The molecular mechanisms underlying the regulation of Pin1 in the induction and maintenance of pluripotency are likely to be highly complex given that Pin1 interacts with multiple substrates in pluripotent stem cells, as revealed by our proteomics analysis. However, our current findings also indicate that Pin1 is involved in the growth and maintenance of pluripotency in stem cells through its phosphorylation-dependent prolyl isomerization of substrates such as Oct4. In this regard, a recent report by Moretto-Zita *et al.* (30) has demonstrated that Pin1 can also associate with another pluripotent transcription factor, Nanog, in murine ES cells and sustain the self-renewal and teratoma formation of these cells in immunodeficient mice. These results indicate that Pin1 is a crucial modulator of the transcription factor network governing cellular stemness. It is possible also that Pin1 could regulate this process by modulating the function of other substrates. Further studies of Pin1 function in stem cells at various stages might shed new light on the underlying molecular pathways and factors that control self-renewal and multipotency.

It has been demonstrated that Pin1 knock-out mice develop normally but display some proliferation abnormalities, including a decreased body weight, retinal degeneration, and impaired mammary gland development (31, 32). Pin1 knock-out mice also exhibit testicular atrophy with a significantly impaired proliferation of primordial germ cells and the progressive loss of spermatogenic cells (33). These phenotypes can now be attributed to the impaired maintenance and proliferation of germ-related stem cells due to the loss of Pin1 function.

In many circumstances, Pin1 acts as either a repressor or an enhancer of the degradation of substrate proteins (15–17, 34). Our current data now additionally demonstrate that Pin1 can also prolong the protein half-life of Oct4, thereby enhancing its transcriptional activity. Oct4 has been shown to be regulated by post-translational modifications such as SUMOlylation (35). Our current findings reveal that Oct4 is also regulated by phosphorylation and subsequent prolyl isomerization. Identification of the kinase(s) responsible for the association of Pin1 and Oct4 will enhance our understanding of the regulatory pathways that operate during and after the induction of pluripotency.

It is desirable to utilize pluripotent stem cells such as iPS cells for future regenerative medicine applications. However, there are already concerns surrounding the use of iPS cells in a clinical setting because prior studies have suggested that they are likely to develop cancers (4, 36). Our current findings suggest, however, that the Pin1 inhibition could effectively block the proliferation of iPS cells in an undifferentiated state. Pin1 could therefore act as a molecular switch that can reversibly control the proliferation and survival of iPS cells, thereby reducing the risk of cell transformation and tumor formation.

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REFERENCES

1. Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M. (1998) *Science* **282,** 1145–1147
2. Watt, F. M., and Hogan, B. L. (2000) *Science* **287,** 1427–1430
3. Lewitzky, M., and Yamanaka, S. (2007) *Curr. Opin. Biotechnol.* **18,** 467–473
4. Takahashi, K., and Yamanaka, S. (2006) *Cell* **126,** 663–676
5. Boyer, L. A., Lee, T. I., Cole, M. F., Johnstone, S. E., Levine, S. S., Zucker, J. P., Guenther, M. G., Kumar, R. M., Murray, H. L., Jenner, R. G., Gifford, D. K., Melton, D. A., Jaenisch, R., and Young, R. A. (2005) *Cell* **122,** 947–956
6. Eiselleova, L., Matulka, K., Kriz, V., Kunova, M., Schmidtova, Z., Neradil, J., Tichy, B., Dvorakova, D., Pospisilova, S., Hampl, A., and Dvorak, P. (2009) *Stem Cells* **27,** 1847–1857
7. Dvorak, P., Dvorakova, D., Koskova, S., Vodinska, M., Najvirtova, M., Krekac, D., and Hampl, A. (2005) *Stem Cells* **23,** 1200–1211
8. Li, J., Wang, G., Wang, C., Zhao, Y., Zhang, H., Tan, Z., Song, Z., Ding, M., and Deng, H. (2007) *Differentiation* **75,** 299–307
9. Sun, H., and Tonks, N. K. (1994) *Trends Biochem. Sci.* **19,** 480–485
10. Brill, L. M., Xiong, W., Lee, K. B., Ficarro, S. B., Crain, A., Xu, Y., Terskikh, A., Snyder, E. Y., and Ding, S. (2009) *Cell Stem Cell* **5,** 204–213
11. Prudhomme, W., Daley, G. Q., Zandstra, P., and Lauffenburger, D. A. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101,** 2900–2905
12. Hunter, T. (2009) *Curr. Opin. Cell Biol.* **21,** 140–146
13. Lu, K. P., Hanes, S. D., and Hunter, T. (1996) *Nature* **380,** 544–547
14. Ryo, A., Liou, Y. C., Lu, K. P., and Wulf, G. (2003) *J. Cell Sci.* **116,** 773–783
15. Ryo, A., Suizu, F., Yoshida, Y., Perrem, K., Liou, Y. C., Wulf, G., Rottapel, R., Yamaoka, S., and Lu, K. P. (2003) *Mol. Cell* **12,** 1413–1426
16. Ryo, A., Nakamura, M., Wulf, G., Liou, Y. C., and Lu, K. P. (2001) *Nat. Cell Biol.* **3,** 793–801
17. Lu, K. P., and Zhou, X. Z. (2007) *Nat. Rev. Mol. Cell Biol.* **8,** 904–916
18. Esnault, S., Shen, Z. J., and Malter, J. S. (2008) *Crit. Rev. Immunol.* **28,** 45–60
19. Takahashi, K., Okita, K., Nakagawa, M., and Yamanaka, S. (2007) *Nat. Protoc.* **2,** 3081–3089
20. Yamada, M., Hamatani, T., Akutsu, H., Chikazawa, N., Kuji, N., Yoshimura, Y., and Umezawa, A. (2010) *Hum. Mol. Genet.* **19,** 480–493
21. Liu, Y., and Labosky, P. A. (2008) *Stem Cells* **26,** 2475–2484
22. Masui, S., Nakatake, Y., Toyooka, Y., Shimosato, D., Yagi, R., Takahashi, K., Okochi, H., Okuda, A., Matoba, R., Sharov, A. A., Ko, M. S., and Niwa, H. (2007) *Nat. Cell Biol.* **9,** 625–635
23. Yang, H. M., Do, H. J., Oh, J. H., Kim, J. H., Choi, S. Y., Cha, K. Y., Chung, H. M., and Kim, J. H. (2005) *J. Cell. Biochem.* **96,** 821–830
24. Takahashi, K., Ichisaka, T., and Yamanaka, S. (2006) *Methods Mol. Biol.* **329,** 449–458
25. Hennig, L., Christner, C., Kipping, M., Schelbert, B., Rücknagel, K. P., Grabley, S., Küllertz, G., and Fischer, G. (1993) *Biochemistry* **37,** 5953–5960
26. Shen, Z. J., Esnault, S., Schinzel, A., Borner, C., and Malter, J. S. (2009) *Nat. Immunol.* **10,** 257–265
27. Yaffe, M. B., Schutkowski, M., Shen, M., Zhou, X. Z., Stukenberg, P. T., Rahfeld, J. U., Xu, J., Kuang, J., Kirschner, M. W., Fischer, G., Cantley, L. C., and Lu, K. P. (1997) *Science* **278,** 1957–1960
28. Lu, P. J., Zhou, X. Z., Liou, Y. C., Noel, J. P., and Lu, K. P. (2002) *J. Biol. Chem.* **277,** 2381–2384
29. Niwa, H., Miyazaki, J., and Smith, A. G. (2000) *Nat. Genet.* **24,** 372–376
30. Moretto-Zita, M., Jin, H., Shen, Z., Zhao, T., Briggs, S. P., and Xu, Y. (2010) *Proc. Natl. Acad. Sci. U.S.A.* **107,** 13312–13317
31. Fujimori, F., Takahashi, K., Uchida, C., and Uchida, T. (1999) *Biochem. Biophys. Res. Commun.* **265,** 658–663
32. Liou, Y. C., Ryo, A., Huang, H. K., Lu, P. J., Bronson, R., Fujimori, F., Uchida, T., Hunter, T., and Lu, K. P. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99,** 1335–1340
33. Atchison, F. W., and Means, A. R. (2003) *Biol. Reprod.* **69,** 1989–1997
34. Ryo, A., Hirai, A., Nishi, M., Liou, Y. C., Perrem, K., Lin, S. C., Hirano, H., Lee, S. W., and Aoki, I. (2007) *J. Biol. Chem.* **282,** 36671–36681
35. Zhang, Z., Liao, B., Xu, M., and Jin, Y. (2007) *FASEB J.* **21,** 3042–3051
36. Knoepfler, P. S. (2009) *Stem Cells* **27,** 1050–1056

Laboratory and Epidemiology Communications

First Detection of Measles Virus Genotype G3 in a Japanese Woman: an Imported Case

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Since 2007, the number of measles patients in Japan had continued to decrease because of regular and widespread measles immunization program (1). However, 450 cases of measles including the suspected cases were reported in 2010 (1). Epidemiological data suggests that most of these cases were imported into Japan, but domestic cases have also been reported (1). Recent molecular epidemiological studies reported the detection of measles virus (MV) genotypes D3, D4, D5, D9, and H1 in Japan (2–4). The D4 and D9 genotypes have usually been detected in imported cases, while the D3, D5, and H1 genotypes have been detected in domestic cases (2–4). Here, we describe the detection of another genotype, G3, in an imported case of measles in a Japanese woman. To the best of our knowledge, this is the first report on the detection of MV genotype G3 in Japan.

