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and its affiliates, the diagnosis of pulmonary cryptococcosis was confirmed in 151 patients. Of these patients, only the present subject exhibited pulmonary cryptococcosis and candidal skin abscess co-infection (0.66%, unpublished data).

Approximately two decades have passed since the introduction of the serum β-D-glucan assay for the clinical diagnosis of deep-seated mycosis in Japan (33). The assay is now widely accepted in Japan and other countries as an indispensable tool for managing febrile episodes in immunocompromised hosts. In addition, its use is included in the guidelines for the diagnosis and treatment of deep-seated mycosis (9, 34, 35). The presence of β-D-glucan in the serum signifies the presence of fungal invasion; however, the results are not specific for *Candida* species (36). False-positive findings can occur for a variety of reasons, including the use of glucan-contaminated blood collection tubes, gauze and depth-type membrane filters for blood processing, as well as *in vitro* tests using various antibiotics (e.g., some cephalosporins, carbapenems and ampicillin-sulbactam) (34, 37). Therefore, a serum β-D-glucan level exceeding the cutoff value even slightly may indicate the absence of deep-seated mycoses. A high serum β-D-glucan level is associated with cryptococcal meningitis (38) and cryptococcemia (39). The present patient was diagnosed with pulmonary cryptococcosis in addition to a moderate immunosuppressive state. A lumbar puncture did not reveal cryptococcal meningoencephalitis, and no microorganisms were cultured from his blood. On admission, the serum β-D-glucan level was approximately twice the cutoff value. Therefore, we interpreted this to be a false-positive result. However, a cautious medical examination revealed that he had candidal skin abscess co-infection.

Cryptococcus was not cultured in this case; therefore, we were unable to perform antifungal susceptibility tests for *Cryptococcus* isolates. It is difficult to distinguish between *Cr. neoformans* and *Cr. gattii* infections based on the results of histopathological examinations. Nevertheless, the first case report of a patient in Japan infected with *Cr. gattii* genotype VGIIa noted that the patient had no recent history of travel to any disease endemic areas (40), suggesting that the virulent strain may have spread to regions outside North America. *Cr. gattii* is generally geographically restricted. Furthermore, among cryptococcal infections in Japan, *Cr. neoformans* (serotype A) is the most common, with a frequency exceeding 95% (41). In addition, no azole-resistant *Cr. neoformans* isolates have been detected, even in the latest reports (42).

The *C. albicans* isolated from this patient was found to be susceptible to all antifungals tested. In the updated practical guidelines for the management of candidiasis issued by the Infection Disease Society of America, treatment with FLCZ or echinocandin is recommended for candidemia in patients with neutropenia as the initial therapy (43). The administration of a combination of the lipid formulation AMPH-B (LFAmB) or AMPH-B deoxycholate with 5-FC is recommended in cases of central nervous system (CNS) candidiasis, candidal endophthalmitis, candidal infection of the cardiovascular system, endocarditis and others (43). We chose the combination of FLCZ and 5-FC as the initial antifungal therapy in the present case based on the antifungal activity against both *Cr. neoformans* and *C. albicans* infection (44, 45). On the other hand, if a non-albicans *Candida* species e.g., *C. glabrata* or *C. krusei*, had been identified, these antifungal agents would have been changed to LFAmB. However, the patient's treatment was successful, as originally prescribed.

In conclusion, we herein reported, to the best of our knowledge, the first published case of pulmonary cryptococcosis and candidal skin abscess co-infection in an immunocompromised patient.

Author's disclosure of potential Conflicts of Interest (COI).
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Efficacy of Combination Therapy with Oseltamivir Phosphate and Azithromycin for Influenza: A Multicenter, Open-Label, Randomized Study

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Abstract

Background: Macrolides have antibiotic and immunomodulatory activities, which may have a favorable effect on the clinical outcome of patients with infections, including influenza. This study aimed to evaluate the effects of combination therapy with an anti-influenza agent, oseltamivir, and a single-dose formulation of azithromycin (AZM), which has been used for influenza-related secondary pneumonia, on influenza patients. The primary endpoint was a change in the expression levels of inflammatory cytokines. Secondary endpoints were the time required for resolution of influenza-related symptoms, incidence of complications, and adverse reactions.

Methods: Patients with seasonal influenza were enrolled in this multicenter, open-label, randomized study. Patients were stratified according to the presence of a high risk factor and were randomized to receive combination therapy with oseltamivir plus an extended-release formulation of AZM (combo-group) or oseltamivir monotherapy (mono-group).

Results: We enrolled 107 patients and randomized them into the mono-group (56 patients) or the combo-group (51 patients). All patients were diagnosed with influenza A infection, and none of the patients had comorbid pneumonia. Statistically significant differences were not observed in the expression levels of inflammatory cytokines and chemokines between the 2 groups. The maximum temperature in the combo-group was lower than that in the mono-group on day 3 through day 5 ($p = 0.048$), particularly on day 4 ($p = 0.037$).

Conclusion: To our knowledge, this is the first prospective, randomized, clinical trial of oseltamivir and AZM combination therapy for influenza. Although the difference in inflammatory cytokine expression level was not statistically significant, combination therapy showed an early resolution of some symptoms.

Name of registry: University hospital Medical Information Network (UMIN).

Trial Registration no.: UMIN000005371

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Introduction

Influenza virus infection is a major respiratory infectious disease that generally induces bronchitis, and occasionally leads to fatal pneumonia in the elderly when bacterial infections are involved [1]. Comorbid or secondary bacterial pneumonia is a severe complication related to the influenza virus infection, which suggests the importance of the latter infection in the morbidity and mortality in elderly patients with this disease [1,2]. High mobility group B1 (HMGB1), a known proinflammatory cytokine

and cytotoxic factor, is suggested to be involved in the development of influenza-related pneumonia [3]. In addition, increase in the levels of proinflammatory cytokines and monokines, including interleukin 1 (IL-1), IL-6, and IL-8, have been observed in the sera of patients and in the lungs of mice infected with the influenza virus [4]. These factors are suggested to be associated with the pathogenesis and severity of influenza virus infection [5].

Azithromycin (AZM), a 15-membered ring macrolide, is an azalide and is structurally related to the macrolide family of antibiotics. It binds to the 50S ribosomal subunit of susceptible organisms, thereby interfering with protein synthesis. AZM is approved worldwide as a broad-spectrum antibiotic for the treatment of a variety of community-acquired infections. A recently developed novel microsphere formulation of AZM (Zithromax SR 2 g) enables oral administration of high doses of AZM as a part of a single-dose regimen while maintaining tolerability.

Macrolides, including AZM and clarithromycin (CAM), a 14-membered ring macrolide, exert immunomodulatory effects on the host and antibacterial effects against the targeted microorganisms [6].

Viasus et al. reported that immunomodulatory therapies using corticosteroids and macrolides did not prevent the development of severe disease in patients with pandemic influenza A (H1N1) 2009 infection complicated by pneumonia [7]. Similarly, macrolide-based treatment has not been associated with improved survival in critically ill H1N1 patients with primary pneumonia in an intensive care unit (ICU) setting [8].

However, for patients with mild influenza, the duration of cough in patients without cough at the onset of pyrexia is significantly shorter with combined therapy with CAM and oseltamivir (Tamiflu) than that with oseltamivir monotherapy [9]. In addition, Kido et al. reported that while administration of CAM to influenza A virus (IAV)-infected mice decreases the production of tumor necrosis factor alpha (TNF-α) and increases the production of IL-12 in the blood, which results in the alleviation of flu symptoms [10], oral treatment with oseltamivir attenuates the induction of respiratory anti-IAV-specific secretory immunoglobulin A (S-IgA) immune responses [11]. Furthermore, a recent study showed that oral CAM increases the nasopharyngeal mucosal immune responses in IAV-infected children, while oseltamivir suppresses the production of mucosal anti-IAV S-IgA [12].

AZM may thus modulate airway inflammation induced by influenza virus infection. Basic studies have shown that AZM is effective against secondary bacterial pneumonia after influenza virus infection because of its inhibitory effect on the expression of various cytokines and its antibacterial activity [13].

In this study, we evaluated the efficacy of combination therapy with an anti-influenza agent, oseltamivir, and a single administration of an extended-release formulation of AZM and compared it with the efficacy of oseltamivir monotherapy in patients with influenza.

Methods

The protocol for this trial and supporting CONSORT checklist are available as supporting information (see Checklist S1 and Protocol S1).

Participants

We enrolled patients with influenza from the Nagasaki University Hospital and 13 of its affiliated hospitals and clinics. Patients aged 20 years and older with influenza A or B virus infection diagnosed by a positive rapid antigen test (RAT) for influenza were considered for enrollment. Patients had to have signs or symptoms of a seasonal flu or influenza A (H1N1) pdm 2009 virus infection with an axillary temperature ≥38.0°C and at least 2 of the following signs or symptoms at a moderate-to-severe degree: headache, muscle or joint pain, fever or chills, fatigue, cough, sore throat, and nasal stuffiness caused by influenza.

In addition, patients had to have accepted the treatment within 48 h from the onset of influenza symptoms, which were defined as follows: initial temperature elevation ≥1°C from the patient's normal body temperature or experience of at least 1 symptom included in the Influenza Symptom Severity scale (ISS) [14].

Patients with a history of hypersensitivity to AZM or oseltamivir and patients with bacterial infections were excluded. At screening, a complete history was recorded from all patients, including notes on flu vaccination, physical examination, chest radiographs, and blood chemistry. Assessment of clinical symptoms of influenza, including vital signs (body temperature, blood pressure, and pulse rate), was performed at baseline (day 0) and on days 2 and 5. Blood samples were collected on days 0, 2, and 5 for measurement of the levels of inflammatory cytokines and chemokines, HMGB1, and procalcitonin (PCT). Patients recorded their own influenza symptoms, maximal temperature, and activities of daily living using a 7-symptom ISS and a visual analogue scale (Influenza Impact Well-Being Score [IIWS]) ranging from 0 to 10.

Study design

This prospective, randomized, open-label, controlled, multicenter study was performed between December 2010 and March 2011.

Ethics

The trial was conducted in accordance with the Declaration of Helsinki and in compliance with the ethical guidelines for clinical studies issued by the Health, Labour and Welfare Ministry. Written informed consent was obtained from all patients before enrollment. The protocol, amendments, and informed consent documentation were approved by the institutional review board and/or independent ethics committee at each facility.

The project approval date for each Research Ethics Board is listed in brackets: Nagasaki University (October 13, 2010), The Japanese Red Cross Nagasaki Genbaku Isahaya Hospital (December 24, 2010), The Japanese Red Cross Nagasaki Genbaku Hospital (October 25, 2010), Hokusho Central Hospital (November 16, 2010), Sasebo General Hospital (January 17, 2011), NHO Ureshino Medical Center (November 22, 2010), Koseikai Hospital (November 22, 2010), Isahaya Health Insurance General Hospital (Not approved until the end of this study), Nagasaki Municipal Hospital (November 4, 2010), Onitsuka Naika Clinic (October 13, 2010), Hayashida Naika Clinic (March 12, 2011), Tomonaga Naika Clinic (October 13, 2010), Irihune Clinic (October 13, 2010), Kawamura Clinic (October 13, 2010).

