

## Original Article

# Human Parainfluenza Virus Type 3 Infections in Patients with Hematopoietic Stem Cell Transplants: the Mode of Nosocomial Infections and Prognosis

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**SUMMARY:** There have been a few prospective and comprehensive surveillance studies on the respiratory viral infections (RVIs) among patients undergoing hematopoietic stem cell transplantation (HSCT). A 2-year prospective cohort surveillance study of symptomatic and asymptomatic RVIs was performed in hospitalized HSCT patients. Oropharyngeal (OP) swab samples were serially collected each week from 1 week before and up to 100 days after HSCT and were tested for virus isolation with cell culture-based viral isolation (CC-based VI) and a multiplex PCR (MPCR). A total of 2,747 OP swab samples were collected from 250 HSCT patients (268 HSCT procedures). Among these patients, 79 had RVIs (CC-based VI,  $n = 63$ ; MPCR,  $n = 17$ ). The parainfluenza virus type 3 (PIV3) accounted for 71% (57/80) of the cases of RVIs. Some PIV3 infections were asymptomatic and involved a longer virus-shedding period. The PIV3 was often cultured from samples taken before the onset of a respiratory disease. The PIV3 infections were attributed to the transmission of nosocomial infections. PIV3 infections before engraftment will more likely result in the development of lower respiratory tract infections and worse outcomes. A real-time monitoring of respiratory viral infections in the HSCT ward among patients with or without respiratory symptoms is required for the prevention of nosocomial RVIs, especially of PIV3 infections.

## INTRODUCTION

Patients who undergo hematopoietic stem cell transplantation (HSCT) suffer from severe immune impairment and are extremely susceptible to infectious diseases, including respiratory viral infections (RVIs). A detailed research revealed that respiratory syncytial virus (RSV), human parainfluenza virus (PIV), influenza virus (Flu), human rhinovirus (HRV), human coronavirus (HCoV), and human metapneumovirus were the major pathogens of RVI in HSCT patients (1–11). Nosocomial outbreaks in HSCT units are not uncommon (12–16). RVIs that occur after HSCT were reported to result in lower respiratory tract infections (LRTIs), which are associated with a high rate of morbidity and mortality, especially when they occurred within 100 days after HSCT (8,17–19). However, most studies were based on retrospective case series.

Thus, this 2-year prospective cohort study was aimed to identify the different respiratory virus infections in an HSCT ward using multiple cell culture-based virus isolation (CC-based VI) and to clarify the characteristics of the virus infection in severely immunocompromised HSCT patients. To ensure that both symptomatic and asymptomatic infections were included, oropharyngeal (OP) swab samples were collected from HSCT patients on a weekly basis, regardless of their respiratory symptoms.

## MATERIALS AND METHODS

**Patients:** Patients who underwent HSCT from June 2010 to May 2012 in the Department of Hematology, Toranomon Hospital, Tokyo, Japan, were recruited for this study. The underlying disease risks were assessed according to the criteria reported previously (20); the conditioning regimen was described in the previous study, which reported the association between the emergence of acyclovir-resistant herpes simplex virus 1 and the prognosis in HSCT patients (21). Conditioning regimens with  $> 8$  mg/kg of busulfan or  $> 6$  gray of total body irradiation were defined as myeloablative conditioning, whereas other regimens were described as reduced intensity conditioning (22). Tacrolimus or cyclosporin A, with or without mycophenolate mofetil, or short-term methotrexate was administered as prophylaxis against graft-versus host

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disease (GVHD) in patients receiving human leukocyte antigen (HLA)-mismatched transplants. Acute GVHD was classified according to the consensus criteria (23). All patients were admitted to the same hospital ward and were isolated in a single room equipped with positive air pressure using an HEPA filter after HSCT until the engraftment of the transplanted cells were confirmed. The engraftment day was defined as the third consecutive day, on which an absolute neutrophil count of  $> 500/\mu\text{l}$  was achieved. When respiratory symptoms appeared, the patients were strictly isolated to avoid nosocomial infections. A comparison between LRTIs and upper respiratory tract infections was made by clinicians based on the patients' symptoms, laboratory data, and/or X-ray findings. The onset and duration of symptoms were also recorded prospectively. Patients who were positive for respiratory virus pathogens, but without any symptoms, were considered asymptomatic.

