrophil cytoplasmic antibody-associated vasculitis

ANCA: antineutrophil cytoplasmic antibody

a.u.: arbitrary units

BVAS: Birmingham Vasculitis Activity Score

cit-H3: citrullinated-histone H3

DNA: deoxyribonucleic acid

DNase: deoxyribonuclease

EGPA: eosinophilic granulomatosis with polyangiitis

ELISA: enzyme-linked immunosorbent assay

GPA: granulomatosis with polyangiitis

MPA: microscopic polyangiitis
MPO: myeloperoxidase

NETosis: neutrophil extracellular trap cell death

NETs: neutrophil extracellular traps

OMAAV: otitis media with antineutrophil cytoplasmic antibody-associated vasculitis

OME: otitis media with effusion

PBS: phosphate-buffered saline

SLE: systemic lupus erythematosus

Declarations

Author contributions

SM designed the study, performed the experiments, and drafted the manuscript. YN designed the study and edited the manuscript. AF, KF, MS, KH, AH and AN provided scientific advice, clinical samples and data. AH supervised the research and revised the final manuscript.

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We have no conflicts of interest to declare.

References


Tables

Table. Characteristics of patients with otitis media with antineutrophil cytoplasmic antibody-associated vasculitis and controls
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**Figures**
Figure 1

Hematoxylin-eosin staining of the middle ear lavage samples in patients with OMAAV (A) and OME (B). Immunofluorescent staining by DNA, cit-H3, and MPO of the middle ear lavage samples in patients with OMAAV (C) and OME (D).

OMAAV; otitis media with antineutrophil cytoplasmic antibody-associated vasculitis, OME; otitis media with effusion, DNA; deoxyribonucleic acid, cit-H3; citrullinated-histone H3, MPO; myeloperoxidase
Figure 2

The levels of cell-free DNA (A), cit-H3-DNA complex (B) and MPO-DNA complex (C) in the patients with OMAAV at onset and those with OME. The levels of cell-free DNA (D), cit-H3-DNA complex (E) and MPO-DNA complex (F) in the patients with OMAAV at onset and remission.

DNA; deoxyribonucleic acid, cit-H3; citrullinated-histone H3, MPO; myeloperoxidase, OMAAV; otitis media with antineutrophil cytoplasmic antibody-associated vasculitis, OME; otitis media with effusion
Figure 3

The serum DNase I activity in the patients with OMAAV and OME (A). The serum DNase I activity in the patients with OMAAV at onset and remission (B).

DNase I; deoxyribonuclease I, OMAAV; otitis media with antineutrophil cytoplasmic antibody-associated vasculitis, OME; otitis media with effusion
Figure 4

The correlations between DNase I activity and cell-free DNA (A), cit-H3-DNA complex (B) or MPO-DNA complex (C).

DNase I; deoxyribonuclease I, DNA; deoxyribonucleic acid, cit-H3; citrullinated-histone H3, MPO; myeloperoxidase