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Short Communication

Histopathological Findings of Lung with A/H1N1pdm09 Infection-Associated Acute Respiratory Distress Syndrome in the Post-Pandemic Season

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SUMMARY: We herein report the pulmonary histopathological findings of an autopsy case of post-pandemic season A/H1N1pdm09 infection-associated acute respiratory distress syndrome (ARDS). The lung histology predominantly exhibited findings indicative of the exudative phase of diffuse alveolar damage, with similar inflammation severity observed in all sections. Furthermore, the lung sections only showed a few A/H1N1pdm09 antigen-positive cells along with a low viral RNA copy number. The sequence of the viral hemagglutinin receptor binding site identified a preference for α-2,6 linked sialic acid, suggesting low alveolar epithelial cell infectivity. The pathological findings, in this case, differed in several aspects from those of the first autopsy case of A/H1N1pdm09 infection-associated ARDS in Japan, reported during the 2009 pandemic season. In conclusion, pathological and molecular biological examinations suggested that in the post-pandemic season A/H1N1pdm09 infection, the infection-associated ARDS was not caused by direct infection-induced damage to the alveolar epithelial cells but was rather a result of indirect sepsis-mediated endothelial cell damage.

The novel A/H1N1pdm09 influenza virus (A/H1N1pdm09) was first reported in Mexico in March 2009, and has since rapidly spread worldwide through human-to-human transmission (1). According to the World Health Organization, a global A/H1N1pdm09 pandemic occurred from June 2009 to August 2010 (2). Several fatal cases of A/H1N1pdm09 infection-associated acute respiratory distress syndrome (ARDS) were reported during the 2009–2010 pandemic season (3–5), and among them, the first autopsy case in Japan was examined in detail using pathological and molecular biological techniques (6). Pathologically, the case (hereafter referred to as ''case 2009'') was complicated by viral pneumonia that developed into ARDS, and the patient subsequently died from a respiratory failure.

The A/H1N1pdm09 virus continues to circulate as a seasonal influenza virus in the post-pandemic period. The seasonal influenza viruses predominantly affect the epithelial cells of the upper respiratory tract. Consequently, the seasonal influenza-associated pneumonia generally manifests as a secondary bacterial pneumonia and pathologically, rarely develops into primary viral pneumonia (7). Indeed, the frequency of A/H1N1pdm09 infection-associated ARDS has decreased in the post-pandemic season (8). Furthermore, the pathogenic mechanism of A/H1N1pdm09 infection-associated ARDS in the post-pandemic season is speculated to differ from that reported in the pandemic season (case 2009). Herein, we report the pulmonary histopathological findings of an autopsy case with post-pandemic season A/H1N1pdm09 infection-associated ARDS, and draw comparisons with those reported in case 2009.

A 50-year-old man with impaired glucose tolerance and severe obesity (body mass index: 36 kg/m^2) complained of cough and fever (day 1). Three days later (day 4), he developed further symptoms of progressive dyspnea, a high fever (39.5°C), tachycardia (150 beats/min), and altered consciousness. He was admitted to the intensive care unit in a state of septic shock (blood pressure: 76/40 mmHg). A rapid test conducted on tracheal lavage aspirate (TLA), collected at the time of admission, tested positive for influenza A antigen. The A/H1N1pdm09 infection diagnosis was confirmed using a real-time reverse transcriptase-polymerase chain reaction (RT-PCR). A chest radiograph showed diffuse bilateral infiltrates. Laboratory findings at admission showed: white blood cell count as 10,300/μL, C-reactive protein concentration as 37.65 mg/dL, lactate dehydrogenase concentration as 1,930 U/L, partial pressure of oxygen (PaO_2) as 38.9 mmHg, and partial pressure of carbon dioxide ($PaCO_2$) as 55.3 mmHg. The blood and TLA bacterial cultures were negative. The patient was subsequently diagnosed with an influenza virus infection, sepsis, ARDS, and multiple organ dysfunction syndrome. He received mechanical ventilation and was administered intravenous peramivir for the influenza virus infection, and methylprednisolone pulse therapy for the ARDS. However, his condition worsened despite the treatment, and he developed disseminated intravascular coagulation on day 5. Although extracorporeal membrane oxygenation and continuous hemodialysis were initiated on day 5 and

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day 6, respectively, he died from multiple organ failure on day 7.

An autopsy was performed, with the exception of the brain. No specific findings were noted in the extra-respiratory organs. The formalin-fixed paraffin-embedded (FFPE) samples from the trachea and bronchi and 50 lung sections were analyzed as previously described (5,6). Briefly, hematoxylin and eosin staining was performed for histological examination; immunohistochemical staining to detect influenza virus type A nucleoprotein antigen (InfA-NP); sequencing to analyze the receptor binding site of the viral hemagglutinin (HA) protein; and a real-time RT-PCR to quantify A/H1N1pdm09 RNA copy number, in the FFPE lung sections.