**Ethical consideration:** This study was approved by the ethical review boards of Toranomon Hospital, National Sendai Medical Center (Sendai, Japan), and the National Institute of Infectious Diseases (NIID, Tokyo, Japan). A written informed consent was obtained from each patient, in accordance with the Declaration of Helsinki.

**Microbiological procedures:** OP swab samples were collected from all patients on a weekly basis, regardless

of their respiratory symptoms that developed from day 6 to day 100 after HSCT (the day of transplantation was day 0) (Fig. 1). All the samples were subjected to CC-based VI. If no pathogens were found with CC-based VI, the samples were then further tested with a multiplex PCR (MPCR) using the residues of the OP swab samples, which were used for CC-based VI. In patients with respiratory symptoms and suspected RVI, respiratory secretion samples were also collected independently and tested for an MPCR as described below.

After sample collection, the OP swab specimens were immediately stored at  $4^{\circ}\text{C}$  and were sent to the Virus Research Center, Sendai Medical Center, for CC-based VI on the same day that the sample was collected. The CC-based VI procedure involves the inoculation of specimen into 6 types of cultured cells: African green monkey kidney (Vero), human embryonic lung fibroblast (HEL), Madin-Darby canine kidney (MDCK), human epidermal carcinoma (HEp2), continuous rhesus monkey kidney (LLC-MK2), and human malignant melanoma (HMV-II) cells (24–26). After inoculation, the residues of the OP swab samples were stored at  $-80^{\circ}\text{C}$ . When a bacterial/fungal contamination occurred, the samples were filtered with  $0.22\text{-}\mu\text{m}$  pores and were re-subjected for CC-based VI. The cells were observed for up to 21 days. When the cytopathic effect appeared, the samples were further tested for virus identification. Hemadsorption tests were