The patient was a 28-year-old Japanese woman who resided in Chiba Prefecture, Japan. She did not have a history of measles and had not been immunized against measles. She had visited Indonesia for 10 days (from January 31 to February 9, 2011) with six colleagues. On February 14, she developed common cold-like symptoms such as cough and shivering, and consulted a local physician, who made a diagnosis of common cold. On February 22, she developed clinical symptoms including high fever (39°C), cough, conjunctivitis, Koplik's spots, and rashes on the face and neck. She then consulted another physician at a general hospital. The physician suspected her to have contracted measles, and suggested that she got admitted to the hospital. Informed consent was obtained, and her whole blood sample was collected on the next day. Viral RNA was extracted from the blood sample using the High Pure Viral RNA Kit (Roche, Indianapolis, Ind., USA), and was suspended in DNase/RNase-free water. Thereafter, reverse transcriptase-polymerase chain reaction (RT-PCR) and nested PCR were performed as previously described (2–4). Amplicons were purified using the High Pure PCR Product Purification Kit (Roche), and the nucleotide sequence was determined using direct se-

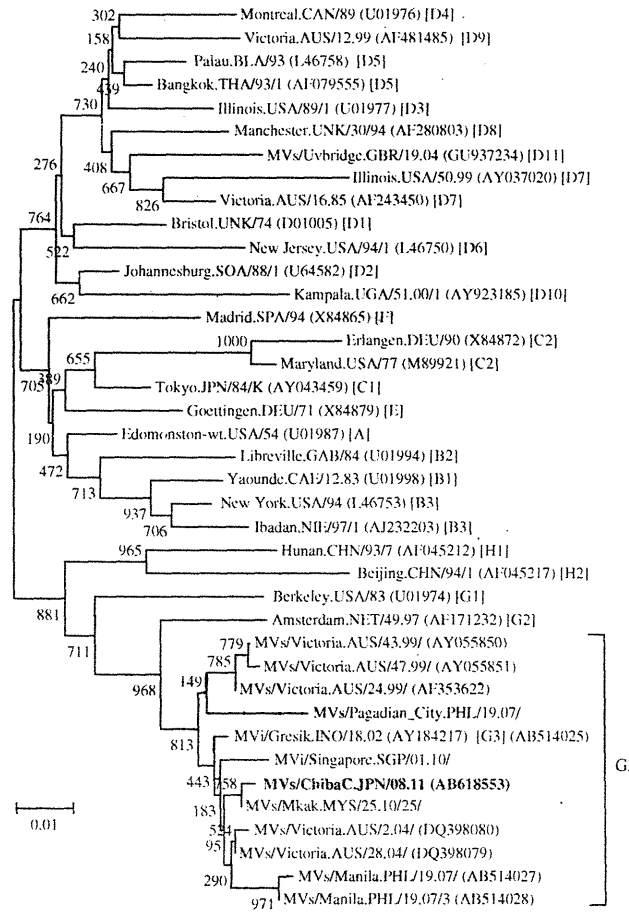

Fig. 1. Phylogenetic tree based on the nucleotide protein (*N*) gene sequences of various strains of the measles virus. The evolutionary distance was calculated using Kimura's two-parameter method, and the tree was plotted using the neighbor-joining method. Numbers at each branch indicate the bootstrap values of the clusters supported by that branch. Genbank accession numbers are given in parentheses. The present strain is represented in bold type.

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quencing method (4). The nucleotide sequence of the partial *N* gene (450 bp) of MVs was analyzed phylogenetically using Molecular Evolutionary Genetics Analysis (MEGA) software (version 4) (2–4). Evolutionary distances were estimated using Kimura's two-parameter method and the phylogenetic tree was constructed using the neighbor-joining (NJ) method (2–4). Reliability of the phylogenetic tree was estimated by 1,000 bootstrap replications.

We constructed a phylogenetic tree based on the *N* gene of the detected MV strain and the reference strains (Fig. 1). The strain was genotyped as MV G3 in the phylogenetic tree. The homology between the reference strain (MVi/Gresik.INO/18.02 [G3], GenBank accession no. AY184217) and the present strain was 99.1% at the nucleotide level and 98.7% at the amino acid level. Epidemiological investigations have not reported occurrence of measles among the patient's family and colleagues.

To the best of our knowledge, this is the first report on MV G3 detection in Japan. The genotype G3 was first detected in Australia and East Timor in 1999 (5). Infection with G3 has not been frequently reported in these countries after 1999. However, this may be attributed to the lack of aggressive MV surveillance in these countries. At present, a small number of the population in Chiba Prefecture may be susceptible to measles because of lack of immunization against the disease. However, as measles is highly contagious in humans (6), and spreads rapidly from one area to another, up-to-date information on the epidemiological status of this disease in our country is needed.

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Conflict of interest None to declare.

REFERENCES

1. National Institute of Infectious Diseases. Infectious Agents Surveillance Report in Japan. Online at 〈http://idsc.nih.go.jp/disease/measles-e/index.html〉.
2. Morita, Y., Suzuki, T., Shiono, M., et al. (2007): Sequence and phylogenetic analysis of the nucleoprotein (*N*) gene in measles viruses prevalent in Gunma, Japan, in 2007. Jpn. J. Infect. Dis., 60, 402–404.
3. Aoki, Y., Mizuta, K., Suto, A., et al. (2009): Importation of the evolving measles virus genotype D9 to Yamagata, Japan from Thailand in 2009. Jpn. J. Infect. Dis., 62, 481–482.
4. Taira, K., Nakamura, M., Okano, S., et al. (2008): Phylogenetic analysis of nucleoprotein (*N*) gene of measles viruses prevalent in Okinawa, Japan, during 2003–2007. Jpn. J. Infect. Dis., 61, 248–250.
5. Chibo, D., Riddell, M., Catton, M., et al. (2002): Novel measles virus genotype, East Timor and Australia. Emerg. Infect. Dis., 8, 735–737.
6. Griffin, D.E. (2001): Measles virus. 4th ed. p. 1401–1441. *In* D.M. Knipe and P.M. Howley (ed.). Lippincott Williams & Wilkins, Philadelphia, USA.

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Laboratory and Epidemiology Communications

Detection of Human Metapneumovirus Genomes during an Outbreak of Bronchitis and Pneumonia in a Geriatric Care Home in Shimane, Japan, in Autumn 2009

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Human metapneumovirus (HMPV), which belongs to the family *Paramyxoviridae*, genus *Metapneumovirus*, is an important causative agent of acute respiratory infections (ARIs) in humans (1). Despite this, the molecular epidemiology of HMPV in Japan is not well understood. We described herein an outbreak of HPMV infection in a geriatric care home in Shimane, Japan in autumn 2009 and the results of genetic analyses of the HMPV detected in samples obtained from residents of this home. An epidemiological investigation in late September 2009 found that 2 of the 99 residents of this home exhibited symptoms such as high fever (>38°C), cough, and inflammation of the lower respiratory tract. Other residents were identified with similar symptoms up until late October 2009. The overall prevalence during this outbreak was around 30% (27/99 persons), although the infection route could not be determined. Nine throat swab samples were collected from these patients after obtaining verbal informed consent and attempts made to detect and/or isolate influenza virus subtype A, human rhinovirus, enteroviruses, respiratory syncytial virus, parainfluenza viruses, and/or adenoviruses using previously reported reverse transcriptase-polymerase chain reaction (RT-PCR) and cell culture methods (Vero E6, RD, MDCK, and HEp-2 cells) (2–5). Viral nucleic acid was extracted from the samples using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany) and suspended in DNase/RNase-free water. After RNA extraction, RT-PCR was performed as described previously (6,7). Amplicons were purified using a QIAquick PCR Purification Kit (Qiagen, Germantown, Md., USA) and the nucleotide sequences were determined by direct sequencing (6). Phylogenetic analysis based on the fusion (*F*) gene of HMPV strains was then performed using the Molecular Evolutionary Genetics Analysis (MEGA) software version 4 (8). Evolutionary distances were estimated using Kimura's two-parameter method and a phylogenetic tree was constructed using the neighbor-joining method (9,10). The reliability of the tree was estimated on the basis of 1,000 bootstrap replications.