Histological analysis revealed desquamation of the epithelial cells and submucosal inflammation and edema in the trachea and bronchi. In addition, findings similar to the exudative phase of diffuse alveolar damage (DAD), with hyaline membrane formation, intra-alveolar edema, and interstitial inflammatory cell infiltration were observed in each lung section (Fig. 1a–d). Immunohistochemical staining for the InfA-NP antigen showed only a few viral antigen-positive cells in several of the 50 lung sections (Fig. 1e). A sequence analysis of the *HA* gene showed aspartic acid at the amino acid position 222 in all 10 A/H1N1pdm09 clones. This characteristic residue is found predominantly in the human-adapted H1N1 viruses with a receptor preference for α-2,6 linked sialic acid (α-2,6 SA) (9) (Fig. 1f). A mutation at position 222 from aspartic acid (D) to glycine (G) (D222G) alters the receptor preference to α-2,3 SA and is reported in the fatal pandemic season cases (10). The A/H1N1pdm09 RNA copy number was calculated relative to the β-actin mRNA copy number. The average A/H1N1pdm09 RNA copy number in the lung sections was 5.3 copies/cell (range: 0.2–20.8 copies/cell, median: 2.9 copies/cell).

The pathological findings of the present case differed in several aspects from those of case 2009. Each lung section from case 2009 showed different stages of DAD, from minor changes to a more progressive proliferative DAD stage (Fig. 2a–d). In addition, many antigen positive alveolar epithelial cells were observed in the lung sections (Fig. 2e). The average copy number of A/H1N1pdm09 RNA in the sections was 834 copies/cell (range: under the detection limit–16,262 copies/cell, median: 3,861 copies/cell). A correlation was seen between the observed histological changes and the number of viral antigen positive cells, and the viral copy number in each lung section.

The majority of A/H1N1pdm09 clones in the frozen lung tissue of case 2009 reportedly harbored the D222G mutation, which enables the virus to infect alveolar epithelial cells (11). We reexamined the HA gene sequence of the 10 A/H1N1pdm09 clones from the FFPE lung sections of case 2009 and noted that 9 of the 10 clones harbored the D222G mutation (Fig. 2f).

The clinical and histopathological findings of the A/H1N1pdm09 infection-associated ARDS cases are summarized in Table 1. Both patients were obese, with mildly increased glycated hemoglobin levels, factors previously reported to be associated with increased severity of A/H1N1pdm09 infection.

ARDS damages the epithelial-endothelial barrier of the pulmonary alveoli (12). An influenza virus with a receptor preference for α-2,3 SA, such as the H5N1 and H7N9 avian influenza viruses or the A/H1N1pdm09 virus with D222G mutation, primarily infects the alveolar epithelial cells and injures them directly (13). The damage to the endothelium; however, occurs indirectly during sepsis (14).

The pathological and molecular biological analysis of

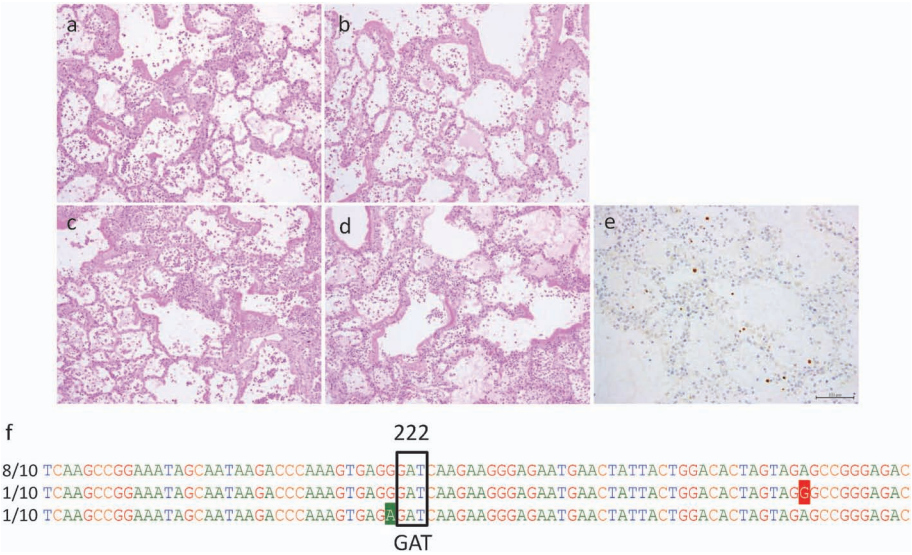

Fig. 1. Histopathological findings, viral distributions and sequences of the hemagglutinin (HA) gene of 10 A/H1N1pdm09 clones detected in lung sections from the present case. Hematoxylin and eosin staining of the representative sections from the upper right lung (a), upper left lung (b), lower right lung (c), and lower left lung (d) showed similar findings of diffuse alveolar damage (DAD) with hyaline membrane. Immunohistochemistry for influenza A nucleoprotein presented a few infA-NP-positive cells in restricted lung sections (e). Hemagglutinin sequences of 10 clones showed D222 (f). Original magnifications: a–d, 100×; e, 200×. scale bar = 100 μm.