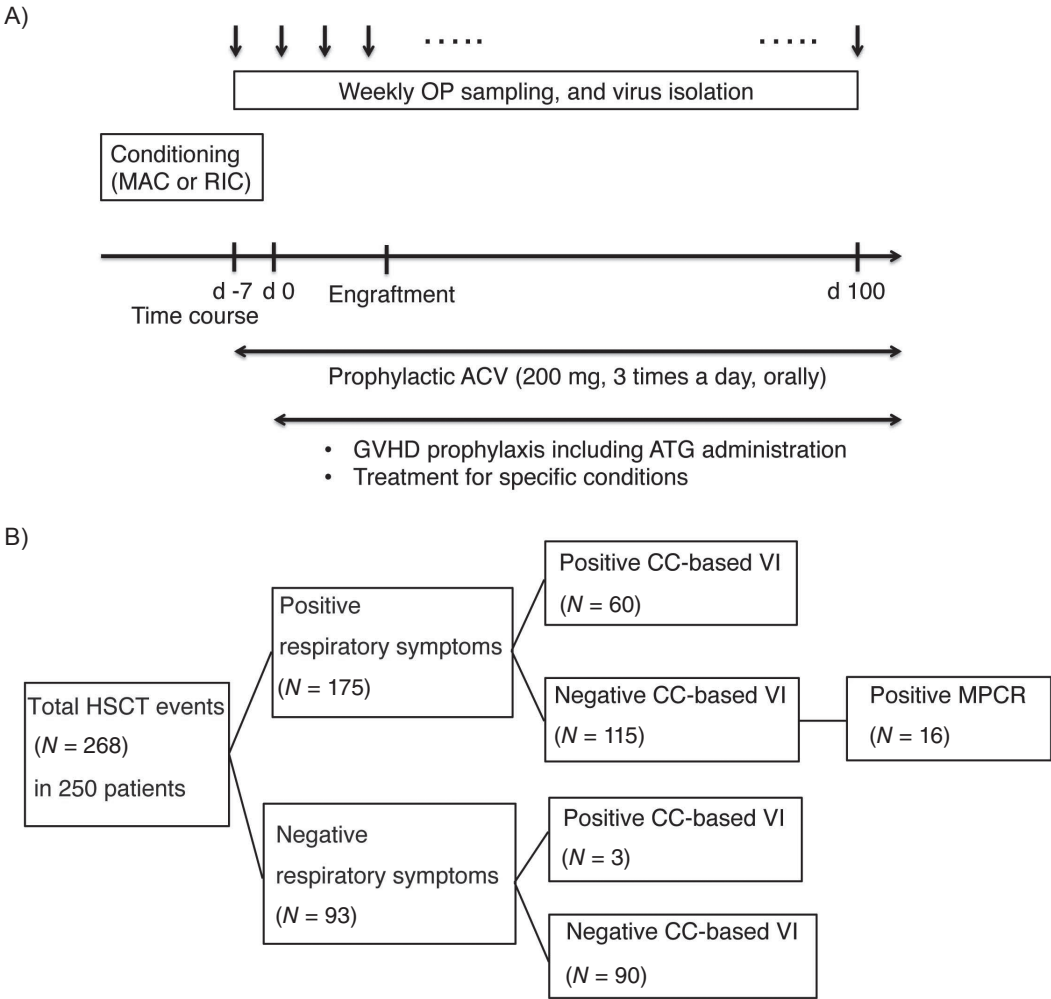


Fig. 1. A schematic representation of the OP sample collection procedure and the standard clinical course of HSCT, and the day for transplantation and engraftment in each patient (A). Categorization of the patients enrolled by detection of respiratory viruses with CC-based VI and MPCR (B).

performed on days 7 and 14 after the inoculation of samples to detect hemagglutination activity-positive viruses. The isolated viruses were identified as previously reported (24).

MPCRs were performed as follows. The nucleic acid was extracted from the residues of OP swab samples stored using the High Pure Viral Nucleic Acid Kit (Roche, Basel, Switzerland). A complemented DNA (cDNA) from the purified RNA was then produced with a 1st-strand cDNA synthesis kit (TAKARA, Tokyo, Japan). The cDNA was subjected to MPCR using 2 MPCR systems: COSMO<sup>®</sup> respiratory-associated virus set targeting HRV, RSV, HCoV, Flu, and adenovirus (ADV) (Maxim Biotech, Rockville, CA) and a combination of primer sets for PIV1, PIV2, and PIV3 (27) and hMPV (28). The cycling conditions were described previously (29). When a positive band appeared in agarose gel electrophoresis, the amplicons were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA). They were, subsequently, sequenced through cloning into pcDNA (Promega, Madison, WI) using the universal primers for the vector, and the virus type was determined.

**Determination of the partial hemagglutinin-neuraminidase (HN) gene of PIV3:** The viral RNA was extracted from the PIV3 stock isolated using a High Pure Viral RNA Kit (Roche), and a reverse-transcription PCR was conducted using an Illustra Ready-To-Go RT-PCR Beads kit (GE, Fairfield, CT, USA) with primers targeting part of the HN gene of PIV3 (30), which was used in previous epidemiological studies of PIV3 outbreaks (14). Amplicons were sequenced directly and bi-directionally using the PCR primers with BigDye reagents (Applied Biosystems, Carlsbad, CA).

**Molecular epidemiology:** A phylogenetic analysis was conducted using the nucleotide sequences of the isolated PIV3 strains. The following software programs were used in the analysis: ATGC ver. 6 was used for base calling and end-trimming; Clustal-X, for determining the nucleotide sequence data alignment; MEGA ver. 6, for creating the phylogenetic trees using the most-likelihood technique (31). The PIV3 strains isolated from pediatric patients with URTI treated in the outpatient clinic of Sendai Medical Center were analyzed in the same manner for comparison.

**Statistical analysis:** The two-sided Fisher's exact test (using  $2 \times 2$  tables) was performed to compare the clin-

ical characteristics of the patients. The Mann-Whitney U test was used to compare the age at transplantation and the days to engraftment of the transplanted stem cells. A univariate analysis was performed to determine the risk factors for RVI and viral LRTI acquisition. The factors with  $P$  values of  $< 0.2$  in the univariate analysis were included in the multivariate stepwise analysis. The factors with  $P$  values of  $< 0.05$  in the multivariate analysis were considered to be statistically significant. All the statistical analyses were conducted using the JMP 11 software program (SAS institute, Cary, NC).