The trial was first approved in October, 2010 by the ethics committee of Nagasaki University (accession number, 100100130), but was only finally approved by the other branch hospitals in March, 2011. Additionally, the trial was registered in the University Hospital Medical Information Network (UMIN) Center system. The UMIN accession number is UMIN000005371. The trial began in December, 2010.

Study intervention and randomization

Patient enrollment was performed using a central registration system through a computer-generated random listing of the two

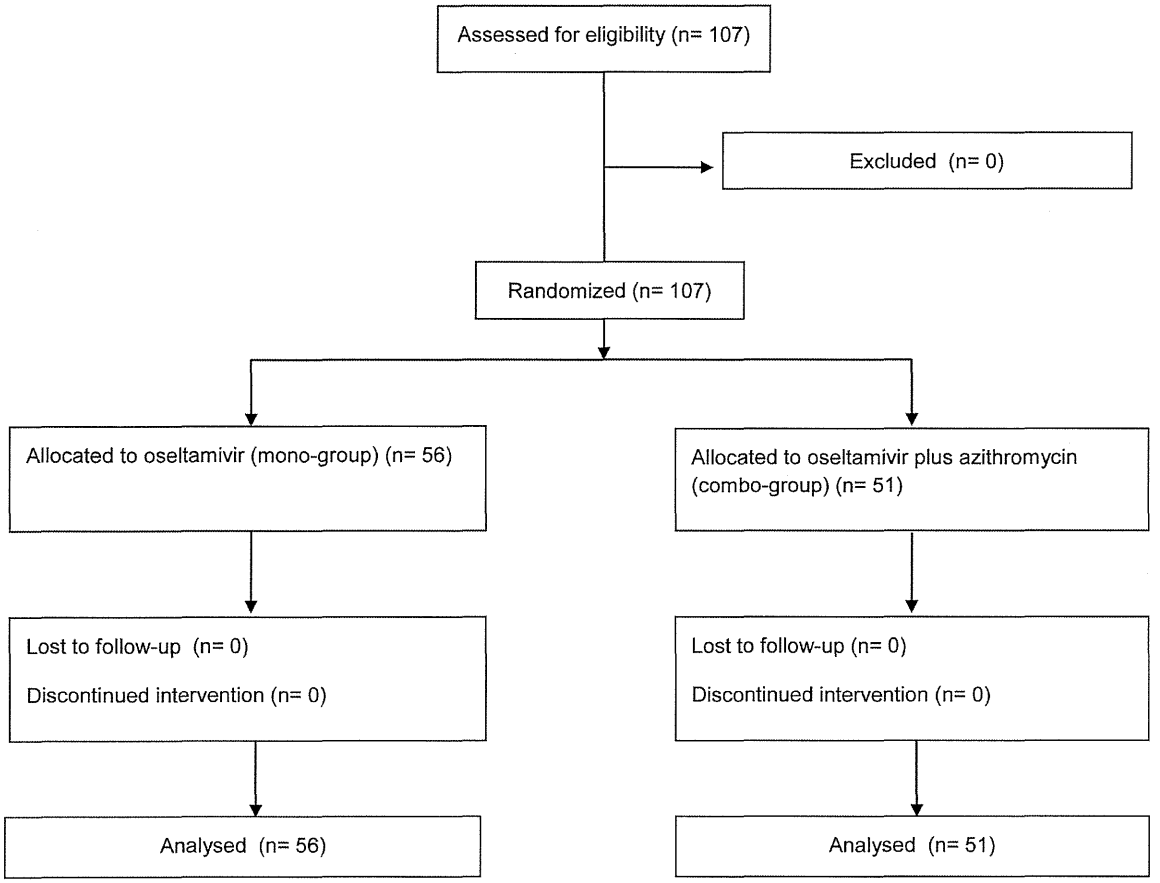

Figure 1. Trial Profile.
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treatment allocations. A minimization method [15] was used to randomize patients in a 1:1 ratio to receive oral oseltamivir 75 mg alone (mono-group) every 12 h or oral oseltamivir 75 mg every 12 h in combination with an extended-release formulation of single-dose oral AZM 2,000 mg (combo-group). For randomization, patients were stratified according to the presence of high risk factors such as age (≥65 years), underlying respiratory diseases (e.g., chronic obstructive pulmonary disease, bronchial asthma), use of steroids (equivalent to prednisolone >10 mg/day), and uncontrolled diabetes mellitus (hemoglobin A1c [HbA1c] level > 7.4; national glycohemoglobin standardization program [NGSP]). Oral oseltamivir was to be administered for 5 days in both groups.

Outcome measures

The purpose of this study was to evaluate the efficacy and safety of combination therapy with an anti-influenza agent, oseltamivir, and AZM in patients with influenza.

The intent-to-treat (ITT) population was used prospectively for analysis. The ITT population included all patients who received 1 or more doses of the study medication. The primary endpoint was defined as variations in the levels of inflammatory markers (i.e., inflammatory cytokines and chemokines, HMGB1, PCT). Secondary endpoints were defined as follows: (1) the duration of influenza; (2) the incidence of influenza-related complications (sinusitis, otitis media, bronchitis, and pneumonia); (3) the time to alleviation of influenza symptoms; and (4) adverse events and adverse drug reactions.

Inflammatory marker assays

The levels of the cytokines IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TGF-β, interferon γ (IFN-γ), and TNF-α were measured using the cytokine bead array.

Clinical laboratory tests

We performed hematological (measurements of red blood cell [RBC] count, Hb level, hematocrit [Ht] level, platelet count, white blood cell [WBC] count, and WBC fraction); biochemical (measurement of the levels of aspartate aminotransferase [AST], alanine aminotransferase [ALT], total bilirubin [T-Bil], blood urea nitrogen [BUN], creatinine [Cre], total protein [T-P], albumin [Alb], sodium [Na], chloride [Cl], and potassium [K]); and immunological (measurement of C-reactive protein [CRP] level) tests on days 1, 2, and 5.

The differences in values on day 2 (ΔDay2) and day 5 (ΔDay5) from those observed on day 0 were evaluated.

Statistical methods

The statistical analyses were performed by an expert biostatistician experienced in the subject studied. The mono-group included 56 patients, while the combo-group included 51 patients, which was the number estimated as the appropriate sample size (see the protocol).

This was an exploratory trial to assess the efficacy of oseltamivir plus AZM in the treatment of patients with influenza. Statistical analyses were performed using PASW Statistics 18 (SPSS Inc., Chicago, IL, USA). All tests were two-tailed, and a p value <0.05

Table 1. Study population in the azithromycin-oseltamivir combination therapy and oseltamivir monotherapy groups.

		Azithromycin		
		-	+	p value
No. of patients		56	51	
Age (years)	Range	20–87	20–91	0.734 (*t* test)
	Median	42	39	
	Mean ± SD	44.1±17.3	42.9±17.3	
Gender	M (%)	25 (39.3)	25 (49.0)	0.398 (Fisher)
	F (%)	31 (60.7)	26 (51.0)	
Chronic Lung Disease (%)		6 (10.7)	5 (9.8)	0.566 (Fisher)
Diabetes		3 (5.4)	0	0.140 (Fisher)
Steroid use		3(5.4)	2(3.9)	0.545(Fisher)
Maximal body temperature(mean± SD)		38.6±0.7	38.8±0.7	0.202 (*t* test)
Influenza Symptom Severity scale (ISS)				
Headache	None	8 (14.3)	7 (13.7)	0.985 (t test)
	Mild	17 (30.4)	15 (29.4)	
	Moderate	24 (42.9)	19 (37.3)	
	Severe	5 (8.9)	5 (9.8)	
Muscle/Joint pain	None	7 (7.1)	5 (9.8)	0.735 (*t* test)
	Mild	11 (19.6)	13 (25.5)	
	Moderate	24 (42.9)	19 (37.3)	
	Severe	12 (21.4)	9 (17.6)	
Heat sensation	None	3 (5.4)	2 (5.9)	0.135 (*t* test)
	Mild	11 (19.6)	6 (11.8)	
	Moderate	24 (42.9)	17 (33.3)	
	Severe	16 (28.6)	21 (41.2)	
Feeling of fatigue	None	2 (3.6)	2 (3.9)	0.738 (*t* test)
	Mild	10 (17.9)	5 (9.8)	
	Moderate	25 (44.6)	25 (49.0)	
	Severe	17 (30.4)	14 (27.5)	
Cough	None	1 (1.8)	3 (5.9)	0.014 (*t* test)
	Mild	10 (17.9)	16 (31.4)	
	Moderate	30 (53.6)	21 (41.2)	
	Severe	12 (21.4)	5 (9.8)	
Sore throat	None	11 (19.6)	10 (19.6)	0.852 (*t* test)
	Mild	26 (46.4)	19 (37.3)	
	Moderate	13 (23.2)	14 (27.5)	
	Severe	4 (7.1)	3 (5.9)	
Nasal congestion	None	16 (28.6)	11 (21.6)	0.732 (*t* test)
	Mild	12 (21.4)	17 (33.3)	
	Moderate	22 (39.3)	16 (31.4)	
	Severe	4 (7.1)	2 (3.9)	

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was considered statistically significant. The significance of differences in the expression levels of cytokines and chemokines and in influenza-related symptoms between the mono-group and combo-group were examined using the Mann–Whitney *U* test. In addition, significance of differences in the maximum temperature between the mono- and combo-groups on days 3 through 5 were using a mixed-design analysis of variance (mixed-design ANOVA).

Results

Study population

A total of 107 patients were enrolled in the study between December 2010 and March 2011.