A summary of patient and viral data is shown in Table 1. HMPV was detected in samples from 7 patients; no other viruses were detected. In addition, serum IgG against HMPV was detected in 2 patients using an indirect immunofluorescence assay (11), with significantly higher levels being found in the convalescent phase. Nucleotide sequence analysis of different HMPV genes, with *F* gene being the most common, allows the virus to be divided into two major genetic groups (A and B) and four subgroups (A1, A2, B1, and B2) (12,13). The phylogenetic tree determined here showed that all strains detected in the patient samples were clustered in subgroup B2 (Fig. 1). The nucleotide identity among the present strains was 100%, with a nucleotide identity of 99.7% with respect to the Yamaguchi 09-15 strain detected in Yamaguchi Prefecture during the same season. A very recent study suggested that HMPV subgroups A2 and B2 are the major types circulating in Japan (14). Indeed, subgroups A2, B2, and B1 were found in 3, 4, and 2 strains, respectively, of the 9 HMPV strains detected by the sentinel surveillance system for viral diseases in Shimane Prefecture from March 2009 to January 2010. Furthermore, a high degree of nucleotide identity (98.7–100%) was seen between the subgroup B2 strains.

It is suggested that HMPV infection mainly occurs in children, although recent reports indicate that outbreaks of HMPV infection also occur in the elderly (15). Indeed, a similar outbreak to the present case occurred in another geriatric care home in Japan (16). However, despite these occurrences, the epidemiology of HMPV infection still remains unclear. A high prevalence (around 30%) of HMPV infection was seen in the present study, with some patients presenting with severe infections such as pneumonia. HMPV infection should therefore be considered in outbreaks among elderly peo-

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Table 1. Patient and human metapneumovirus data

Patient	Age (y)	Sex	Diagnosis	Onset date	Sampling date	Strain	Subgroup	GenBank accession no.
1	65	F	Pneumonia	14 Oct. 2009	23 Oct. 2009	Shimane 09-17	B2	AB594742
2	55	M	Pneumonia	16 Oct. 2009	23 Oct. 2009	Shimane 09-18	B2	AB594743
3	69	M	Fever, Cough	17 Oct. 2009	23 Oct. 2009	Shimane 09-16	B2	AB594741
4	56	F	Fever, Cough	20 Oct. 2009	23 Oct. 2009	Shimane 09-15	B2	AB594740
5	65	F	Pneumonia	21 Oct. 2009	23 Oct. 2009	Shimane 09-19	B2	AB594744
6	56	F	Fever, Cough	22 Oct. 2009	23 Oct. 2009	Shimane 09-14	B2	AB594739
7	61	M	Fever, Cough	29 Oct. 2009	30 Oct. 2009	Shimane 09-20	B2	AB594745

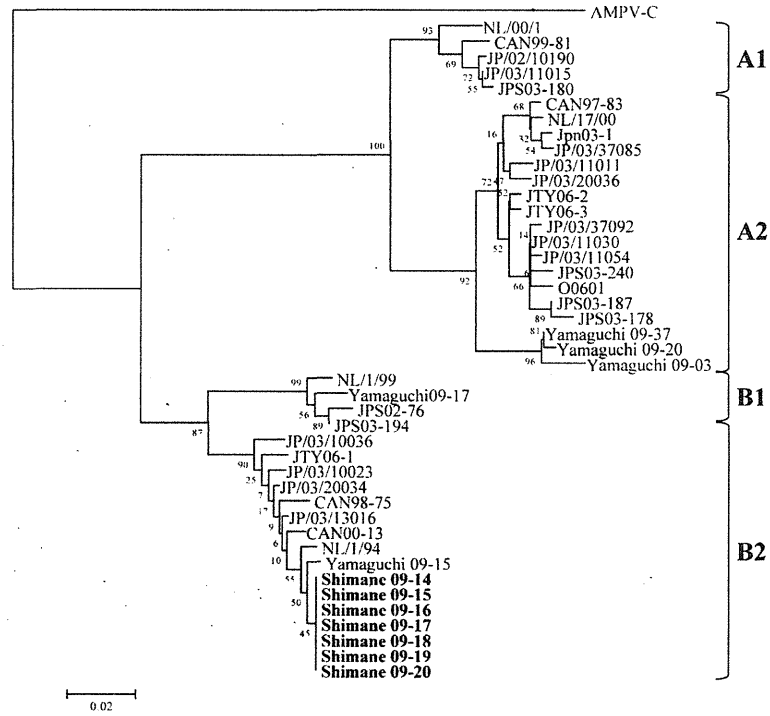

Fig. 1. Phylogenetic tree constructed on the basis of partial sequences of the human metapneumovirus *F* gene. Distance was calculated using Kimura's two-parameter method, and the tree was plotted using the neighbor-joining method. Numbers above the branches are bootstrap probabilities (%). Reference strains were NL/00/1 (AF371337), CAN99-81 (AY145294), JP/02/10190 (AB113377), JP/03/11015 (AB113372), JPS03-180 (AY530092), CAN97-83 (AY145296), NL/17/00 (AY304360), Jpn03-1 (AB503857), JP/03/37085 (AB119485), JP/03/11011 (AB113371), JP/03/20036 (AB126612), JTY06-2 (EU127918), JTY06-3 (EU127919), JP/03/37092 (AB119486), JP/03/11030 (AB119489), JP/03/11054 (AB119491), JPS03-240 (AY530095), O0601 (EF589610), JPS03-187 (AY530093), JPS03-178 (AY530091), Yamaguchi 09-37 (AB533251), Yamaguchi 09-20 (AB533245), Yamaguchi 09-03 (AB533239), NL/1/99 (AY304361), Yamaguchi09-17 (AB533244), JPS02-76 (AY530089), JPS03-194 (AY530094), JP/03/10036 (AB126611), JTY06-1 (EU127917), JP/03/10023 (AB126608), JP/03/20034 (AB119493), CAN98-75 (AY297748), JP/03/13016 (AB126607), CAN00-13 (AY145298), NL/1/94 (AY304362), and Yamaguchi 09-15 (AB533243). Avian metapneumovirus type C (AMPV-C, AY579780) was also included as an outgroup.

ple with severe ARIs.

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Conflict of interest None to declare.

REFERENCES

1. Collins, P.L. and Crowe, J.E, Jr. (2007): Respiratory syncytial virus and metapneumovirus. p. 1601-1646. *In* D.M. Knipe and P.M. Howley (ed.), Fields Virology. vol. 1. 5th ed. Lippincott Williams & Wilkins, Philadelphia.
2. World Health Organization. WHO Information for Laboratory Diagnosis of Pandemic (H1N1) 2009 Virus in Humans—revised. Online at 〈http://www.vihda.gov.ar/DocumentosGripePorcina/10_WHO_Diagnostic_RecommendationsH1N1_20090521.pdf〉. Accessed 21 May 2009.
3. Savolainen, C., Blomqvist, S., Mulders, M.N., et al. (2002): Genetic clustering of all 102 human rhinovirus prototype strains: serotype 87 is close to human enterovirus 70. J. Gen. Virol., 83, 333-340.
4. Osiowy, C. (1998): Direct detection of respiratory syncytial virus, parainfluenza virus, and adenovirus in clinical respiratory specimens by a multiplex reverse transcription-PCR assay. J. Clin. Microbiol., 36, 3149-3154.
5. Aguilar, J.C., Pérez-Breña, M.P., García, M.L., et al. (2000):

Detection and identification of human parainfluenza viruses 1, 2, 3, and 4 in clinical samples of pediatric patients by multiplex reverse transcription-PCR. J. Clin. Microbiol., 38, 1191-1195.
6. Toda, S., Kimura, H., Noda, M., et al. (2010): Phylogenetic analysis of human metapneumovirus from children with acute respiratory infection in Yamaguchi, Japan, during summer 2009. Jpn. J. Infect. Dis., 63, 139-140.
7. Takao, S., Shimozono, H., Kashiwa, H., et al. (2003): Clinical study of pediatric cases of acute respiratory diseases associated with human metapneumovirus in Japan. Jpn. J. Infect. Dis., 56, 127-129.
8. Tamura, K., Dudley, J., Nei, M., et al. (2007): MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol., 24, 1596-1599.
9. Saitou, N. and Nei, M. (1987): The neighbor-joining method: a new method for reconstructing phylogenetic trees. Biol. Evol., 4, 406-425.
10. Kimura, M. (1980): A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol., 16, 111-120.
11. Ebihara, T., Endo, R., Kikuta, H., et al. (2003): Seroprevalence of human metapneumovirus in Japan. J. Med. Virol., 70, 281-283.
12. Mizuta, K., Abiko, C., Aoki, Y., et al. (2010): Endemicity of human metapneumovirus subgenogroups A2 and B2 in Yamagata, Japan, between 2004 and 2009. Microbiol. Immunol., 54, 634-638.
13. van den Hoogen, B.G., Herfst, S., Sprong, L., et al. (2004): Antigenic and genetic variability of human metapneumoviruses. Emerg. Infect. Dis., 10, 658-666.
14. Boivin, G., Mackay, I., Sloots, T.P., et al. (2004): Global genetic diversity of human metapneumovirus fusion gene. Emerg. Infect. Dis.,10, 1154-1157.
15. Osbourn, M., McPhie, K.A., Ratnamohan, V.M., et al. (2009): Outbreak of human metapneumovirus infection in a residential aged care facility. Commun. Dis. Intell., 33, 38-40.
16. Honda, H., Iwahashi, J., Kashiwagi, T., et al. (2006): Outbreak of human metapneumovirus infection in elderly inpatients in Japan. J. Am. Geriatr. Soc., 54, 177-180.