## RESULTS

**Patients:** During the study period, 269 consecutive HSCTs were performed in 251 patients (26 re-transplantations were performed in 24 patients). Eight of the 26 re-transplants were performed within 100 days of the previous HSCT because of graft failure ( $n = 5$ ) or relapse ( $n = 3$ ). Each of these re-transplants was regarded as part of a single HSCT session. The remaining 18 re-transplants were conducted more than 100 days after the previous transplant and were regarded as independent HSCT procedures. The patient who was transferred to another hospital within 100 days was excluded from the analysis. Consequently, the data regarding the 268 HSCTs performed in 250 patients were analyzed. A total of 2,747 OP swab samples (mean, 10.3 per transplantation) were collected. The information on these patients was described previously (21).

**RVIs within 100 days after HSCT:** An overview of the study cohort is shown in Fig. 1A. This figure shows that 175 of 268 patients presented respiratory symptoms within 100 days after transplantation. Moreover, 63 of the 175 HSCT patients were diagnosed with RVIs caused by bacterial and/or fungal pathogens, based on the clinical and/or laboratory test results (data not shown). In the remaining 112 cases, respiratory symptoms were not caused by bacterial and/or fungal pathogens.

The detailed results of the virus isolation tests and virus detection with MPCR are summarized in Table 1. This table shows that PIV3, PIV2, Flu A, RSV, PIV1, ADV, and echovirus were isolated from 63 HSCT patients, and majority of the isolates was PIV3 (51/63).

Respiratory virus genomes were detected in 17 additional patients with RVIs using the MPCR tests. One

Table 1. The number of the patients, from whom each respiratory virus was detected with CC-based VI and MPCR with the classification 1 of the patients according to the clinical diagnoses of LRTI, URTI, and asymptomatic infection

Categories		Number of the HSCT patients, from whom viruses were detected								Total
		PIV1	PIV2	PIV3	RSV	RSV+ADV	ADV	FluA <sup>1)</sup>	EV	
Methods	CC-based VI	2	4	51	2	0	1	2	1	63
	MPCR	0	0	6	9	1	0	1	NT <sup>2)</sup>	17
Total		2	4	57	11	1	1	3	1	80
Diagnosis	LRTI	1	3	34	8	1	1	1	0	49
	URTI	0	1	21	3	0	0	2	1	28
	Asymptomatic	1	0	2	0	0	0	0	0	3
	Total	2	4	57	11	1	1	3	1	80

<sup>1)</sup>: "FluA" indicates "influenza virus A."

<sup>2)</sup>: "NT" indicates "not tested."

patient was positive for both ADV and RSV (Table 1). MPCRs were used to test the OP swab samples of 69 patients, in whom no pathogens were detected. The PIV3 genome was detected in 6 additional patients. Forty-nine of the 80 respiratory virus-positive patients (61%) were diagnosed with LRTIs, whereas 3 patients had asymptomatic PIV1 or PIV3 infections (Table 1).

**Monthly distribution and the phylogenetic analysis of the PIV3 strains:** Because PIV3 was the most dominant RVI pathogen detected, the monthly distribution of PIV3 infections was studied in greater detail (Fig. 2). The peak of the PIV3 isolation in the cohort was observed 2 months later than that reported in the Infectious

Agent Surveillance Report (IASR), distributed by the NIID (available online: <http://www.nih.go.jp>) in each year (Fig. 2A). The monthly distribution of PIV3 in the 1st year of the study was especially intriguing; there were twin peaks and the outbreak lasted until winter, when virtually no PIV3 cases were observed in the community. The hospital stay, the presence of respiratory symptoms, and the PIV3 status of the 34 PIV3-positive patients in the 1st year of the study are shown (Fig. 2B). All but 1 (ID-76) of the PIV3-positive patients showed respiratory symptoms. The isolation of PIV3 preceded the onset of respiratory symptoms in 11 of the 34 patients. Four patients (ID-33, ID-34, ID-37, and ID-38)