The number of patients enrolled at each hospital was as follows: 4, Nagasaki University; 12, The Japanese Red Cross Nagasaki Genbaku Isahaya Hospital; 8, The Japanese Red Cross Nagasaki Genbaku Hospital; 2, Hokusho Central Hospital; 6, Sasebo

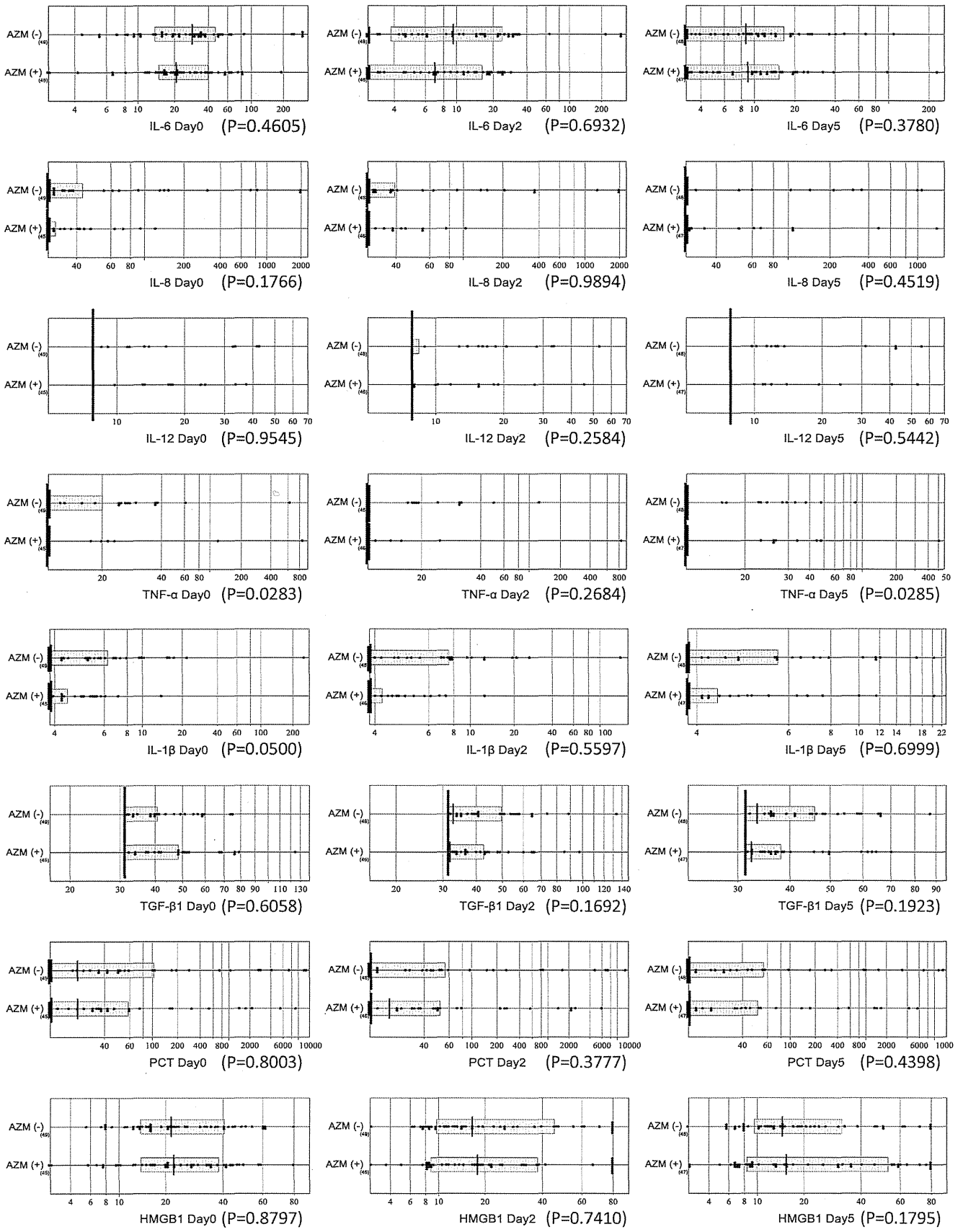
AZM (-)
AZM (+)
IL-6 Day0 (P=0.4605)
IL-6 Day2 (P=0.6932)
IL-6 Day5 (P=0.3780)
IL-8 Day0 (P=0.1766)
IL-8 Day2 (P=0.9894)
IL-8 Day5 (P=0.4519)
IL-12 Day0 (P=0.9545)
IL-12 Day2 (P=0.2584)
IL-12 Day5 (P=0.5442)
TNF-α Day0 (P=0.0283)
TNF-α Day2 (P=0.2684)
TNF-α Day5 (P=0.0285)
IL-1β Day0 (P=0.0500)
IL-1β Day2 (P=0.5597)
IL-1β Day5 (P=0.6999)
TGF-β1 Day0 (P=0.6058)
TGF-β1 Day2 (P=0.1692)
TGF-β1 Day5 (P=0.1923)
PCT Day0 (P=0.8003)
PCT Day2 (P=0.3777)
PCT Day5 (P=0.4398)
HMGB1 Day0 (P=0.8797)
HMGB1 Day2 (P=0.7410)
HMGB1 Day5 (P=0.1795)

Figure 2. Comparison of inflammatory marker levels in the azithromycin-oseltamivir combination therapy and oseltamivir monotherapy groups. Serial test results (on days 0, 2, and 5) for the 8 inflammatory markers were compared between the groups with and without AZM. The horizontal and vertical lines depicted in the scattergram represent the central 50% range (25–75 percentiles) and the median, respectively. In the graphs for interleukin 8 (IL-8), IL-12, and tumor necrosis factor α (TNF-α), missing central boxes or concordance of the left end of the box with the median indicate that the majority of the test results were lower than the limit of detection.
doi:10.1371/journal.pone.0091293.g002

General Hospital; 6, NHO Ureshino Medical Center; 5, Koseikai Hospital; 0, Isahaya Health Insurance General Hospital (Not approved until the end of the study); 6, Nagasaki Municipal Hospital; 20, Onitsuka Naika Clinic; 0, Hayashida Naika Clinic; 20, Tomonaga Naika Clinic; 13, Irihune Clinic; and 5, Kawamura Clinic. All patients were recruited after the relevant project approval date.

The patients were randomized into the mono-group (56 patients) or combo-group (51 patients), and all enrolled patients were included in the ITT population (Figure 1). All patients were diagnosed with influenza A virus infection, and none of the patients had comorbid pneumonia. The participants included 50 male patients and 57 female patients, and their mean age was 43.5 years. The 2 treatment groups did not differ significantly in terms of their clinical characteristics, sex, age, underlying diseases, or disease severity (Table 1).

Inflammatory markers

The baseline values of IL-6, IL-8, IL-12, TNF-α, IL-1β, TGF-β1, PCT, and HMGB1 on day 0 before treatment allocation did not differ between the groups (Figure 2). However, the baseline values of TNF-α were statistically significantly higher in the combo-group than in the mono-group ($p = 0.03$). No statistically significant differences were observed between the 2 groups in the expression of any of the inflammatory cytokines or chemokines on days 2 and 5.

Although TNF-α levels were statistically different between the 2 groups on day 5, its value decreased below measurable limits in almost all patients, and thus no clear difference was observed.

Resolution time of influenza-related symptoms

The baseline maximal temperature on day 0 did not differ between the 2 groups ($p = 0.984$). Comparison of the maximum temperatures on days 1 to 5 showed no significant differences on

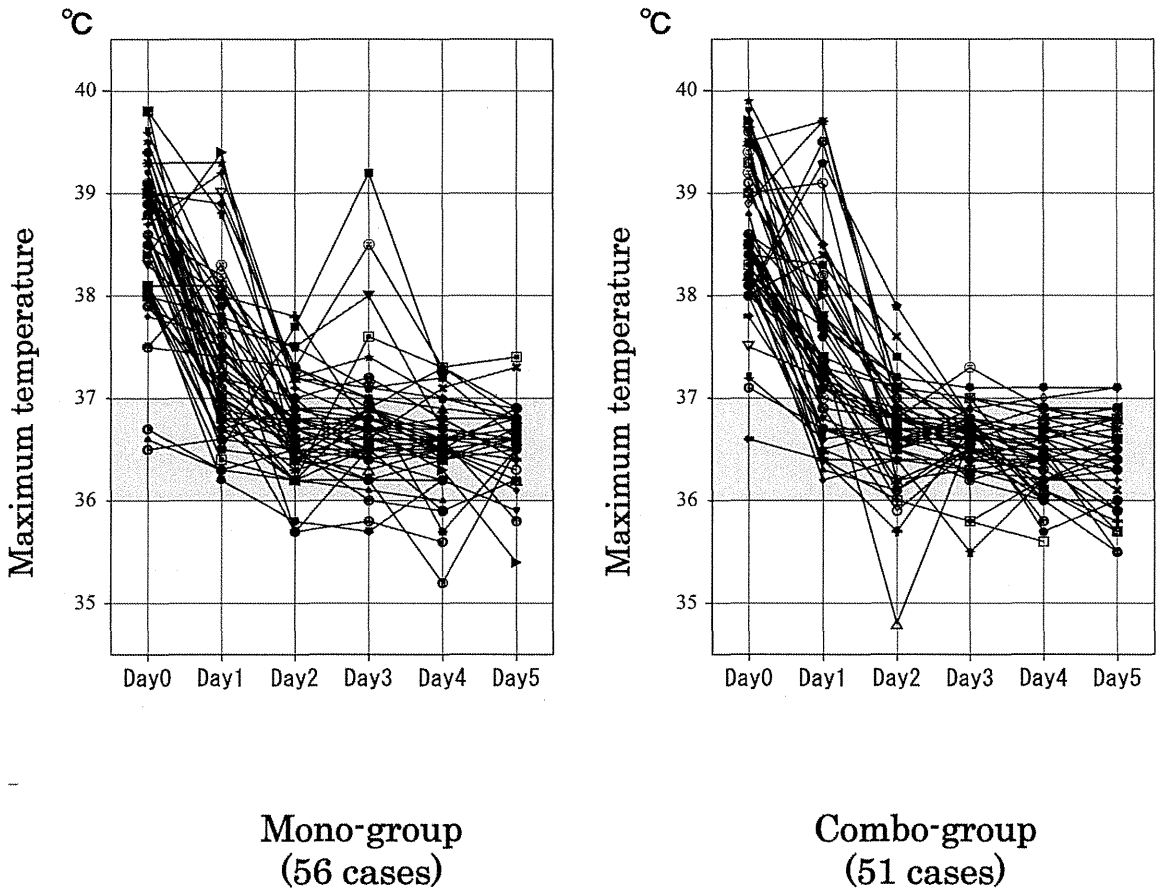

Figure 3. Comparisons of maximum temperature in the azithromycin-oseltamivir combination therapy and oseltamivir monotherapy groups. The maximum temperatures on day 1 through day 5 were compared between groups. No significant differences were detected between the groups on days 1, 2, 3, and 5. However, our analysis revealed a significant decrease in the maximum temperature on day 4 in the combo-group compared to that in the mono-group ($p = 0.037$). In addition, a mixed-design ANOVA indicated that the maximum temperature on days 3 through 5 was significantly lower in the combo-group than in the mono-group ($p = 0.048$).
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Table 2. Improvement of influenza-related symptoms in the azithromycin-oseltamivir combination therapy and oseltamivir monotherapy groups.