ORIGINAL ARTICLE LOWER AIRWAYS

Different cytokine profile and eosinophil activation are involved in rhinovirus- and RS virus-induced acute exacerbation of childhood wheezing

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Abstract

Because little information is available on eosinophil activation and cytokine response in virus-induced wheezing, we attempted to detect respiratory viruses and measure eosinophil cationic protein (ECP), and 27 types of cytokines/chemokines in both serum and nasal secretions from children with wheezing. This study was an observational, case–control investigation of 267 subjects, who were visited and/or hospitalized with acute respiratory symptoms (with wheezing: men, 115; women, 59; mean/median age, 3.6/3.0 years) or who were visited for regular physical examination and treatment (non-symptomatic wheezing: men, 48; women, 31; mean/median, 5.0/4.7 years), and 14 control subjects (controls: men, 9; women, 5; mean/median, 3.6/3.7 years). We detected viruses in nasal secretions from 174 patients with acute exacerbations of wheezing using antigen detection kits or reverse transcription-polymerase chain reaction, followed by direct DNA sequencing analysis. We measured peripheral eosinophil counts, and serum concentrations of ECP and 27 cytokines/chemokines using a multiplex bead-based assay in patients with wheezing or non-symptomatic wheezing. We also examined nasal ECP and 27 cytokines/chemokines in patients with wheezing. Of 174 samples from wheezing exacerbations, rhinovirus was detected in 59; respiratory syncytial (RS) virus in 44; enterovirus in 17; other viruses in 19; and no viruses in 35. Serum concentrations of ECP, IL-5, IL-6, IL-1ra, and IP-10 were significantly elevated in rhinovirus-induced wheezing compared with non-symptomatic wheezing. Similarly, serum ECP, IL-5, and IP-10 were significantly higher in rhinovirus-induced wheezing than in controls. On the other hand, IL-1ra and IP-10, but not ECP and IL-5 were significantly higher in RS virus-induced wheezing than in controls. Furthermore, only IL-5 was significantly elevated in the rhinovirus group compared with the RS virus group in both serum and nasal secretions. Different cytokine profile and eosinophil activation might be involved in rhinovirus- and RS virus-induced acute exacerbation of childhood wheezing.

Respiratory infections can cause wheezing illnesses in children of all ages and can also influence the causation and disease activity of asthma (1). For years, it has been recognized that respiratory syncytial (RS) virus infections often produce the first episode of wheezing in children who go on to develop chronic asthma (1). Furthermore, respiratory viral

Abbreviations:
ECP, eosinophil cationic protein; IL, interleukin; IFN, interferon; IP, interferon-γ-induced protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; RS, respiratory syncytial; PI, parainfluenza.

infections are detected in the majority of asthma exacerbations in both children (80–85%) (2) and adults (75–80%) (3), and of these, approximately 60% are rhinoviruses. However, at the present time, there is no specific treatment for virus-induced exacerbations of wheezing/asthma, and the precise mechanisms responsible are still unclarified.

Two studies compared responses to experimental rhinovirus infections in asthmatic or allergic rhinitic subjects and normal subjects, and both implicated an increased bronchial eosinophil infiltrate in the pathogenesis of virus-induced exacerbations of asthma (4, 5). Eosinophils are important effector cells in host defense against parasites in allergic diseases such as bronchial asthma (6). In allergic inflammation, mediators released from epithelial cells or inflammatory cells induce migration of eosinophils from the blood into affected tissues. After migration, eosinophils are activated by appropriate stimuli resulting in the release of inflammatory mediators, including arachidonic acid metabolites such as platelet-activating factor, or cystenyl leukotrienes, oxygen radicals, and toxic cationic granule proteins such as major basic protein, eosinophil peroxidase, eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (6). Eosinophils also play a critical role on airway remodeling (7).

The aims of this study were to assess the occurrence of viral infections in acute exacerbations of wheezing and to evaluate the association of these viral findings with cytokine/chemokine profiles and eosinophil activation in wheezing children.

Methods

Patients and study setting

This study was an observational, case–control investigation of 267 subjects, who were visited and/or hospitalized with acute respiratory symptoms (with wheezing: men, 115; women, 59; mean/median age, 3.6/3.0 years) or who were visited for regular physical examination and treatment (non-symptomatic wheezing: men, 48; women, 31; mean/median, 5.0/4.7 years), and 14 control subjects (controls: men, 9; women, 5; mean/median, 3.6/3.7 years) at the Gunma Children's Medical Center from November 1, 2003, through October 31, 2006.

All recruited patients had a history of three or more different episodes of recurrent wheezing and documented evidence of wheezing by auscultation. The subjects with asthma were diagnosed according to the criteria of the National Institutes of Health (8). In this study, we analyzed the patients as wheezing children including recurrent wheezing and asthma because the diagnosis of asthma in children <5 years old presents particularly difficult problems (8). Also, studies of the natural history asthma have established that in almost 80% of cases, it begins during the first 6 years of life (9). We excluded children with congenital heart disease, cystic fibrosis, or other chronic lung disease, presence of foreign body or signs of severe infection or who were immunosuppressed that could interfere with the assessment of wheezing/asthma-related outcome measures. An acute exacerbation of wheezing/asthma was diagnosed by the emergency department physician based on the presence of wheezing with increased difficulty of breathing and had a history of a cold before the onset of the exacerbation. The criterion of 'non-symptomatic wheezing' patients was defined as physician-diagnosed wheezing/asthma and is currently stable at least 3 months before and at the timing of examinations. The patients were prescribed short-acting β2 agonists and/or long-term controller medications. The control group included 14 healthy children with no symptoms of wheezing at the time of investigation. Exclusion criteria for the controls included immunosuppression, the presence of other respiratory tract symptoms or a history of previous wheezing and asthma. This study was approved by the Ethics Committee of Gunma Children's Medical Center. Informed consent was obtained from parents, and assent was obtained from older children (usually over 9 years old).

Virus detection

Nasal aspirates were obtained from 174 patients during acute exacerbation of wheezing. However, we could not take these samples from patients with non-symptomatic wheezing patients and controls. Nasal secretions were aspirated into a mucus trap (attached to wall suction) by inserting the tip of a flexible 5F Argyle suction catheter (Nippon Sherwood, Tokyo, Japan) into the anterior nares. Nasal samples were analyzed by antigen detection kits (Becton Dickinson, Franklin Lakes, NJ, USA) for RS virus, influenza types A and B, and adenoviruses. The remaining secretions were frozen at −80°C until examination by further reverse transcription-polymerase chain reaction (RT-PCR), and then by direct DNA sequencing analysis. Viral RNA was purified from nasopharyngeal samples diluted in virus transport medium. The samples were centrifuged at 3000 *g* at 4°C for 30 min. RNA extraction from samples was performed using a commercial RNA isolation procedure (QIAamp viral RNA purification kit; QIAGEN, Hilden, Germany). RNA solution was treated with DNase I (Takara, Tokyo, Japan), and the reverse transcription reaction mixture was incubated with random hexamers at 42°C for 90 min, followed by incubation at 99°C for 5 min, and then amplification by thermal cycling. The RT-PCR method for rhinoviruses and enteroviruses, developed by Blomqvist et al., was used for rhinovirus and enterovirus identification (10). The primers for RT-PCR were as follows: 5′-GAA ACA CGG ACA CCC AAA GTA-3′ (human rhinovirus primer 1; sense) and 5′-TCC TCC GGC CCC TGA ATG-3′ (human rhinovirus primer 2; antisense). The PCR protocol was as follows: incubation for 3 min at 94°C, followed by 40 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 2 min, followed by an additional 7 min for elongation at 72°C after the last cycle. The RT-PCR method used for RS virus and parainfluenza (PI) virus types 1, 2, and 3 is as previously described with minor modifications (11). The primers for RT-PCR were as follows: 5′-GGG AGA GGT GGC TCC AGA ATA CAG GC-3′ (RS virus N3; sense), 5′-AGC ATC ACT TGC CCT GAA CCA TAG GC-3′ (RS virus N5; antisense), 5′-TCT GGC GGA

GGA GCA ATT ATA CCT GG-3′ (PI virus type 1 PR3; sense), 5′-ATC TGC ATC ATC TGT CAC ACT CGG GC -3′ (PI virus type 1 PR5; antisense), 5′- AAC TAT GTC CAG AGG AGA GGT GCT GG -3′ (PI virus type 2 PR3; sense), 5′-CCA TGC CTG CAT AAG CAC ACT GTA GC -3′ (PI virus type 2 PR5; antisense), 5′-ACC AGG AAA CTA TGC TGC AGA ACG GC-3′ (PI virus type 3 PR3; sense), and 5′-GAT CCA CTG TGT CAC CGC TCA ATA CC-3′ (PI virus type 3 PR5; antisense). To avoid cross-contamination in RT-PCR, procedures for the extraction of viral RNA were carried out in a room physically separated from that used for performing RT-PCR. Furthermore, positive and negative controls were included in all PCR assays. The size of the amplified DNA fragment was determined by electrophoresis on 3% agarose gel. The DNA fragments were purified using a QIAquick PCR Purification kit (QIAGEN), and the nucleotide sequence was determined with an automated DNA sequencer ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems). For identification of the virus, newly determined sequences were compared with those available in the sequences using the GenBank DNA databases (http://www.ncbi.nlm.nih.gov) and the standard nucleotide–nucleotide BLAST algorithm. The identities of the sequences were determined on the basis of the highest percentage of total nucleotide match in GenBank.