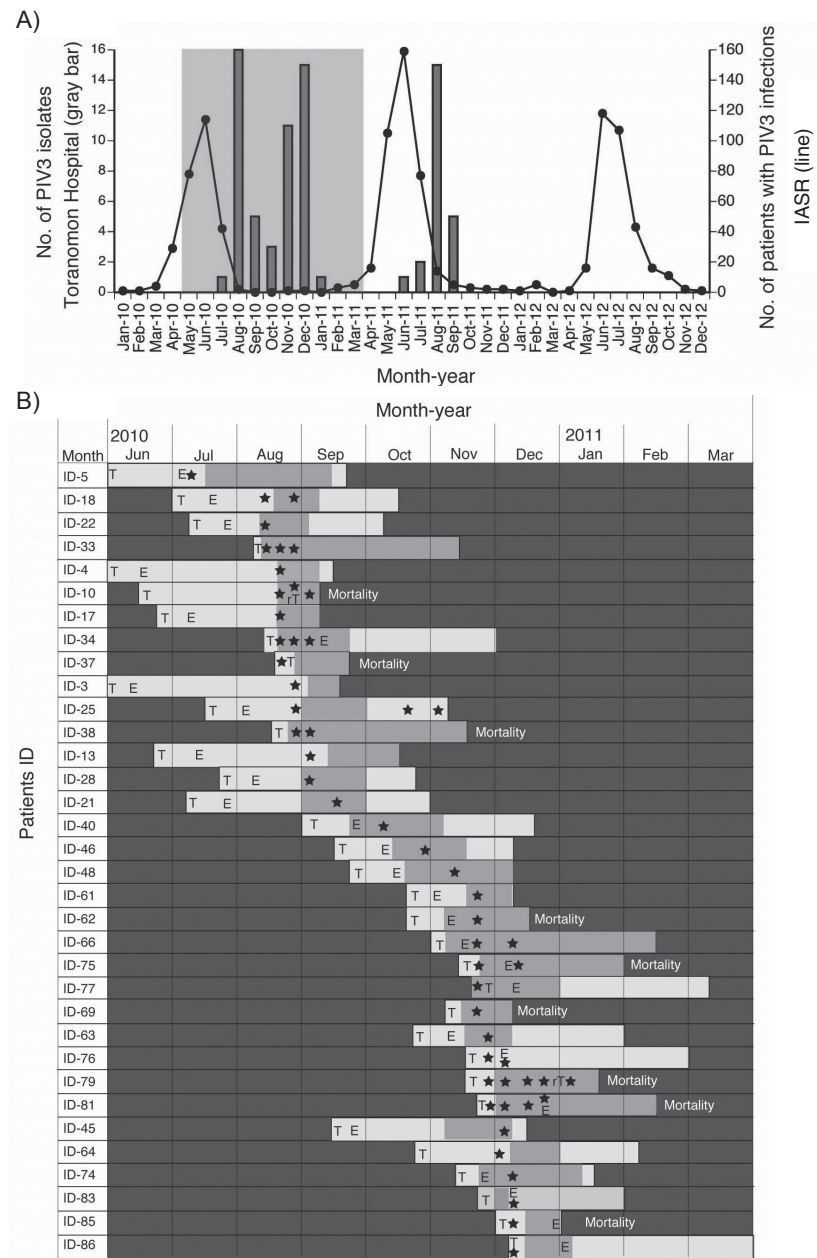


Fig. 2. The monthly distribution of the number of PIV3 isolates, which were detected in the ward and those reported in the same season in the IASR distributed by the NIID, Tokyo, Japan (available online; <http://www.nih.go.jp/niid/images/iasr/table/virus/inf10.pdf> for 2010 data; <http://www.nih.go.jp/niid/images/iasr/table/virus/inf11.pdf> for 2011 data; <http://www.nih.go.jp/niid/images/iasr/table/virus/inf12.pdf> for 2012 data) (A) and the hospital stay, presence of respiratory symptoms and PIV3 isolation status of the 34 PIV3-positive patients in the 2010–2011 outbreak (B). The part gray-colored in each patient's hospitalization indicates the duration with respiratory tract symptoms. The abbreviations are as follows: T, transplantation; E, engraftment; RT, re-transplantation; Mortality indicates the discharge due to the fatal outcome. The black star (★) indicates positive PIV3 isolation.