		Azithromycin		
		−mean ± S.D	+ mean ± S.D	p value
Headache	Day0	1.48±0.86	1.48±0.89	0.9648
	ΔDay2	0.68±0.68	0.74±0.95	0.7890
	ΔDay5	1.23±0.99	1.30±0.96	0.8649
Muscle/Joint pain	Day0	1.76±0.95	1.70±0.92	0.6611
	ΔDay2	1.08±0.92	1.15±0.99	0.7968
	ΔDay5	1.60±0.93	1.6±0.95	0.9970
Heat sensation	Day0	1.98±0.86	2.24±0.85	0.1039
	ΔDay2	1.43±0.91	1.78±1.11	0.0506
	ΔDay5	1.8±0.94	2.13±1.00	0.0609
Feeling of fatigue	Day0	2.06±0.81	2.11±0.77	0.7373
	ΔDay2	0.98±0.84	1.20±1.07	0.2211
	ΔDay5	1.58±0.93	1.76±1.04	0.1871
Sore throat	Day0	1.19±0.85	1.20±0.86	0.8020
	ΔDay2	0.34±0.73	0.70±0.87	0.0323
	ΔDay5	0.81±0.83	1.02±0.88	0.2138
Nasal congestion	Day0	1.26±0.97	1.2±0.86	0.2138
	ΔDay2	0.43±1.05	0.44±0.89	0.8630
	ΔDay5	0.79±1.12	0.93±1.04	0.3890
Cough	Day0	2.00±0.71	1.62±0.78	0.0143
	ΔDay2	0.52±0.83	0.60±0.86	0.6645
	ΔDay5	1.06±0.78	1.07±0.93	0.8783

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days 1 ($p = 0.864$), 2 ($p = 0.864$), 3 ($p = 0.741$), or 5 ($p = 0.068$). However, a significant decrease in the maximum temperature was observed on day 4 between the combo-group and the mono-group ($p = 0.037$; Figure 3). In addition, the maximum temperature on days 3 through 5 was significantly lower in the combo-group than in the mono-group ($p = 0.048$).

Improvements in sore throat were observed more frequently on day 2 among patients in the combo-group than in the mono-group ($p<0.05$). No significant differences were observed between the 2 groups in the resolution time of other influenza-related symptoms (headache, muscle or joint pain, heat sensation, feeling of fatigue, sore throat, nasal congestion, and cough). However, compared to the mono-group, the combo-group showed a trend toward earlier resolution of fever ($p = 0.05$ on day 2 and $p = 0.06$ on day 5, Table 2).

Laboratory tests

The baseline hematological test values (hemoglobin, Ht, WBC, neutrophil count, lymphocyte count, total protein and albumin) on day 0 before treatment allocation were not significantly different between the 2 groups. Only the baseline RBC was statistically significantly higher in the combo-group than in the mono-group ($p<0.05$). In addition, the combo-group showed statistically significant increases in the RBC and hemoglobin and Ht values on days 2 and 5 and a statistically significant decrease in the levels of Alb and T-P on day 2 ($p<0.05$ and $p<0.01$, respectively; Table 3).

Safety

Adverse events (AEs) occurred in 11 of the 56 patients (19.6%) in the combo-group and in 9 of the 51 patients (17.6%) in the mono-group (Table 4). There was no significant difference in the incidence of AEs between the 2 groups. AEs for which a causal relationship with the study drugs could not be ruled out (known plus unknown causal relationships) occurred in 9 patients (16.1%) in the combo-group and 4 patients (7.8%) in the mono-group. No severe AE occurred in either group and no patients discontinued treatment because of an AE. The most common AEs were diarrhea ($n = 3$ in the combo-group) and decreased WBC ($n = 5$ in the combo-group and $n = 3$ in the mono-group). Only 1 patient in the mono-group developed secondary pneumonia.

Discussion

In this study, we present the findings of a randomized clinical trial of combination therapy with oseltamivir and AZM in patients with influenza virus infection. The primary endpoint of this study was variation in the expression of inflammatory markers (i.e., inflammatory cytokines and chemokines). Although the combination of oseltamivir plus AZM did not show any early reduction in the levels of inflammatory markers compared to that with oseltamivir alone, the combination treatment showed a potential early resolution of influenza-related symptoms such as fever and sore throat.

Macrolides have antibiotic and immunomodulatory activities *in vitro* and *in vivo*, and thus may have a favorable effect on the clinical outcome of patients with severe infection [6]. CAM decreases the

Table 3. Laboratory data for the azithromycin-oseltamivir combined therapy and oseltamivir monotherapy groups.

		Azithromycin		
		- mean ± S.D (median)	+ mean ± S.D (median)	p value
WBC	Day0	4154±3442 (5045)	4137±3430 (5175)	0.7904
	ΔDay2	−1013±2091 (−1400)	−2138±1472 (−2350)	0.0103
	ΔDay5	−614±1648 (−910)	−754±2203 (−1100)	0.5667
Neutrophil	Day0	4720±1707 (4320)	4745±1524 (4540)	0.7565
	ΔDay2	−1692±2154 (−1987)	−2771±1462 (−2635)	0.0081
	ΔDay5	−1503±704 (−1286)	−1729±2143 (−1825)	0.5802
Lymphocyte	Day0	948±429 (847)	851±343 (767)	0.2722
	ΔDay2	633±540 (694)	751±346 (797)	0.1856
	ΔDay5	940±79 (918)	1170±557 (1189)	0.0614
RBC	Day0	474±46 (476)	458±40 (453)	0.0455
	ΔDay2	−3.53±20.0 (−1.36)	14.4±23.3 (13.5)	0.0002
	ΔDay5	−9.53±19.8 (−1.50)	5.9±24.1 (9.0)	0.0008
Hgb	Day0	14.3±1.47 (14.2)	13.8±1.68 (13.8)	0.1744
	ΔDay2	−0.33±0.65 (0.00)	0.43±.71 (0.50)	0.0012
	ΔDay5	−0.30±0.63 (−0.30)	0.16±0.72 (0.20)	0.0010
Hct	Day0	42.8±3.81 (42.6)	41.2±4.36 (41.4)	0.1195
	ΔDay2	−0.36±1.81 (−0.30)	1.36±2.20 (1.45)	0.0001
	ΔDay5	−1.10±1.81 (−1.30)	0.22±2.11 (0.20)	0.0010
Total Protein	Day0	7.3±0.45 (7.3)	7.2±0.44 (7.2)	0.2910
	ΔDay2	−0.22±0.33 (−0.20)	0.01±0.41 (0.05)	0.0026
	ΔDay5	−0.10±0.36 (−0.20)	0.06±0.48 (0.00)	0.0831
Alb	Day0	4.5±0.34 (4.5)	4.4±0.30 (4.5)	0.5207
	ΔDay2	−0.24±0.26 (−0.20)	−0.12±0.24 (−0.10)	0.0155
	ΔDay5	−0.20±0.22 (−0.30)	−0.02±0.29 (0.00)	0.0026
AST	Day0	24.8±9.36 (22.0)	22.6±12.38 (19.5)	0.0204
	ΔDay2	1.36±6.31 (1.0)	0.84±6.10 (2.0)	0.7559
	ΔDay5	−1.79±8.61 (−1.0)	−0.75±9.49 (0.0)	0.0609
ALT	Day0	23.3±15.02 (19.0)	24.0±21.41 (16.0)	0.4339
	ΔDay2	2.36±6.89 (1.0)	0.34±4.78 (1.0)	0.4868
	ΔDay5	1.02±7.50 (0.5)	−1.16±12.58 (0.5)	0.6777
BUN	Day0	11.3±3.60 (10.8)	11.5±4.65 (11.2)	0.9093
	ΔDay2	0.76±3.00 (1.20)	1.20±3.18 (0.80)	0.6140
	ΔDay5	0.51±3.15 (0.95)	1.57±3.11 (1.65)	0.1293
Cr	Day0	0.8±0.20 (0.8)	0.7±0.18 (0.8)	0.7492
	ΔDay2	−0.05±0.10 (−0.05)	−0.04±0.09 (−0.05)	0.5743
	ΔDay5	−0.08±0.08 (−0.06)	−0.07±0.08 (−0.06)	0.4516

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ratio of serum IL-10 to serum TNF-α in patients with ventilator-associated pneumonia (VAP) and sepsis caused by gram-negative bacteria [16]. In addition, AZM significantly reduces the expression of the proinflammatory cytokine IL-1β and the chemokine C-C motif ligand (CCL)-2 and TNF-α in M1-induced cystic fibrosis alveolar macrophages in patients with cystic fibrosis [17]. In addition, AZM decreases acute and chronic airway inflammation in a mouse model of paramyxoviral bronchiolitis without any association with antiviral activity [18]. Azithromycin has a large volume of distribution, although serum concentrations remain low, and its half-life is much longer than that of clarithromycin. Therefore, a single dose of azithromycin is an effective and convenient dosing schedule that improves patient compliance [19]. Although we expected AZM to reduce inflammatory cytokine expression in patients with influenza virus infection, compared to oseltamivir alone, the combination of oseltamivir plus AZM did not result in an early reduction in the levels of inflammatory markers. The baseline values of each inflammatory cytokine differed for each patient, and the median value was relatively low, and therefore we suspect that variability in the patient backgrounds might have affected the study outcomes.

The present study has several potential limitations. Our study was performed during 1 winter season. Further, the number of

Table 4. List of adverse events in the present study.

AZM	Adverse Event	Severity	Causality	Treatment
+	Secondary bronchitis	Mild	No	Continue
+	Bronchitis	Mild	No	Continue
+	Abdominal pain	Mild	Unknown	Continue
+	Abdominal pain/Diarrhea	Mild	Unknown	Continue
+	Diarrhea	Mild	Yes	Continue
+	Diarrhea	Mild	Unknown	Continue
+	Leucopenia	Mild	Unknown	Continue
+	Leucopenia	Moderate	Unknown	Continue
+	Leucopenia	Moderate	Unknown	Continue
+	Leucopenia	Mild	Unknown	Continue
+	Leucopenia	Mild	Unknown	Continue
−	Sinusitis	Moderate	No	Continue
−	Pneumonia	Mild	No	Continue
−	Bronchitis	Mild	No	Continue
−	Leucopenia	Mild	No	Continue
−	Leucopenia	Mild	Unknown	Continue
−	Leucopenia	Mild	Unknown	Continue
−	Eosinophilia	Mild	Unknown	Continue
−	Hepatic dysfunction	Mild	Unknown	Continue
−	Hepatic dysfunction	Mild	No	Continue

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patients was limited. We planned a sub-group analysis of older individuals, particularly patients with underlying respiratory disease. However, the number of patients who met this definition was limited, and thus the analysis was not possible. A randomized controlled trial of such patients should be performed in the future. Moreover, the enrollment criteria included patients with a wide variety of backgrounds to ensure the feasibility of patient enrollment. The timing of enrollment from the onset of an influenza-related symptom was different for each patient. Although the inclusion criteria stipulated that a patient had to be enrolled within 48 h after the onset of an influenza-related symptom, there were still 48 hours between the symptom onset and enrollment. Additionally, it was difficult to prepare a specially blinded drug for the AZM extended-release formulation because of its unique size and shape. Therefore, the study was conducted in an open-label manner. Although it cannot be denied that AZM may have had some placebo effect, we do not believe that this affected our results.