Serum and nasal cytokines/chemokines and eosinophil cationic protein (ECP)

We measured peripheral eosinophil counts and the concentrations of serum ECP and 27 cytokines/chemokines [interleukin(IL)-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, interferon (IFN)-γ, IFN-γ-induced protein (IP)-10, tumor necrosis factor (TNF)-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, eotaxin, regulated upon activation, normal T expressed and secreted (RANTES), platelet-derived growth factor (PDGF)-bb, fibroblast growth factor (FGF) basic, and vascular endothelial growth factor (VEGF)] from 79 patients with wheezing and 79 non-symptomatic wheezing patients who had not used systemic corticosteroids at the timing of the examinations, and 14 control subjects. ECP contents in serum and nasal secretions were measured with a fluoroenzyme immunoassay kit (Pharmacia, Uppsala, Sweden). Serum or nasal cytokines/chemokines were determined by the multicytokine detection system, Bio-Plex (Bio-Rad, Hercules, CA, USA), following the manufacturer's instructions, measured using a Luminex System, Bio-Plex Pro Assay (Austin, TX, USA), and calculated using Bio-Plex software (Bio-Rad).

Statistical analyses

Patient characteristic data were evaluated by the Pearson X^2 test statistic and Fisher's exact test for categorical variables. Multivariate analyses were conducted by using multivariate linear regression or multivariate logistic regression analysis, allowing assessment simultaneously for each factor from patients with acute wheezing group, non-symptomatic wheezing group, or controls. The age, sex, and atopic status were potential confounders, and adjustments were made for the multivariate analyses. Unpaired data were analyzed using the Mann–Whitney *U* test. Differences between more than three groups were analyzed by the Kruskal–Wallis test, followed by the Dunn's multiple comparison test. Correlation coefficients for the parameters were calculated by using Spearman rank correlation coefficient analysis. A statistically significant result was defined by a value of $p < 0.05$ (two-sided). All analyses were performed using a statistical software package (SPSS for Windows, version 14.0; SPSS Japan Inc., Tokyo, Japan; or for multiple group comparison, GraphPad Prism for Macintosh, version 4.0, GraphPad Software, San Diego, CA, USA).

Results

Virus detection

Patient characteristics in viral detection are shown (Table 1). No significant differences for age, sex, or atopic status between each group were found. We detected 59 rhinoviruses, 44 RS viruses, 17 enteroviruses, and 19 other viruses, and no viruses were detected in 35 samples in a total of 174 patients with acute exacerbations of wheezing (Table 1). Of the 17 enteroviruses, 15 were enterovirus type 68 and 2 were coxsackievirus, or an echovirus. The other virus group consisted of 11 positive for both rhinovirus and RS virus, five for two other viruses, two for influenza A, and one for influenza B. The monthly changes in acute exacerbations of wheezing were shown (Fig. 1). Two-thirds of the patients were hospitalized or treated for wheezing/asthma attacks from September through December. RS viruses and enteroviruses were frequently detected from October through December, or October and November, respectively. In contrast, rhinoviruses were detected in almost all months of the year.

Serum and nasal cytokines/chemokines and eosinophil cationic protein (ECP)

Patient characteristics in relation to cytokine/chemokine measurements are shown (Table 2). No significant differences for age, sex, or atopic status between each group were found. We compared peripheral eosinophil counts, the concentrations of serum ECP and 27 cytokines/chemokines in each virus group against non-symptomatic wheezing. Table 3 showed the significantly elevated parameters among them. In comparison with each virus group, the concentrations of serum ECP, IL-5, IL-6, IL-1ra, and IP-10 were significantly elevated in rhinovirus-induced wheezing compared with non-symptomatic wheezing. On the other hand, peripheral eosinophil counts and any other cytokines/chemokines including IL-1β, IL-2, IL-4, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-γ, TNF-α, GM-CSF, G-CSF, MCP-1,

Table 1 Characteristics of patients in virus detection

Virus	Number (% of total)	Mean/median age year (range)	Gender, % male	≥1 positive aeroallergen CAP-RAST, % positive
Rhinovirus	59 (33.9)	3.1/2.8 (0.3–9.2)	64.4	71.4
Respiratory syncytial virus	44 (25.3)	3.3/2.4 (0.8–11.2)	72.7	67.5
Enterovirus	17 (9.8)	5.5/5.4 (2.1–11.3)	52.9	88.2
Others	19 (10.9)	3.9/3.0 (1.3–9.8)	73.7	52.6
Not detected	35 (20.1)	3.8/3.1 (0.4–11.8)	68.6	65.6
Total	174 (100)	3.6/3.0 (0.3–11.8)	67.2	68.5

CAP-RAST, capsulated hydrophilic carrier polymer-radioallergosorbent test.

MIP-1α, MIP-1β, eotaxin, RANTES, PDGF-bb, FGF basic, and VEGF were not significantly elevated in the same comparison. By contrast, IL-6, IL-8, IL-1ra, and IP-10, but not ECP and IL-5 were significantly higher in RS virus-induced wheezing than in non-symptomatic wheezing. Notably, only IP-10 was significantly elevated in all wheezing groups compared with non-symptomatic wheezing. On the other hand, IL-5 was significantly higher in the rhinovirus, enterovirus, and other virus groups, but not in the RS virus group, than in non-symptomatic wheezing. Next, we compared each group against controls. Table 3 and Figure 2 showed the significantly elevated parameters among them. Serum ECP and IL-5 were significantly elevated in the rhinovirus, enterovirus, and other virus groups but not in the RS virus group, compared with controls. By contrast, serum IL-1ra was significantly higher in only RS virus group than in controls. Furthermore, we compared these parameters between non-symptomatic wheezing patients and controls. As a result, only ECP was significantly elevated in non-symptomatic wheezing patients than in controls ($p < 0.01$). Finally, we compared these parameters among three major viruses in acute exacerbations of wheezing, that is, rhino-, RS- and enterovirus groups, and only IL-5 was significantly elevated in the rhinovirus group compared with the RS virus group, in both serum and nasal secretions (Fig. 3). In contrast, only serum IL-1ra was significantly elevated in the RS virus group compared with the rhinovirus and enterovirus groups. Nasal IL-1ra was higher in some patients with RS virus group but not significantly elevated in the same comparison (Fig. 3).

Discussion

Johnston et al. (2) reported that in 9- to 11-year-old children with asthma, viral infections were associated with more than 80% of asthma exacerbations, and rhino/enteroviruses were responsible for 50% of these episodes, as detected by PCR or conventional methods including cell culture, immunofluorescence, and ELISA. Viruses such as corona, influenza, parainfluenza 1, 2, and 3, RS virus, and other viruses are responsible for 13.0, 7.2, 7.2, 4.1, and 1.0% of exacerbations, respectively. Brouard et al. (12) reported that in 118 children hospitalized for acute asthma, rhinoviruses (45%) were prevalent, followed by RS viruses (28%) and enteroviruses (8.5%), as detected by conventional techniques, such as viral culture and immunofluorescence, and molecular techniques, such as PCR. They also noted that rhinoviruses and RS viruses have a similar prevalence (42% and 36%) before two years of age, when compared with 66% and 27%, respectively, in older children. Our methods, using a combination of an antigen detection kit and PCR followed by direct sequence analysis, revealed rhinoviruses (33.9%), RS viruses (25.4%), enteroviruses (9.8%), other viruses (10.9%), and no

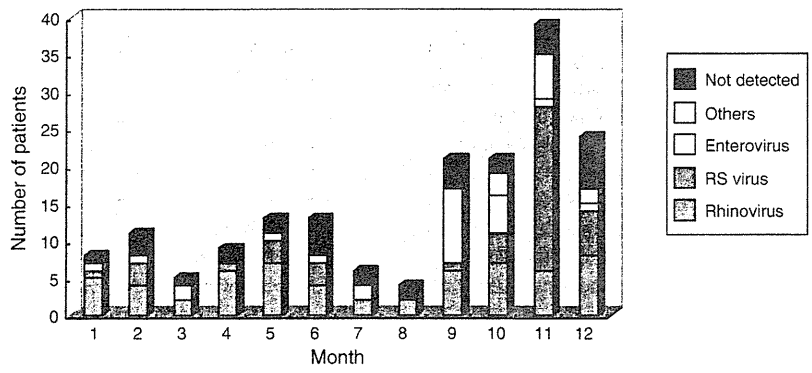

Figure 1 Monthly changes of virus detection in acute exacerbation of wheezing. Two-thirds of the patients were hospitalized or treated for wheezing attacks from September through December. Respiratory syncytial viruses or enteroviruses were frequently detected from October through December, or October and November, respectively. In contrast, rhinoviruses were detected in almost every month of the year.