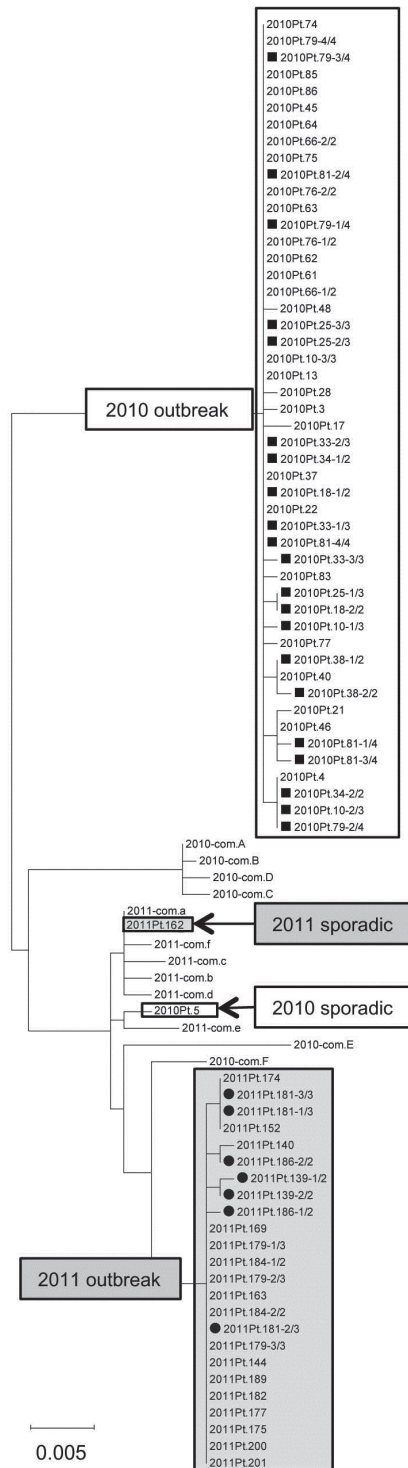


Fig. 3. The phylogenetic trees of the PIV3 isolates evaluated with the hemagglutinin-neuraminidase (HN) gene nucleotide sequences constructed from the PIV3 isolates and the sequences (community isolates) isolated from children with respiratory symptoms in the same study period. These community isolates were the viruses isolated from children in Sendai City, approximately 350 km north of Tokyo. The PIV3s with the name, in which “com” is inserted, indicates the community isolates. The number in the name indicates the year of isolation. The name of each PIV3 isolate from HSCT patients was defined as year of isolation-Patient ID (Pt-ID number)-order of isolation/number of isolation. For example, “2010Pt79-4/4” indicates the PIV3 isolate recovered from the patient ID-79 on the 4th isolation events among the 4 isolation events in total. Furthermore, the isolate with black box indicates the isolates, which were isolated from a single patient on equal to or more than 2 events during the hospitalization period.