High body temperature is a common influenza-related symptom. Although no statistically significant differences in fever reduction were observed between the 2 groups until day 3, compared to oseltamivir alone, the oseltamivir plus AZM combination group showed a statistically significant early antipyretic effect on day 4. The mechanism of action of the early antipyretic effect associated with AZM is difficult to determine in our patients, but we present 2 hypotheses. The first is an anti-inflammatory effect exerted by AZM [6], and the second is a conventional antibiotic effect giving due consideration to a bacterial superinfection. In this study, we were unable to show that AZM decrease the levels of inflammatory markers in influenza patients compared to those in controls. However, macrolide therapy has been reported to improve the outcomes of patients with VAP [20] and acute lung injury (ALI) [21]. Several studies have also shown that compared to administration of beta-lactams alone, fluoroquinolones alone, or beta-lactams in combination with fluoroquinolones, administration of beta-lactams in combination with macrolides improves the survival of patients with severe community-acquired pneumonia (CAP) [22–24]. These reports suggest that macrolides produce an effect (i.e., anti-inflammatory or immunomodulatory) other than a conventional antibiotic effect. The potential therapeutic value of the anti-inflammatory effects of macrolides is supported by murine models of ALI induced by endotoxin [25,26] as well as murine models of influenza [13,27] and VAP caused by *Pseudomonas aeruginosa* [28]. These studies have shown increased survival after macrolide therapy. The possible involvement of a bacterial superinfection cannot be ruled out because bacteriological examinations such as Gram stains and cultures of respiratory samples were not required to be performed at baseline in this study. Rates of influenza-related pneumonia are generally less than 10% [29,30], and it is very rare in Japan (1-2%) because patients tend to consult physicians at an early stage because of the broad coverage by the health insurance system. In this study, we analyzed about 100 patients, but none developed pneumonia.

The incidence of bacterial pneumonia as a secondary infection after influenza is well known as a major cause of increased morbidity and mortality. Concomitant administration of macrolides, including AZM, to treat influenza may contribute to the prevention of secondary bacterial pneumonia by preventing airway epithelial cell damage because of an overactive immune response. Macrolides exert immunomodulatory effects via inhibition of neutrophil oxidative bursts, decrease of elastase activity, and suppression of granulocyte macrophage-colony stimulating factor [6]. In this study, only 1 patient who received oseltamivir monotherapy developed secondary pneumonia, but the sample size was not sufficiently large to adequately detect secondary infection after influenza.

Decreases in serum albumin and total protein levels were significantly modulated by the addition of AZM to oseltamivir therapy. The relationship between AZM and such modulation is unclear, but AZM could have contributed to early improvement in the general condition of the patients.

In conclusion, to our knowledge, this is the first prospective, randomized, clinical trial of oseltamivir and AZM combination therapy for influenza. Although no statistically significant difference was observed in the expression levels of inflammatory cytokines and chemokines, the combination therapy showed a trend toward the earlier resolution of some symptoms.

Supporting Information

Checklist S1 CONSORT checklist.
(DOC)

Protocol S1 Trial Protocol.
(DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: HK, MS, KI, KY, TT, S. Kohno. Performed the experiments: KK, YM, S. Kurihara, SN, YI, TM, MT. Analyzed the data: KI, KK, YM, S. Kurihara, SN, YI, TM, MT, KI, KY, TT. Wrote the paper: HK, MS, S. Kohno.

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Original article

Pathogenesis and clinical features of chronic pulmonary aspergillosis – Is it possible to distinguish CNPA and CCPA clinically?

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Chronic progressive pulmonary aspergillosis

ABSTRACT

Background: The pathogenesis of chronic pulmonary aspergillosis (CPA) including chronic necrotizing pulmonary aspergillosis (CNPA), chronic cavitary pulmonary aspergillosis (CCPA), and simple aspergilloma (SA) has been poorly investigated. We examined all types of CPA cases with histopathological evidence to clarify the differences in pathogenesis and clinical features.

Method: We searched for cases diagnosed as pulmonary aspergillosis by histopathological examination in Nagasaki University Hospital between 1964 and September 2010. All available clinical information including radiological findings were collected and analyzed.

Result: We found 7, 5, 8, and 7 cases of proven CNPA, probable CNPA, CCPA, and SA, respectively. The radiograph of proven and probable CNPA was initially infiltrates or nodules that progress to form cavities with or without aspergilloma, whereas the radiograph of CCPA showed pre-existed cavities and peri-cavitary infiltrates with or without aspergilloma. The patients with proven and probable CNPA exhibited not only respiratory symptoms but also systemic symptoms and malnutrition. *Aspergillus fumigatus* was the most frequently isolated *Aspergillus* species ($n = 14$), however, *Aspergillus niger* was the predominant isolated species in proven CNPA cases ($n = 4$).

Conclusion: Our data indicate that the cases with chronic infiltration, progressive cavitation, and subsequent aspergilloma formation should be diagnosed as CNPA, and the cases with pre-existed cavities showing peri-cavitary infiltrates with or without aspergilloma would mean CCPA. However, it may be difficult to distinguish the two subtypes if a series of adequate radiography films are not available. We propose the term "chronic progressive pulmonary aspergillosis (CPPA)" for the clinical syndrome including both CNPA and CCPA.

1. Introduction

Chronic pulmonary aspergillosis (CPA) was originally established in the early 1980s by Binder as chronic necrotizing pulmonary aspergillosis (CNPA) [1] and semi-invasive aspergillosis (SIA) by Gefter [2]. CNPA/SIA is characterized by a slow progressive cavitating process in the lungs due to *Aspergillus* spp. infection. In the last decade, new clinical nomenclature and definition of chronic forms of aspergillosis have been proposed [3–5], and recent guidelines from the Infectious Diseases Society of America (IDSA) have indicated 3 major subtypes of chronic forms of pulmonary aspergillosis, namely CNPA (categorized in subacute invasive form of aspergillosis; subacute IPA), chronic cavitary pulmonary

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aspergillosis (CCPA), and aspergilloma [6]. Aspergilloma was traditionally classified as simple or complex in the surgical literature, and complex aspergilloma is considered CCPA by current IDSA guidelines [6,7]. Updated IDSA guidelines and textbook have indicated that the differences between CNPA and CCPA include prolonged time frame (CNPA, 1–3 months vs. CCPA, >3 months) [8]. The original CNPA cases defined by Binder are not equal to those of CNPA cases defined by Denning. Establishing the precise subclassifications of CPA, while challenging, is important for clinical trials as well as the development of tools for its diagnosis and treatment. The pathogenesis of each type of CPA examined by pathological samples has been poorly investigated. In this study, we investigated all types of CPA cases including CNPA, CCPA, and simple aspergilloma (SA) diagnosed by histopathological examination to clarify the differences in the pathogenesis and clinical features of each type of CPA. In particular, revealing the difference between CNPA and CCPA is the most important of goal of our study. This study was performed in a single medical teaching hospital in Japan.

2. Material and methods

2.1. Case collection and analysis

We searched for cases diagnosed as "pulmonary aspergillosis" by histopathological examination of respiratory specimens acquired by biopsy, surgical resection, or autopsy in Nagasaki University Hospital. Nagasaki University Hospital is a Japanese teaching hospital that contains 860 beds and is located in the southwestern part of Japan. All cases registered in the database between 1964 and September 2010 were screened, and patient data such as sex, age, underlying diseases, clinical symptoms, clinical course, laboratory and radiological findings, and treatment was collected from medical records and analyzed. This retrospective study was approved by the ethics committee of Nagasaki University Hospital.

2.2. Definition of pulmonary aspergillosis subtypes by histopathological and clinical aspects

The histopathological and clinical definition of invasive pulmonary aspergillosis (IPA), CNPA, CCPA, and SA in this study is indicated in Fig. 1. A diagnosis of IPA and CNPA requires extensive hyphal invasion in the lung parenchyma and localized hyphal invasion in cavity walls and/or destroyed lung tissue, respectively. Non-invasive CPA was subdivided into three groups as Group A, CCPA and SA. CCPA requires pre-existing cavities and disease progression within 3 months. SA requires a solitary cavitary legion with an aspergilloma and no disease progression within 3 months. Group A does not match to either of CNPA and CCPA. This group requires a newly developed necrotic lung cavity and no hyphal invasion in any lung tissue. The existence of pre-existing cavity was confirmed by X-ray films taken prior to the disease onset.

2.3. Statistical analysis

The chi-square test was used for qualitative variables. Multivariate logistic regression analysis was performed using the variables that were selected from univariate analysis ($P < 0.1$). This analysis was performed by SPSS ver. 16 software. P values <0.05 were considered statistically significant.

3. Results

3.1. Characters of CPA cases and patients

A total of 60 cases of which histopathological findings indicated pulmonary aspergillosis were found in the database. Then the cases of IPA and allergic bronchopulmonary aspergillosis (ABPA) were excluded. We searched the patients' medical records including X-ray films and accepted 27 cases of CPA for further analysis due to their availability of medical information and X-ray films. We found 7, 5, 8, and 7 cases of CNPA, Group A, CCPA, and SA, respectively. They were histopathologically diagnosed by surgical resection in 19, biopsy in 4, and autopsy in 4 cases, respectively.

Table 1 shows sex, age, body mass index, symptoms, and disease duration prior to diagnosis. The cases of CNPA, Group A and CCPA demonstrated a male predominance, and the body mass index values of patients with CNPA and Group A were statistically lower than those of patients with SA. Several patients with SA complained of cough, hemoptysis, and sputum; however, the symptoms or radiographic findings did not indicate disease progression within 3 months. All cases with CNPA, Group A and CCPA complained respiratory symptoms. Progressive fever and weight loss were found

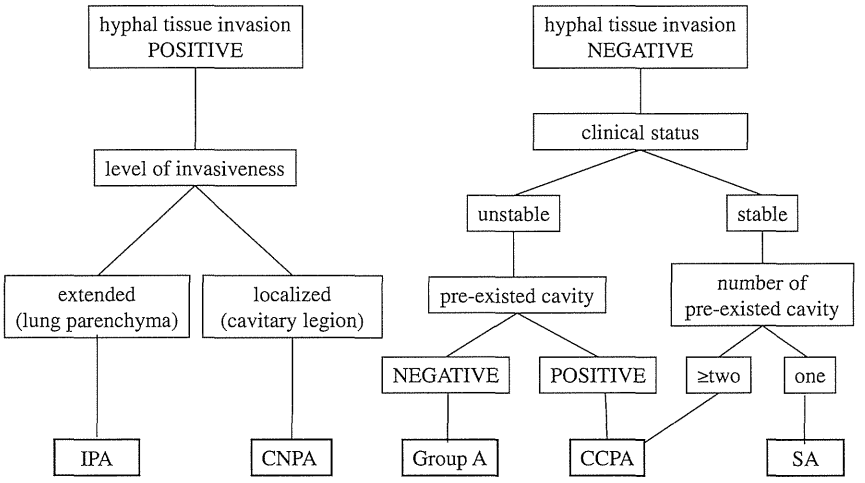

Fig. 1. Histopathological and clinical diagnosis of pulmonary aspergillosis in this study. A diagnosis of invasive pulmonary aspergillosis and chronic necrotizing pulmonary aspergillosis (CNPA) requires extensive hyphal invasion in the lung parenchyma and localized hyphal invasion in cavity walls and/or destroyed lung tissue, respectively. Group A, chronic cavitary pulmonary aspergillosis (CCPA), and simple aspergilloma (SA) require no hyphal invasion in any lung tissue; however, Group A requires new cavity formation and clinical deterioration without a pre-existing cavitary lesion in the lung. CCPA requires pre-existing cavities and disease progression and SA requires a solitary cavitary legion with an aspergilloma and no clinical deterioration within 3 months.