Table 2 Patient characteristics in cytokines/chemokines measurement

	Number	Mean/median age year (range)	Gender (% male)	≥1 positive aeroallergen CAP-RAST, % positive
Acute wheezing exacerbation group				
Rhinovirus-induced wheezing	28	2.9/2.7 (0.3–9.2)	60.7	70.4
Respiratory syncytial virus-induced wheezing	17	3.4/2.1 (0.8–11.2)	76.5	71.4
Enterovirus-induced wheezing	9	6.0/6.2 (2.8–11.3)	66.7	88.9
Other viruses-induced wheezing	10	3.5/1.9 (1.3–9.8)	60.0	40.0
Not detected viruses-induced wheezing	15	4.6/4.3 (0.4–11.8)	53.3	76.9
Non-symptomatic wheezing	79	5.0/4.7 (1.1–13.6)	60.8	78.2
Control	14	3.6/3.7 (1.3–6.4)	64.3	0.0

CAP-RAST, capsulated hydrophilic carrier polymer-radioallergosorbent test.

Table 3 Elevated serum eosinophil cationic protein (ECP) and cytokines/chemokines in acute wheezing groups compared with non-symptomatic wheezing or control

	Compared with non-symptomatic wheezing (n = 79)	Compared with control (n = 14)
Rhinovirus-induced wheezing (n = 28)	ECP*, IL-5***, IL-6**, IL-1ra**, IP-10***	ECP†, IL-5††, IP-10††
Respiratory syncytial virus-induced wheezing (n = 17)	IL-6*, IL-8*, IL-1ra***, IP-10***	IL-1ra††, IP-10††
Enterovirus-induced wheezing (n = 9)	IL-5**, IL-8*, IP-10*	ECP††, IL-5†
Other viruses-induced wheezing (n = 10)	IL-5**, IL-6**, IL-8*, IL-10**, IL-1ra*, G-CSF**, IP-10***	ECP†, IL-5†, IL-10†, IP-10††
Not detected viruses-induced wheezing (n = 15)	IL-8**, IL-1ra*, IP-10**	ECP†, IP-10†

*p < 0.05; **p < 0.01; ***p < 0.001 compared with non-symptomatic wheezing: †p < 0.05; ††p < 0.01 compared with control.

viruses detected (20.1%) in 174 acute exacerbations of patients with wheezing aged 3.6 years (mean). Comparing this study to the report by Brouard et al., the mean age in the rhinovirus and RS virus groups is almost the same, namely, 3.2 vs. 3.3 years, respectively. Indeed, Kotaniemi-Syrjänen et al. (13) reported that rhinoviruses were identified in 33% of wheezing children aged 1–23 months. They also found that rhinoviruses were associated with the development of subsequent asthma, suggesting that rhinovirus, as well as the RS virus, are important inducers of wheezing even in infancy and play an important role in the development of asthma. In addition, our results showed much higher RS virus detection than the previous report. This could be attributable to improved detection by the high-sensitivity antigen detection kit. We also used DNA direct sequence analysis for more accurate and complete virus detection, and specifically, to distinguish between rhinovirus and enterovirus, compared with the previous report (2). The limitation of this study is the absence of the data from nasal samples in asymptomatic patients and control subjects. We thought that these children would be difficult to recruit to an intensive and invasive study of this type. Indeed, in Japan it is ethically difficult to do such procedure in these children.

Although previous reports have shown virus detection and eosinophil activation and/or cytokine profiles in virus-induced acute exacerbations of wheezing/asthma (14–19), this is the first report to show ECP and various cytokine/chemokine profiles in nasal and serum samples from patients with virus-induced, particularly rhinovirus-induced, acute exacerbations of wheezing. While there were some reports regarding serum or nasal ECP in asthma and RS virus infection (14, 15), Rakes et al. (16) showed that rhinovirus infection in wheezing children is associated with positive serum nasal eosinophilia, elevated nasal ECP, and positive radioallergosorbent testing, suggesting that wheezing attacks by rhinovirus infection are most likely in conjunction with evidence of eosinophilic airway inflammation or atopy. The same group also reported that experimental rhinovirus infection in mild adult asthma induced higher total blood eosinophil counts and increased nasal ECP (17). Furthermore, Johnston's group showed that nasal major basic protein was elevated in acute exacerbations of childhood asthma induced by virus infection, and most of the viruses were rhinoviruses (18). Previously, Oymar et al. (14) reported that serum IL-5 was elevated in childhood asthma and RS virus-induced bronchiolitis. In our study, ECP and IL-5 showed a significant association in both serum and nasal samples (data not shown).

These results collectively suggest that virus-induced wheezing/asthma, particularly by rhinovirus, might enhance eosinophil activation through IL-5 production. However, at the present time, we do not know the exact mechanism by which

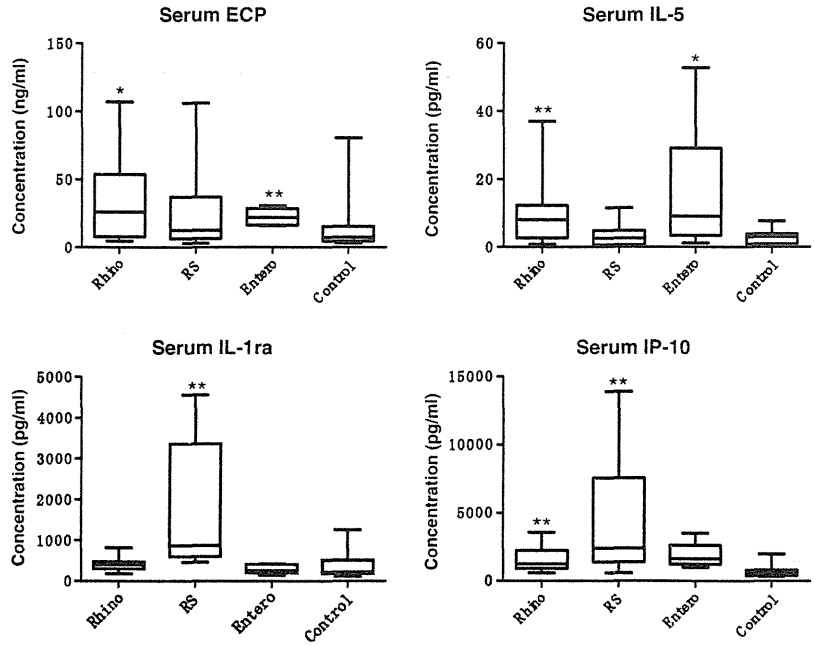

Figure 2 Elevated serum eosinophil cationic protein and/or cytokines/chemokines in rhinovirus-, respiratory syncytial virus-, and enterovirus-induced acute exacerbation of wheezing compared with controls. The median is represented as horizontal bars. Data were analyzed using the Mann–Whitney *U* test, *p < 0.05; **p < 0.01.

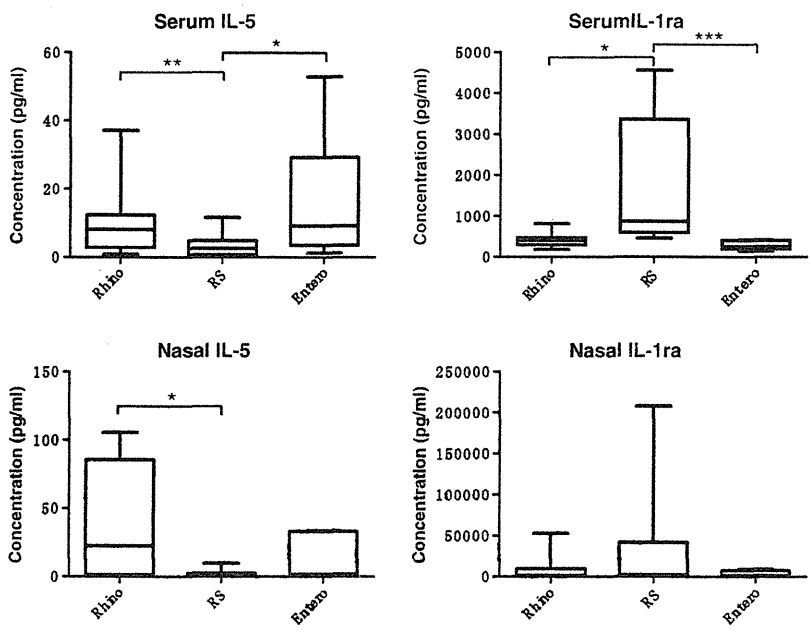

Figure 3 Elevated IL-5 and IL-1ra in serum and nasal secretions among rhinovirus-, respiratory syncytial (RS) virus-, and enterovirus-induced acute exacerbation of wheezing. Only IL-5 was significantly elevated in the rhinovirus group compared with the RS virus group in both serum and nasal secretions. In contrast, only serum IL-1ra was significantly elevated in the RS virus group compared with the rhinovirus or enterovirus groups. The median is represented as horizontal bars. Data were analyzed using the Kruskal–Wallis test, followed by Dunn's multiple comparison test, *p < 0.05; **p < 0.01; ***p < 0.001.