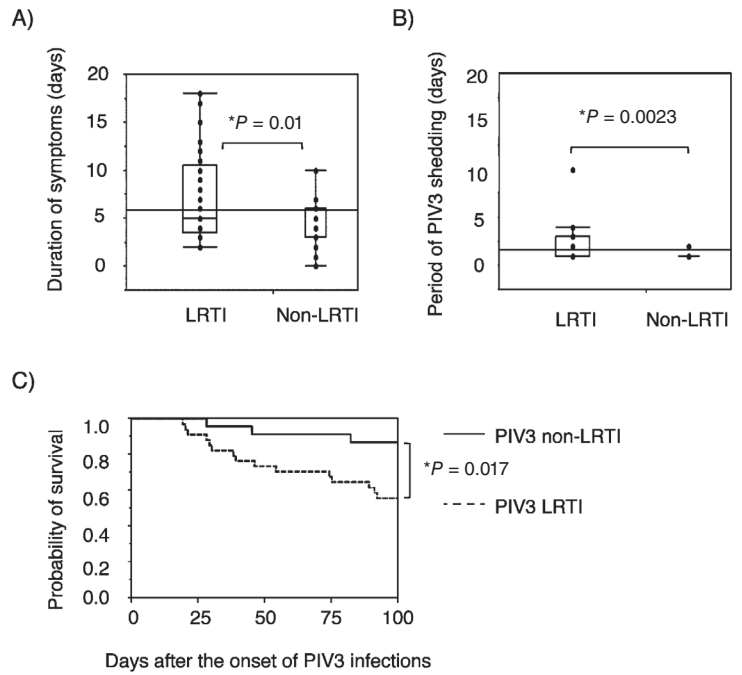


Fig. 4. A comparison of the clinical characteristics of the symptoms and outcomes, duration of symptoms (A), PIV3-shedding period (B), and 100-day survival rate (C) among the PIV3-positive subjects with and without LRTIs.

were hospitalized in August (Fig. 2B). One patient (ID-25) shed PIV3 9 weeks later from the first PIV3 isolation when the patient was asymptomatic.

**The phylogenetic analyses of the PIV3 isolates:** The phylogenetic trees of the PIV3 isolates are shown in Fig. 3. The partial HN gene of PIV3 obtained in this study was deposited in the DDBJ with the following accession numbers: LC189373-LC189456. Among the isolates, 1 isolate (2010Pt 5) in the 2010–2011 season infections formed an independent cluster compared with those isolates in the 2011–2012 season and those isolates from community-acquired infections (Fig. 3). A minor variability of up to 2 nucleotide sequences in 881 sequences was detected between the isolates in each major cluster.

**The risk factors for PIV3 infections and their morbidity and mortality:** The symptoms and outcomes of the PIV3-positive patients with LRTIs and non-LRTIs are shown in Fig. 4. The duration of symptoms in patients with LRTIs was significantly longer than that of the PIV3-positive non-LRTI patients (Fig. 4A). Furthermore, the PIV3-shedding period in the patients with LRTIs was longer than that of the patients with non-LRTIs (Fig. 4B). The 100-day survival rate was significantly lower in the PIV3-positive patients with LRTIs than in those with non-LRTIs (Fig. 4C). The risk factors for LRTI development in PIV3-positive patients were evaluated (Table 2). The time point of the infection was crucial: the infection that occurred before the engraftment was associated with a higher risk of LRTI development. The conditioning type, donor type, and HLA match were not correlated with LRTI-development.

## DISCUSSION

Two independent PIV3 infection clusters were observed in the HSCT ward within the 2-year-study peri-

Table 2. The risk factors for LRTI development among patients with PIV3 infections

Category	LRTI due to PIV3 infection (N = 34)	Non-LRTI due to PIV3 infection (N = 23)	P value (univariate analysis)	P value (multivariate analysis)	odds ratio (95% CI)
Age, median (range)	54 (20–72)	45 (24–69)	0.20		
Myeloablative conditioning	28 (82%)	19 (83%)	1.0		
Mismatched donor	4 (12%)	3 (13%)	1.0		
Cord blood transplant	29 (85%)	18 (78%)	0.50		
Days to engraftment	19 (12–33, n = 29)	17 (11–61, n = 22)	0.86		
Infection before engraftment	23 (67%)	5 (22%)	0.0011	0.00067	7.52 (2.21–25.6)
GVHD grade $\geq 2$	21/29 (72%)	15/22 (68%)	0.77		
Steroid use	25 (74%)	16 (70%)	0.77		

od. The peak of the infections occurred approximately 2 months after the community outbreaks (Fig. 2A). Each of the PIV3 infection clusters was caused by the introduction of a single strain of PIV3 and lasted for a longer period (Figs. 2 and 3). These results indicate that the PIV3 infections developed as a nosocomial infection.