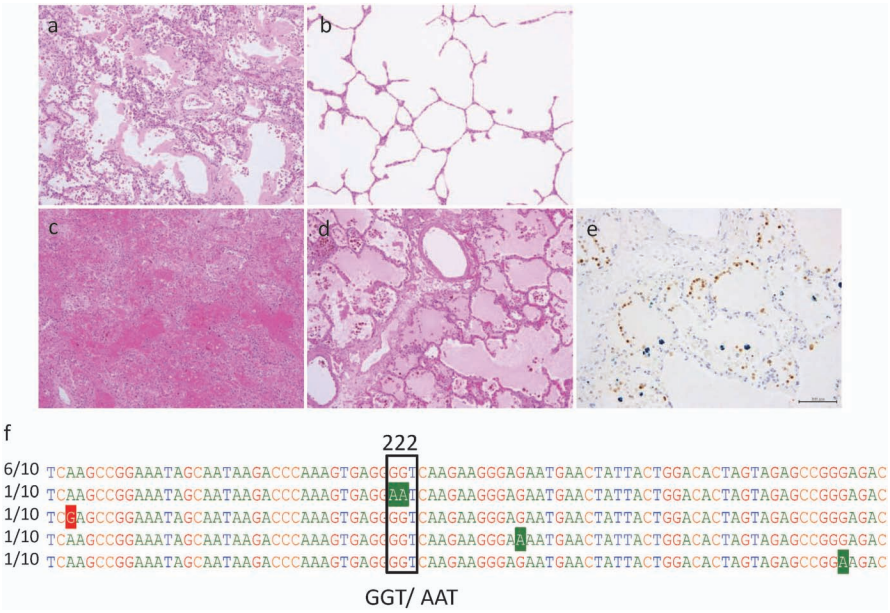

Fig. 2. Histopathological findings, viral distributions and sequences of the hemagglutinin (HA) gene of 10 A/H1N1pdm09 clones detected in lung sections from case 2009. Hematoxylin and eosin staining of representative sections from the upper right lung (a), upper left lung (b), lower right lung (c), and lower left lung (d) showed different stages of DAD in every lung section from minor changes to a more progressed stage. Immunohistochemistry for influenza A nucleoprotein presented the most infA-NP-positive cells in a lower left lung (e). Hemagglutinin sequences of 9 clones showed D222G and one clone showed D222N (f). Original magnifications: a–d, 100×; e, 200×. scale bar = 100 μm.

Table 1. Clinical and histopathological findings of A/H1N1pdm09 infection-associated ARDS

	Case 2009 (ref 6)	Present case
Season	Pandemic season	Post-pandemic season
Onset (mo, yr)	August, 2009	January, 2011
Age (yr)/gender	33 yr/male	50 yr/male
Underlying conditions	Obesity (*BMI = 38) Diabetes mellitus (HbA1c = 6.8%) Dilated cardiomyopathy Atopic dermatitis Asthma	Obesity (*BMI = 36) Impaired glucose tolerance (HbA1c = 6.4%)
Intubation day (on admission)	day 6	day 4
PaO_2 (mmHg)/$PaCO_2$ (mmHg) (on admission)	PaO_2 62.5/$PaCO_2$ 26.3	PaO_2 38.9/$PaCO_2$ 55.3
DIC (Disseminated intravascular coagulation)	(−)	day 5～
Death day	day 8	day 7
Histopathology in each lung section	Different	Similar
Viral antigen-positive cells	(−) ～ (卄)	(−) ～ (+/−)
Viral copy numbers/cell	**UDL ～ 16,262	20.2 ～ 20.8
Receptor preference	α2,3 SA	α2,6 SA
Correlation between A/H1N1pdm09 and chemokines	(+)	(−)
Cause of ARDS	Primary viral pneumonia	Severe sepsis with A/H1N1pdm infection

*BMI, Body mass index; **UDL, Under the detection limit.

the FFPE lung sections revealed that A/H1N1pdm09 virus in the present case had a receptor preference for α-2,6 SA. Therefore, the ARDS, in this case, may have resulted primarily from the indirect damage to the endothelial cells through a virus-mediated systemic inflammatory response, rather than because of primary A/H1N1pdm09 pneumonia.

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Conflict of interest None to declare.

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