rhinoviruses might induce acute exacerbations of wheezing/asthma and enhance eosinophil activation. There are a number of articles relating to experimental rhinovirus infection and the induction of a variety of cellular responses. Human rhinoviruses induce an increase in airway reactivity and epithelial (4) or sputum (20) eosinophils in asthmatic patients. Rhinovirus infection in human respiratory submucosal glands up-regulates the expression of intracellular adhesion molecule-1 mRNA, the major rhinovirus receptor, and increased production of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and GM-CSF in supernatants (21). Similarly, rhinovirus infection up-regulates GM-CSF (22), eotaxin, and eotaxin-2 expression in bronchial epithelial BEAS-2B cells (23). In human airway submucosal glands, eosinophil chemotaxis was augmented during rhinovirus infection (24). A very recent article found that rhinovirus infection enhances airway epithelial remodeling through VEGF production (25). Collectively, evidence suggests that rhinoviruses could induce eosinophil activation,

particularly through eosinophil-active cytokines/chemokines, such as IL-5, GM-CSF, and eotaxin, as well as an increase in the number of eosinophils.

In contrast to elevated ECP and IL-5 in rhinovirus group, our results showed that serum IL-1ra was elevated in only RS virus group. IL-1ra has anti-inflammatory properties and contributes to limit the inflammatory process in asthma (26). IL-1ra in bronchial lavage was increased in status asthmatics than in stable asthmatics and control patients (27). Another report found that nasal IL-1ra levels were significantly higher in the non-asthmatics than in asthmatics during experimental rhinovirus infection (28). These reports and our results suggest that IL-1ra might play an anti-inflammatory role in RS virus-induced acute exacerbations of childhood wheezing/asthma. IP-10 (or CXCL10) is a chemokine ligand for CXCR3 receptor that has been shown to be induced in human bronchial epithelial cells after infection with rhinovirus (29). A recent report showed that serum IP-10 levels were increased in acute virus-induced asthma compared with non-virus-induced acute asthma (30). Our results also suggest that serum IP-10 is a novel marker of both rhinovirus- and RS virus-induced acute wheezing/asthma exacerbations. In the differences of cytokine profiles between rhinovirus and RS virus infection, previously, Gern et al. showed that there were significant correlations between nasal IL-8 levels and symptom scores during infections with rhinovirus, but not RS virus, suggesting that the evidence of a close relationship between the generation of IL-8 and symptoms during acute infections with rhinovirus (19).

Collectively, our observations and the previous report clearly indicate that different cytokine profile and eosinophil activation might be involved in rhino- and RS virus-induced wheezing/asthma exacerbations.

Further work is needed to better explore the mechanisms behind the association between wheezing/asthma exacerbations and rhinovirus or RS virus infections. These studies might ultimately lead to treatment modalities to prevent and/or treat the significant burden of wheezing/asthma exacerbations caused by virus infection.

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Financial disclosure and conflict of interest statement

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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References

1. Lemanske RF. Viral infections and asthma inception. *J Allergy Clin Immunol* 2004: **114**: 1023–6.
2. Johnston SL, Pattemore PK, Sanderson G, et al. Community study of role of viral infections in exacerbations of asthma in 9–11 year old children. *BMJ* 1995: **310**: 1225–9.
3. Grissell TV, Powell H, Shafren DR, et al. Interleukin-10 gene expression in acute virus-induced asthma. *Am J Respir Crit Care Med* 2005: **172**: 433–9.
4. Fraenkel DJ, Bardin PG, Sanderson G, Lampe F, Johnston SL, Holgate ST. Lower airways inflammation during rhinovirus colds in normal and asthmatic subjects. *Am J Respir Crit Care Med* 1995: **151** (3 Pt 1): 879–86.
5. Calhoun WJ, Dick EC, Schwartz LB, Busse WW. A common cold virus, rhinovirus 16, potentiates airway inflammation after segmental antigen bronchoprovocation in allergic subjects. *J Clin Invest* 1994: **94**: 2200–8.
6. Gleich GJ. Mechanisms of eosinophil-associated inflammation. *J Allergy Clin Immunol* 2000: **105**: 651–63.
7. Humbles AA, Lloyd CM, McMillan SJ, et al. A critical role for eosinophils in allergic airways remodeling. *Science* 2004: **305**: 1776–9.
8. National Asthma Education and Prevention Program. Expert Panel Report II: Guidelines for the Diagnosis and Management of Asthma. Bethesda, MD: National Institutes of Health, National Heart, Lung, and Blood Institute, 1997. NIH Publication No. 97–4051.
9. Yunginger JW, Reed CE, O'Connell EJ, Melton LJ III, O'Fallon WM, Silverstein MD. A community-based study of the epidemiology of asthma. Incidence rates, 1964–1983. *Am Rev Respir Dis* 1992: **146**: 888–94.
10. Blomqvist S, Skyttä A, Roivainen M, Hovi T. Rapid detection of human rhinoviruses in nasopharyngeal aspirates by a microwell reverse transcription-PCR-hybridization assay. *J Clin Microbiol* 1999: **37**: 2813–6.
11. Osiowy C. Direct detection of respiratory syncytial virus, parainfluenza virus, and adenovirus in clinical respiratory specimens by a multiplex reverse transcription-PCR assay. *J Clin Microbiol* 1998: **36**: 3149–54.
12. Brouard J, Freymuth F, Toutain F, et al. Role of viral infections and Chlamydia pneumoniae and Mycoplasma pneumoniae infections in asthma in infants and young children. Epidemiologic study of 118 children. *Arch Pediatr* 2002: **9** (Suppl. 3): 365S–71S.
13. Kotaniemi-Syrjänen A, Vainionpää R, Reijonen TM, Waris M, Korhonen K, Korppi M. Rhinovirus-induced wheezing in infancy – the first sign of childhood asthma? *J Allergy Clin Immunol* 2003: **111**: 66–71.
14. Oymar K, Elsayed S, Bjerknes R. Serum eosinophil cationic protein and interleukin-5 in children with bronchial asthma and acute bronchiolitis. *Pediatr Allergy Immunol* 1996: **7**: 180–6.
15. Dimova-Yaneva D, Russell D, Main M, Brooker RJ, Helms PJ. Eosinophil activation and cysteinyl leukotriene production in infants with respiratory syncytial virus bronchiolitis. *Clin Exp Allergy* 2004: **34**: 555–8.
16. Rakes GP, Arruda E, Ingram JM, et al. Rhinovirus and respiratory syncytial virus in wheezing children requiring emergency care.

IgE and eosinophil analyses. *Am J Respir Crit Care Med* 1999: **159**: 785–90.
17. Zambrano JC, Carper HT, Rakes GP, et al. Experimental rhinovirus challenges in adults with mild asthma: response to infection in relation to IgE. *J Allergy Clin Immunol* 2003: **111**: 1008–16.
18. Teran LM, Seminario MC, Shute JK, et al. RANTES, macrophage-inhibitory protein 1alpha, and the eosinophil product major basic protein are released into upper respiratory secretions during virus-induced asthma exacerbations in children. *J Infect Dis* 1999: **179**: 677–81.
19. Gern JE, Martin MS, Anklam KA, et al. Relationships among specific viral pathogens, virus-induced interleukin-8, and respiratory symptoms in infancy. *Pediatr Allergy Immunol* 2002: **13**: 386–93.
20. de Kluijver J, Evertse CE, Sont JK, et al. Are rhinovirus-induced airway responses in asthma aggravated by chronic allergen exposure? *Am J Respir Crit Care Med* 2003: **168**: 1174–80.
21. Suzuki T, Yamaya M, Sekizawa K, et al. Effects of dexamethasone on rhinovirus infection in cultured human tracheal epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2000: **278**: L560–71.
22. Sanders SP, Kim J, Connolly KR, Porter JD, Siekierski ES, Proud D. Nitric oxide inhibits rhinovirus-induced granulocyte macrophage colony-stimulating factor production in bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2001: **24**: 317–25.
23. Papadopoulos NG, Papi A, Meyer J, et al. Rhinovirus infection up-regulates eotaxin and eotaxin-2 expression in bronchial epithelial cells. *Clin Exp Allergy* 2001: **31**: 1060–6.
24. Furukawa E, Ohrui T, Yamaya M, et al. Human airway submucosal glands augment eosinophil chemotaxis during rhinovirus infection. *Clin Exp Allergy* 2004: **34**: 704–11.
25. Leigh R, Oyelusi W, Wiehler S, et al. Human rhinovirus infection enhances airway epithelial cell production of growth factors involved in airway remodeling. *J Allergy Clin Immunol* 2008: **121**: 1238–45.
26. Sim TC, Hilsmeier KA, Reece LM, Grant JA, Alam R. Interleukin-1 receptor antagonist protein inhibits the synthesis of IgE and proinflammatory cytokines by allergen-stimulated mononuclear cells. *Am J Respir Cell Mol Biol* 1994: **11**: 473–9.
27. Tillie-Leblond I, Pugin J, Marquette CH, et al. Balance between proinflammatory cytokines and their inhibitors in bronchial lavage from patients with status asthmaticus. *Am J Respir Crit Care Med* 1999: **159**: 487–94
28. de Kluijver J, Grünberg K, Pons D, et al. Interleukin-1β and interleukin-1ra levels in nasal lavages during experimental rhinovirus infection in asthmatic and non-asthmatic subjects. *Clin Exp Allergy* 2003: **33**: 1415–8.
29. Spurrell JC, Wiehler S, Zaheer RS, Sanders SP, Proud D. Human airway epithelial cells produce IP-10 (CXCL10) in vitro and in vivo upon rhinovirus infection. *Am J Physiol Lung Cell Mol Physiol* 2005: **289**: L85–95.
30. Wark PA, Bucchieri F, Johnston SL, et al. IFN-γ-induced protein 10 is a novel biomarker of rhinovirus-induced asthma exacerbations. *J Allergy Clin Immunol* 2007: **120**: 586–93.