Table 1
Characteristics of CPA patients.

	CNPA ($n = 7$)	Group A ($n = 5$)	CCPA ($n = 8$)	SA ($n = 7$)
Sex; Male/Female	6/1	4/1	7/1	3/4
Age; mean (range)	59.1 (45–75)	67.0 (50–77)	69.7 (58–80)	56.6 (40–68)
Body mass index;	18.0*	16.9*	20.2	21.3
mean (range)	(13.0–21.4)	(13.9–18.8)	(15.7–25.7)	(18.0–23.9)
Symptoms				
Cough	5	5	7	4
Sputum	5	2	3	3
Hemoptysis	1	5	6	4
Dyspnea	3	1	2	0
Fever	4	2	0	0
Malaise	3	0	0	0
Weight loss	1	2	0	0
Duration; median	3 m	24 m	29 m	24 m
(range)	(1 m–6 y)	(17 m–6 y)	(6 m–5 y4 m)	(6 m–4 y)

CNPA, chronic necrotizing pulmonary aspergillosis; CCPA, chronic cavitary pulmonary aspergillosis; SA, simple pulmonary aspergilloma; m, months; and y, years.
*: Statistical difference between CNPA, Group A and SA was indicated ($p < 0.05$).

in only CNPA and Group A cases, which may reflect progressive tissue destruction and more direct inflammatory responses than CCPA and SA does.

The underlying conditions of patients with CPA are indicated in Table 2. Patients with CNPA most frequently had chronic obstructive pulmonary disease (COPD) or emphysema, whereas patients with CCPA and SA most frequently had prior tuberculosis. A history of pneumonia was detected in 3/7 and 4/5 cases of CNPA and Group A, respectively, and may have been caused by a direct or indirect response to *Aspergillus* spp. A history of thoracic surgery or pneumothorax was also detected in CNPA and Group A cases. Diabetes mellitus was the predominant underlying systemic disease in all subtypes of CPA cases. Cigarette smoking was also seen in all subtypes of CPA cases. Malnutrition was seen in 4/7, 4/5 and 3/8 cases of Group A, CNPA and CCPA, respectively.

3.2. Radiological findings of patients with CPA

Typical radiographic images of each subtypes of CPA are presented in Fig. 2. The radiograph of CNPA and Group A cases was initially infiltrates that progress to form cavities with or without aspergilloma. The radiograph of CCPA cases showed pre-existed cavities and peri-cavitary infiltrates with or without aspergilloma, and SA cases showed a single cavity with aspergilloma. Radiological findings at the time of diagnosis are summarized in Table 3. The upper lobes were affected in all cases of CPA, while both the right and left upper lobes were affected in 4/7 and 1/5 cases of CNPA and Group A, respectively. Although the upper lobes were most commonly affected, the middle and the lower lobes were also affected in CCPA and SA cases. Cavitary infiltrates with aspergilloma was the predominant finding in CNPA and Group A cases. Cavitary nodules with aspergilloma were also recognized in CNPA cases. Cavities with aspergilloma were most commonly found in CCPA cases. All SA cases showed a single cavity with aspergilloma. Aspergillomas were recognized in 4/7, 5/5, 6/8, and 7/7 cases of CNPA, Group A, CCPA, and SA, respectively.

Table 2
Underlying conditions of CPA patients.

	CNPA ($n = 7$)	Group A ($n = 5$)	CCPA ($n = 8$)	SA ($n = 7$)
Respiratory conditions				
Prior tuberculosis	2	1	3	4
Bronchiectasis	1	3	2	3
Prior pneumonia	3	4	0	2
Pneumothorax or bullae	1	3	2	2
COPD or emphysema	5	0	1	0
Prior thoracic surgery	2	2	1	1
Other respiratory conditions[a]	2	4	9	4
Systemic conditions				
Diabetes Mellitus	3	2	2	2
Steroid usage	2	0	2	0
Other systemic conditions[b]	4	0	3	8
Smoking (>20 y)	5	2	7	2
Malnutrition (BMI <18.5)	4	4	3	1

CNPA, chronic necrotizing pulmonary aspergillosis; CCPA, chronic cavitary pulmonary aspergillosis; SA, simple pulmonary aspergilloma; COPD, chronic obstructive pulmonary disease.
[a] Bronchial asthma (4 cases), lung cancer (4 cases), chronic parasinusitis (4 cases), prior pleuritis (2 cases), interstitial pneumonia (2 cases), eosinophilic pneumonia (1 case), non-tuberculous mycobacteriosis (1 case) and pneumoconiosis (1 case).
[b] Gastric cancer (2 cases), colon cancer (1 case), prostate cancer (1 case), rheumatoid arthritis (1 case), Harada disease (1 case), ovarial cyst (2 cases), nasal disease (3 cases), liver disease (1 case), alcoholism (2 cases), and acoustic neuroma (1 case).

3.3. Laboratory findings of patients with CPA

Laboratory findings are presented in Table 4. *Aspergillus* spp. were isolated in 6/7 (86%), 4/5 (80%), 7/8 (88%), and 4/7 (57%) from CNPA, Group A, CCPA, and SA cases, respectively. *A. fumigatus* was the most frequently isolated *Aspergillus* species ($n = 14$), followed by *A. niger* ($n = 5$), *A. flavus* ($n = 2$), and *A. terreus* ($n = 1$) in all CPA cases; while *A. niger* was the predominant isolated species in CNPA cases ($n = 4$). Anti-*Aspergillus* antibody tests were positive in 3/5 (60%), 3/4 (75%), 4/8 (50%), and 1/6 (17%) cases of CNPA, Group A, CCPA, and SA, respectively. *Aspergillus* antigen tests were positive in 5/6 (83%), 4/5 (80%) and 6/6 (100%) cases of CNPA, Group A and CCPA, respectively. However, it was negative in all SA cases. A few CPA cases had a positive β-D-glucan test. The inflammatory marker results indicated a lower positive rate of leukocytosis compared to C-reactive protein and erythrocyte sedimentation rate (ESR), although the number of cases in which ESR was measured was low. The number of cases in which any of the 3 inflammatory markers tested positive was 5/7 (71%), 5/5 (100%), 4/8 (50%), and 2/7 (29%) in CNPA, Group A, CCPA, and SA, respectively.

4. Discussion

The pathogenesis of CPA, especially CNPA and CCPA, has not been widely investigated, and the non-committal definition criteria and overlapping clinical features make it difficult to distinguish the two subtypes. The clinical time course, radiological findings, and defects in innate immunity may be indexes of differences as suggested by Denning and others [3,5,6,8,9], however, subclassification of CPA should be made according to the pathogenesis of the disease on the basis of pathological and radiological features. Although this study is retrospective, we collected a total of 27 CPA cases with complete pathological, radiological, microbiological, and serological findings.

The clinical distinction between SA and other CPA subtypes may not be difficult because of it presents with obvious radiological findings and clinical features. All 7 SA cases in our series showed a solitary cavity with an aspergilloma. On the other hand, discerning the difference between CNPA and CCPA is challenging. Binder et al. described that CNPA is defined as an indolent, cavitating process in the lungs due to the invasion of lung tissue by a fungus of the *Aspergillus* spp. Gefter et al. reported that the radiographic features of SIA include chronic infiltration, progressive cavitation, and subsequent mycetoma formation, and that the absence of a previous cavity distinguishes such cases from secondary non-invasive mycetomas. While, Denning et al. defined CNPA as subacute invasive form of aspergillosis, occurring over 1–3 month, and CCPA as non-invasive chronic cavitary aspergillosis, occurring >3 months.

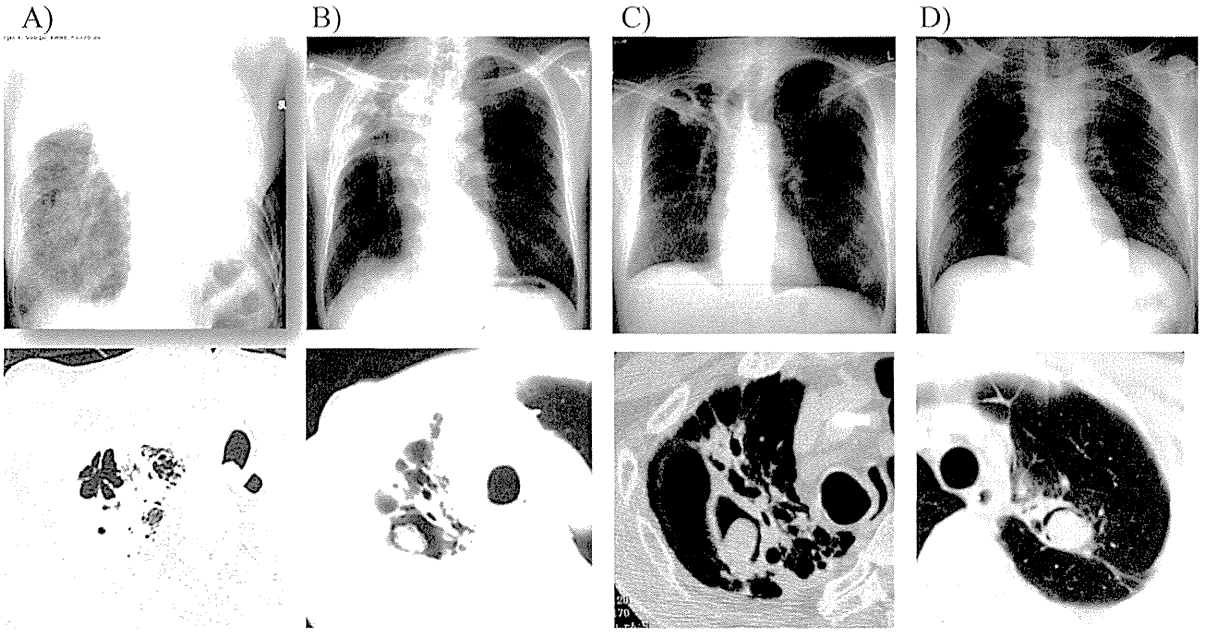

Fig. 2. Chest radiographic images of CPA cases. A) CNPA: the patient (75-year-old man) had chronic obstructive lung disease and a history of left pneumonectomy for intractable pulmonary tuberculosis and aspergilloma 7 years before admission. The radiographs show consolidation around bullae and pleural thickening in the right upper lung. B) Group A: the patient (75-year-old man) had diabetes mellitus and a history of pneumonia 2 years before admission. The radiographs show consolidation around cavities with aspergilloma and pleural thickening in the right upper lung. C) CCPA: the patient (69-year-old man) had a history of pulmonary tuberculosis. The radiographs show multiple cavities with aspergilloma in the right upper lung. D) Simple aspergilloma: the patient (40-year-old man) had a history of bullectomy for left pneumothorax 6 years before admission. The radiographs show a single cavity with an aspergilloma in the left upper lung.