The relationship between the course of the respiratory symptoms and the time point of viral culture positivity was observed, revealing several important findings. First, one unique aspect of PIV3 was that there were asymptomatic cases with prolonged viral shedding in patients, including a patient who shed infectious PIV3 for as long as 9 weeks from the first nosocomial PIV3 infection, bridging the 2 PIV3 peak months of July to November. Second, the positive PIV3 isolation preceded the onset of respiratory symptoms, indicating that the PIV3 replicates in the respiratory tract before the disease onset. Third, detailed analyses of the patients' hospitalization statuses and the molecular epidemiology showed that the PIV3 outbreaks were likely to be attributable to nosocomial transmissions.

Prolonged and/or asymptomatic PIV3 shedding is known to occur in both immunocompetent and immunocompromised individuals (32–34); furthermore, single- and multi-cluster PIV3 outbreaks among HSCT patients were reported (12–15, 35, 36). The difficulty in preventing PIV3 among HSCT patients may be due to the unique, but unidentified features of the virus. Precautions are the most important measures. However, a certain number of patients infected with PIV3 were under strict single-room isolation before engraftment. The members of the medical staff and/or hospital visitors were possibly responsible for the nosocomial transmission. Further studies that include staff members and visitors may assist in the development of better strategies for preventing nosocomial PIV3 transmission. To improve prevention, the use of rapid diagnostic kits for PIV3 with high sensitivity and specificity is needed. However, such kits are not currently available in the clinical setting.

PIVs were an important cause of life-threatening pneumonia in adult HSCT recipients, particularly those patients who had recently undergone allogeneic bone marrow transplantation (17). One study demonstrated that 24 (5.3%) of 456 HSCT patients were diagnosed as having PIV3 infections, and 10 of those PIV3-positive patients had upper RTI and survived, but 8 of the 14 with LRTI died (19). Both upper RTI and LRTI due to PIV3 were associated with overall mortality (34). Moreover, patients with probable and proven PIV-associated LRTI

had significantly worse survival than those with URTI (37). All these studies were carried out in a retrospective manner. On the contrary, the nature of the present study is unique; this study was prospectively performed, and regular virus isolations were done on all patients regardless of the presence of RTI symptoms.

The duration of symptoms and viral shedding were longer in patients with LRTIs, affecting the patients' quality of life. As regards the risk factors for LRTI development in patients with PIV3 infection, the time point of the infection was crucial. Therefore, infection before engraftment was a strong predictor of LRTI development. PIV3 infections that occurred in patients with severe bone marrow suppression resulted in more severe clinical manifestations. Moreover, this study might be the first to report the association between PIV3 infection before engraftment and poor prognosis. However, the significance of the previously reported risk factors (steroid use, neutropenia, lymphocytopenia, and the APACHE II score) for PIV3 LRTI development (18, 34, 38) was not demonstrated in this study.

The present study had several limitations. The patients were all treated at a single center; thus, the possibility of sampling bias cannot be ruled out. Additionally, the sampling policy and clinical definition are other possible limitations. Previous studies reported that the strict definition according to the site of PIV3 detection was critically correlated with patient's prognosis (37); a diagnosis of "proven LRTI," with the detection of PIV3 from lower respiratory samples, was associated with the worst prognosis. It would be more ideal if LRTIs were defined by bronchoalveolar lavage sampling, which was not performed in this study. Furthermore, the OP swab samples were selected for virus isolation, although nasal washing was more ideal for virus isolation. The reason was to reduce the burden and discomfort for the patients enrolled.

In conclusion, PIV3 was the most frequent and important pathogen in hospitalized acute-phase HSCT patients with RVIs. A relatively long asymptomatic period of PIV3 infection, a prolonged period of PIV3 shedding, and a nosocomial transmission due to PIV3 were reported in this study. The enhancement of protective precautions, especially in patients before engraftment, is critical, because pre-engraftment PIV3 infections are more likely to result in LRTIs with a worse outcome. The present study highlighted the importance of monitoring hospitalized HSCT patients for RVIs to implement preventive measures against nosocomial RVIs in the HSCT units.

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**Conflict of interest** None to declare.

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