According to Denning et al., the development of CCPA lesions involves two possible ways of cavity formation: 1) the infiltrates were initially ill-defined areas of consolidation that progressed to form well-defined cavities; and 2) the cavities were pre-existing (i.e., in cases of previous tuberculosis or bronchiectasis) [4]. However, the former radiographic features are concordant with those of Binder's CNPA or Gefter's SIA. Thus, the consensus of definition of CNPA is not established, and some cases of Denning's CCPA may be Binder's CNPA.

In this study, we defined the cases with progressive cavitating processes in the lung without a pre-existing cavity and presence of hyphal invasion into lung parenchyma as CNPA, and the cases with pre-existing cavities and peri-cavitary infiltrates and presence of hyphae in a cavity but not in lung parenchyma as CCPA. However, whether group A in our series is classified into CNPA or CCPA is controversial. The radiographic features of CNPA and Group A cases are similar; they are chronic infiltration, progressive cavitation and subsequent aspergilloma formation. The pathological features of the cavity wall of CNPA and Group A cases are similar except hyphal invasion; they are acute and chronic inflammation, granulation, parenchymal necrosis and subsequent cavity formation with or without fragments of destroyed lung tissue in the cavity. The hyphal invasion into cavity wall was observed only in CNPA cases but not Group A cases. Antifungals treatment before the pathological examinations were conducted in 3/7 and 4/5 cases of CNPA and

Table 3
Radiological findings of CPA patients.

	CNPA ($n = 7$)	Group A ($n = 5$)	CCPA ($n = 8$)	SA ($n = 7$)
Affected sites				
RUL + LUL	4	1	0	0
RUL	3	3	3	2
RML	0	0	2	2
RLL	0	0	1	1
LUL	0	1	0	2
LLL	0	0	2	0
Cavities with aspergilloma	2	1	5	0
Cavity with aspergilloma	1	0	1	7
Cavities without aspergilloma	1	1	0	0
Cavitary infiltrates with aspergilloma	3	4	0	0
Cavitary infiltrates without aspergilloma	1	0	0	0
Cavitary nodules with aspergilloma	2	0	0	0
Multiple nodules	1	0	0	0
Bronchiectasis and infiltrate[a]	0	0	1	0
Bronchiectasis and atelectasis[a]	0	0	1	0

CNPA, chronic necrotizing pulmonary aspergillosis; CCPA, chronic cavitary pulmonary aspergillosis; SA, simple pulmonary aspergilloma. RUL, right upper lobe; LUL, left upper lobe; RML, right middle lobe; RLL, right lower lobe; LLL, left lower lobe.
[a] Intrabronchial aspergilloma.

Table 4
Laboratory findings of CPA patients.

	CNPA ($n = 7$)	Group A ($n = 5$)	CCPA ($n = 8$)	SA ($n = 7$)
Isolation of *Aspergillus* spp.				
A. fumigatus	2	4	5	3
A. niger	4	0	1	0
A. flavus	0	0	1	1
A. terreus	1[a]	0	0	0
Not identified	1	1	1	3
Serological findings				
Anti-*Aspergillus* antibody (+)	3/5	3/4	4/8	1/6
Aspergillus antigen (≥0.5 C.O.I.)	5/6	4/5	6/6	0/5
β-D-glucan (≥20 pg/ml)	2/6	1/4	1/5	1/4
Inflammatory makers				
Leukocytosis (WBC>9000/mm^3)	2	0	1	0
CRP (>0.3 mg/dl)	5	4	4	0
ESR (>20 mm/h)	1/1	2/2	3/3	2/4

CNPA, chronic necrotizing pulmonary aspergillosis; CCPA, chronic cavitary pulmonary aspergillosis; SA, simple pulmonary aspergilloma; CRP, C-reactive protein; and ESR, Erythrocyte sedimentation rate.
[a] *A. terreus* was isolated with *A. fumigatus* from the same patient.

Group A, respectively. Additionally, duration of treatment was relatively longer in Group A cases (6 weeks–8 months) compared to that of CNPA(10 days for two case) except one case (approximately 14 months). It is possible that antifungal treatment prior to the pathological examination may block the hyphal invasion of *Aspergillus* in Group A cases. Moreover, systemic symptoms such as fever and weight loss, and underlying conditions such as history of pneumonia or thoracic surgery are common to both CNPA and Group A, but not to CCPA. Thus, the pathological and clinical features and backgrounds of Group A cases were resemble to those of CNPA cases; which means the pathogenesis of CNPA and Group A cases is considered the same.

It is supposed that *Aspergillus* infects air spaces, such as emphysematous bullae, then destroys lung tissue by invasion and/or mycotic toxins, proteolytic enzymes, and metabolites produced by *Aspergillus*. Proteolytic enzymes and oxidant derived from neutrophils and macrophages may also cause tissue necrosis. Thus, the cavity formation can be developed even though the hyphal invasion is minimal or absent. Therefore, the cases with chronic infiltration, progressive cavitation, and subsequent aspergilloma formation should be diagnosed as CNPA; the cases in which hyphal invasion of lung tissue is demonstrated are proven CNPA and the cases in which the invasion is not demonstrated are probable CNPA. Hence, we diagnose the Group A in our series as probable CNPA. The median duration of the disease was 3 and 24 months in proven CNPA and probable CNPA (Group A) cases, respectively. As Binder et al. mentioned that the disease is usually of 1–6 months duration but can be present for years prior to diagnosis, CNPA is not always subacute (1–3 months).

There were pre-existing cavities such as sequelae of pulmonary tuberculosis, bronchiectasis, COPD, bullae, and interstitial pneumonia in all 8 of CCPA cases, and the wall of these cases was composed of chronic inflammatory, granulation, and fibrous tissue layers, and the inner surface of the cavity is covered by bronchial epithelium or metaplastic squamous cell epithelium in part. Thus, CCPA is synonymous with complex aspergilloma. Our study results indicate that CNPA can be distinguishable from CCPA by careful observation of progression of the cavitary lesion if a series of adequate radiography films are available. In some cases, however, it will be difficult to distinguish the two subtypes because adequate films are not always available.

The limitations of our study include its retrospective nature, the number of cases being insufficient for establishing definite criteria, and the need for additional information. The importance of differentiating between CNPA and CCPA is scientific; however, it may be difficult because of the non-committal definition criteria and existence of overlapping findings. From the management perspective, its elucidation does not provide deeper insight into the differentiation, except for executing clinical trials. Although we respect and follow the original definition of CNPA and the concept of CCPA, there is a limitation to distinguishing these two entities in actual clinical settings as discussed above. Hope et al. indicated that apparent distinct entities do not exist for the CPA subtypes and that these forms usually overlap; therefore, the requirement and importance of the rigorous sub-classifications of CPA are unclear [5]. Hence, we propose the term "chronic progressive pulmonary aspergillosis (CPPA)" for the clinical syndrome including both CNPA and CCPA [10]. Therefore, CPA is consisted of CPPA and SA.

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Original article

Clinical features of pulmonary cryptococcosis in non-HIV patients in Japan

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ABSTRACT

Objective: To clarify the clinical features of pulmonary cryptococcosis in Japanese non-HIV population.
Methods: Retrospective investigation of 151 pulmonary cryptococcosis cases between 1977 and 2012 was executed. The underlying disease (UDs), aggravating factors, radiological characteristics, and treatment were examined.
Results: Sixty-seven patients (44.4%) had no UDs. The common UDs were diabetes (32.1%) followed by hematologic disease (22.6%), and collagen disease (22.6%). Peripherally distributed pulmonary nodules/masses were most commonly seen. Lesions in the right middle lobe ($p = 0.01$) and air bronchogram ($P = 0.05$) were significantly more frequent, respectively, in patients with UDs than patients without them. Azoles were mainly selected for the patients without meningoencephalitis. Mean treatment duration for patients with and without UDs was 6.64 and 2.87 months, respectively. Patients whose pulmonary nodules improved after treatment continued to experience gradual reduction of cryptococcosis antigen titers, even if antigen titers were positive at the time of treatment cessation. The average time for antigen titers to become negative after treatment cessation was 13.1 and 10.7 months for patients with and without UDs, respectively. When groups were compared according to the presence of meningoencephalitis complications, deaths, and survivals, factors contributing to cryptococcosis prognosis included higher age, hypoproteinemia, hypoalbuminemia, steroid use, high C-reactive protein levels, and meningoencephalitis complications.
Conclusions: It is crucial to consider the presence of UDs and meningoencephalitis for the choice of antifungals and treatment duration for cryptococcosis in non-HIV patients. Three- and six months-administration of azoles for pulmonary cryptococcosis with or without UDs, respectively is reasonable.

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1. Introduction

Cryptococcus neoformans is a nonmycelial, budding encapsulated yeast-like fungus found in soil contaminated with pigeon and chicken excreta [1–4]. Inhalation of cryptococcal particles from contaminated soil into the lung is considered the usual route of human infection [2,3]. The organism may cause isolated pulmonary infection or hematogenous dissemination involving the central nervous system (CNS), bones, and skin, mostly depending on the host immunity [2,3]. Although cryptococcal infection can occur in individuals with normal immunity, it most commonly occurs in immunocompromised hosts. Predisposing factors are acquired immune deficiency syndrome (AIDS) and other causes of impaired T cell–mediated immunity, e.g., transplant-related immunosuppression, hematological malignancies, corticosteroid administration, and diabetes mellitus [4–6].

Although the clinical characteristics and natural history of cryptococcosis in HIV patients have been described elsewhere due to its large number, those in non-HIV patients have rarely been reported [7]. To date, few studies have reported comparative data regarding the clinical manifestations, laboratory findings, radiographic findings and survival of patients with pulmonary cryptococcosis in Japan [8]. Additionally, very few research comparing clinical manifestation of cryptococcosis between HIV and non-HIV patients [9–11].

In Japan, the number of HIV/AIDS patients is relatively lower compared to those of other countries. However, it is increasing recently and over 20,000 of the cumulative patients are registered in Japanese government database to date (http://www.nih.go.jp/niid/ja/aids-m/aids-iasrd/2274-kj3888.html). Hence, the study of clinical manifestation of cryptococcal diseases in non-HIV background possess high impact. We reviewed 151 cryptococcal cases among non-HIV background and investigated the clinical features, including clinical manifestations, underlying conditions, laboratory findings, radiological features, treatment, survival, and outcomes.

2. Materials and methods

2.1. Patients

A retrospective cohort study was conducted by reviewing the medical records of patients who had been diagnosed with pulmonary cryptococcosis at Nagasaki University Hospital and its affiliated hospitals during the 35-year period between 1977 and 2012. The patients were grouped into 2 populations based on positivity of underlying diseases. Definite case of pulmonary cryptococcosis requires isolation or detection of *Cryptococcus* by lung specimen culture and/or by histopathological examination, and only definite cases are recruited in this study. This retrospective study including analysis and release of clinical data was approved by the ethical committee of Nagasaki University Hospital.

2.2. Clinical data

All available patient records were reviewed from the time of cryptococcal diagnosis until the patients died or were lost to follow up.

The data included clinical manifestations, underlying conditions, laboratory findings (age, lymphocyte count, neutrophil count, immunoglobulin, serum protein, serum albumin, CD4/8 ratio, CD4 count, C-reactive protein [CRP], cryptococcal serum antigen titers) at the timing of diagnosis, radiological findings, treatment, survival, and outcome were recorded.

Eiken Latex® (Eiken Kagaku Co., Tokyo, Japan) was used for the qualitative and semi-quantitative detection of the *C. neoformans* capsular polysaccharide antigen in serum and CSF according to the manufacturer's instructions.

2.3. Interpretation of chest CT scans

The findings of chest CT scans were assessed for 1) the presence and distribution of parenchymal lesions, including nodules, masses, and consolidation; 2) the characteristics of nodules and masses; and 3) related thoracic abnormalities such as pleural effusion and lymphadenopathy according to previous reports [12]. Based on the predominant parenchymal findings from the CT scans, the morphological characteristics were classified as solitary nodule/mass (type I), multiple nodules/masses (type II), and consolidation (type III). In addition, type II was subdivided into distribution in a single lobe (type IIa) and distribution in multiple lobes (type IIb).

2.4. Statistical analysis

We used FREQ, NPAR1WAY, and ANOVA in SAS. The chi-square test was used to compare the frequency of categorical variables (e.g., underlying disease, steroid usage). Wilcoxon's test was used to compare age, lymphocyte count, neutrophil count, serum protein, serum albumin, CD4/8 ratio, CD4 count, CRP, and cryptococcal serum antigen titers. The Eiken Latex® latex agglutination test was used to detect cryptococcal polysaccharide. Antigen titers were transformed to the logarithm to the base 2 ($Log_2[Ag + 1]$). Ag (cryptococcal antigen titer) is expressed as 0, 1, 2, 4, ... as powers of 2 and Ag + 1 was expressed as $Log_2(0 + 1) = 0$.

For radiographic analysis, a chi-square test was employed to compare the presence and distribution of parenchymal lesions, nodule and mass characteristics except their number, and related thoracic abnormalities between the 2 groups. A Cochran–Armitage test was used to analyze the differences among 4 groups based on the number of nodules and masses, and among 4 morphological types based on the CT classification between the 2 patient populations. For all statistical tests, $p < 0.05$ indicated a significant difference.

3. Results

3.1. Patients

One hundred fifty-one patients were diagnosed with pulmonary cryptococcosis during the 35-year period between 1977 and 2012. Sixty-seven (44.4%) occurred in the patients without underlying diseases. Forty-two were men and 25 were women. Eighty-four cases (56.6%) were the patients with underlying disease. Thirty-eight were men and 46 were women.

3.2. Underlying diseases

Among 84 patients with underlying diseases, diabetes mellitus was most dominant (32.1%) followed by hematological diseases including human T-cell leukemia virus type-I carrier (22.6%), collagen disease including systemic lupus erythematosus, rheumatoid arthritis and others (22.6%), renal failure (16.7%), solid tumor (13.1%), chronic lung diseases including bronchiectasis, sequel pulmonary tuberculosis, and interstitial pneumonia (13.1%), liver disease including cirrhosis or hepatitis (9.5%), renal transplantation (2.4%), and other diseases (9.5%). Treatment with glucocorticoids (5–40 mg/day or pulse therapy) were recorded in 31 (37.0%) patients. Total of 5 patients were administrated glucocorticoids concomitantly with immunosuppressant such as cyclosporine and azathioprine.

3.3. Clinical symptoms

In 67 patients without underlying diseases, 43 (64.2%) patients were asymptomatic and detected accidentally by mass screening examination. Others had pulmonary symptoms such as cough ($n = 15$; 22.3%), sputum ($n = 4$; 6.0%), chest pain ($n = 7$; 10.4%), fever ($n = 2$; 3.0%), and others. In 84 patients with underlying disease, 39 patients (46.4%) were asymptomatic and found by abnormal chest radiograph findings taken as during routine examination of underlying diseases. Others had pulmonary symptoms such as cough ($n = 15$; 17.6%), sputum ($n = 15$; 17.6%), chest pain ($n = 3$; 3.6%), fever ($n = 20$; 23.8%), and other symptoms ($n = 19$; 22.6%).

3.4. Laboratory findings

The laboratory findings of the patients with and without underlying disease at the timing of diagnosis are shown in Table 1. The patients without underlying disease are statistically younger, better nutrition status reflected by higher total protein and albumin value, compared to those with underlying diseases. Serum antigen titers (Log_2[Antigen titer + 1]) were not different statistically in both arms.

Compared to steroid non-usage patients ($n = 114$), steroid usage patients ($n = 36$) were statistically significantly older ($p < 0.0001$), had lower lymphocyte count ($p = 0.03$), higher neutrophil count ($p = 0.02$), lower blood serum protein ($p = 0.0002$), lower blood serum albumin ($p < 0.0001$), and higher CRP ($p = 0.001$). There was no significant difference in IgG, IgA, or IgM between the two groups.

3.5. CT findings

Table 2 shows the detail of CT findings between patients with or without underlying diseases. The CT findings of 81 of 151 pulmonary cryptococcosis patients were analyzed. Forty-two and 39 patients were without and with underlying diseases, respectively. The frequency of the four CT classification types based on predominant parenchymal findings and lobar distribution of the lesions is listed in Table 2. Type IIb and type III lesions occurred more frequently in patients with underlying diseases than in those without underlying diseases. The main finding of this study is the presence of peripherally distributed multiple pulmonary nodules or masses with predominant lower lobe involvement in both patients without and with underlying diseases ($P < 0.0001$, Data not shown). Parenchymal lesions in the right middle lobe ($P = 0.01$), masses and more extensive lung involvement such as multiple lobes in distribution ($P = 0.06$) were more common in patients with underlying disease than those without underlying diseases. The number of masses (>30-mm diameter) ($P = 0.02$) and air bronchogram ($P = 0.05$) was significantly more common in patients with underlying diseases than those without underlying diseases.

3.6. Serum cryptococcal antigen titer and radiological findings

The relationship of serum cryptococcal antigen titer and radiological findings were analyzed. Data from patients with

Table 1
Characteristics of patients with cryptococcosis with or without underlying disease.

		State of underlying conditions						
		Patients without underlying diseases			Patients with underlying diseases			Wilcoxon test
	Criteria	n	Median	IQR	n	Median	IQR	p Value
1	Age	67	41	31	84	63	18.5	<0.0001
2	lymphocyte counts	54	1985.5	573.0	74	1429.0	1218.0	0.03
3	Neutrophil counts	55	3245.0	2403.0	75	4680.0	4273.0	0.02
4	IgG	32	1262.0	435.0	37	1343.0	891.0	0.31
5	IgM	32	142.0	71.3	37	145.0	96.0	0.49
6	IgA	31	249.0	142.0	37	275.0	169.0	0.39
7	Total protein	48	6.90	0.70	65	6.40	1.40	0.0002
8	Serum albumin	42	4.39	0.54	60	3.60	1.29	<0.0001
9	CD4/CD8	31	1.50	0.79	37	1.42	0.90	0.53
10	CRP	40	0.21	0.33	49	0.84	3.60	0.001
11	Cryptococcal antigen	56	16.00	124.00	63	32.00	252.00	0.35
12	CD4 counts	17	874.80	282.20	19	637.00	915.20	0.99

IQR: Inter Quartile Range, CRP, C-reactive protein.

Table 2
Comparison of CT findings of cases with pulmonary cryptococcosis with or without underlying disease.

	Patients without underlying diseases		Patients with underlying diseases		p Value
	n = 42	%	n = 39	%	
Mean of age (range)	47.4 (15–80)		61.4 (19–79)		
Sex (men: women)	26:16:00		18:21		
Presence of parenchymal lesions					
Nodule and masses	42	100.0	39	100	
Consolidation	3	7.1	7	18.0	0.14
Solitary nodules/ mass(type I)	14	33.3	9	23.1	0.30
Multiple nodules	25	59.5	29	74.4	0.15
Single lobe limited (type IIa)	10	23.8	5	12.8	0.06
Multiple lobe limited (type IIb)	15	35.7	24	61.5	
Consolidation (type III)	3	7.1	7	17.9	0.14
Distribution of parenchymal lesions					
Lobar distribution					
Right upper lobe	13	31.0	16	41.0	0.34
Right middle lobe	6	14.3	15	38.5	0.01
Right lower lobe	26	61.9	28	71.8	0.34
Left upper lobe	10	23.8	6	15.4	0.34
Lingura	3	7.1	5	12.8	0.39
Left lower lobe	20	47.6	17	43.6	0.71
Contact with pleura	33	78.6	33	85.0	0.48
Size (mm)					
1–30	31	73.8	16	41.0	0.02
31-	5	11.9	11	28.0	
Number					
1	15	35.7	11	28.0	0.85
2–4	10	23.8	10	26.0	
5–9	7	16.7	7	18.0	
10-	9	21.4	11	28.0	
Border					
Well-defined/ ill-defined	34/8	(81/19)	34/5	(87/13)	0.44
Margin					
Smooth/irregular/ speculated	20/21/19	(48/50/45)	12/27/20	(31/69/51)	0.25
Convergence of bronchi and vessel	35	83.3	31	79.0	0.65
Pleural identification	19	45.2	22	56.0	0.32
Internal characteristics					
Air-bronchogram	21	50.0	28	72.0	0.05
Cavitation	12	28.6	16	41.0	0.24
Calcification	1	2.4	0	0.0	0.33
CT halo sign	25	59.5	18	46.0	0.22
Satellite lesion	27	64.3	26	67.0	0.82