Laboratory and Epidemiology Communications

Sequencing and Phylogenetic Analyses of Saffold Cardiovirus (SAFV) Genotype 3 Isolates from Children with Upper Respiratory Infection in Gunma, Japan

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Saffold cardiovirus (SAFV) is a newly discovered virus that belongs to family *Picornaviridae* and genus *Cardiovirus* (1). Recent studies suggest that SAFV is relatively common in children with various diseases, with PCR-positive rates varying from 0.5–12% (2–6). For example, SAFV has been detected in stool samples from children with respiratory tract infections, gastroenteritis, and non-polio acute flaccid paralysis cases (2–6). Recently, we showed that SAFV genotype 2 was associated with typical exudative tonsillitis in Japanese children (7). However, as this virus is also detected in asymptomatic humans, the pathogenicity of SAFV is not yet clearly understood (5). In addition, Zoll et al. suggested that different SAFV strains may also exhibit different in vitro tropisms (6), thus suggesting that it may be relatively difficult to isolate SAFV using culture methods, which may explain why few studies have been conducted to date on its isolates (6). Herein we report the isolation of two strains of SAFV genotype 3 from children with acute respiratory infections, and the results of sequence and phylogenetic analyses (*VP1* coding region) of these strains.

We analyzed nasopharyngeal samples from two male patients, aged 5 and 6 years, who presented with fever (>38°C), canker sores, and upper respiratory inflammation. A pediatrician had diagnosed upper respiratory infection (URI) with sinusitis, pharyngitis, and laryngitis (8). The samples were obtained by the local health authority of Gunma Prefecture in 2008 for the surveillance of viral diseases in Japan. Informed consent was obtained from the parents of both subjects for donation of the nasopharyngeal samples used in this analysis.

The throat swab samples were centrifuged at 3,000 × g at 4°C for 30 min and the supernatants used for virus isolation. Cell lines (HEp-2, Vero, MDCK, A549, and RD18s cells) were inoculated and subjected to three freeze/thaw cycles.

The observation of enterovirus-like cytopathic effects (CPEs), such as collapsed balloons and small fragmented cells floating in the medium inoculated with HEp-2 cells (Fig. 1), led us to initially suspect another virus such as an enterovirus. However, we failed to amplify the *VP1* and *VP4* coding region of the hypothetical enterovirus in culture supernatants by RT-PCR with broad detectable primers (9,10). Next, we carried out a

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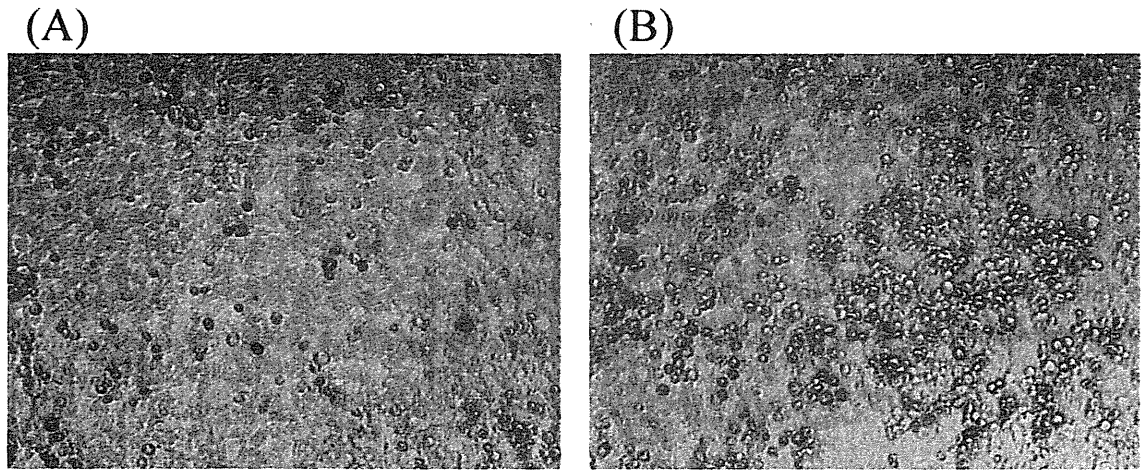

Fig. 1. Cytopathic effects of Saffold cardiovirus (SAFV) type 3 in HEp-2 cells. (A) No infection. (B) SAFV type 3 infected-HEp-2 cells. Images were taken following three passages after specimen (throat swab) inoculation.

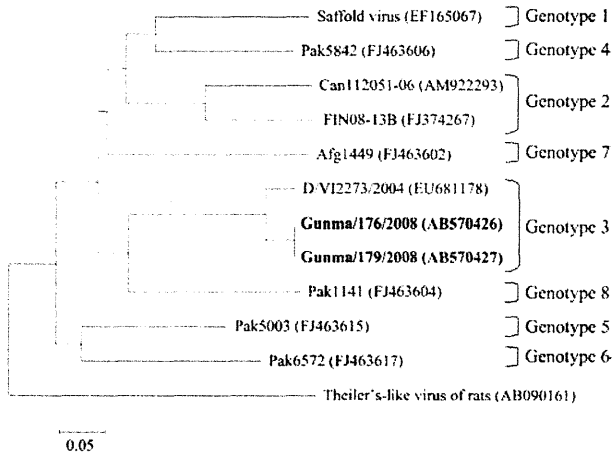

Fig. 2. A phylogenetic tree of SAFV type 2 based on the *VP1* coding region (327 nt). The phylogenetic tree was constructed using the *VP1* coding region. Numbers in parentheses are the GenBank accession numbers.

comprehensive sequencing (using the RDV method) for viral RNA determination, as described previously (11). The partial viral nucleotide sequences obtained upon analysis of the sequence data with BLAST (11) suggested SAFV. Finally, we analyzed the *VP1* coding region of SAFV, as described previously (7). Briefly, viral RNA was extracted from 140 μL of the supernatants using a QIAamp Viral RNA Mini Kit (Qiagen, Germantown, Md., USA) and the RT-PCR procedure performed according to the manufacturer's instructions (One-step RT PCR kit; Qiagen). The primers for RT-PCR (for amplification of the *VP1* coding region) were as follows: 5′-HAA RCA RGR YTG GAR YTT YNT NAT GTT-3′ (primer 315F) and 5′-DGG BCK DGG RCA RWA VAC YCT CAT-3′ (primer 738R) (7). The following protocol was used: incubation for 30 min at 50°C, 15 min at 95°C, followed by 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, ending with elongation for an additional 5 min at 72°C. The DNA fragments were purified using a QIAquick PCR Purification kit (Qiagen) and the nucleotide sequence determined using an automated DNA sequencer (7). Amplicons were sequenced and aligned (327 bp), then analyzed phylogenetically using the CLUSTAL W program on the website of DNA Data Bank of Japan (DDBJ; http://hypernig.nig.ac.jp/homology/clustalw-e.shtml) and TreeExplorer (version 2.12) (http://evolgen.biol.metro-u.ac.jp/TE/). Evolutionary distances were estimated using Kimura's two-parameter method and the phylogenetic trees were constructed using the neighbor-joining (N-J) method (12). The reliability of the tree was estimated using 1,000 bootstrap replications.

Phylogenetic analysis based on the *VP1* coding region revealed two strain clusters in the SAFV genotype 3 (Fig. 2), with an interstrain nucleotide identity of 100%. The high diversity of SAFV is highlighted by the identification of at least eight *VP1* coding regions (5). No pathogen other than SAFV was isolated using the cell-culture method.

Although a wide range of viruses is known to cause respiratory infections, the etiology of 20–30% of these currently remains unidentified (13). This may be due to the lack of sensitivity of some of the detection methods used, and it has also been suggested that unrecognized infectious agents, including other viruses, have yet to be discovered (14). Viral diagnosis is mostly based on clinical symptoms; thus, it is important to collect appropriate clinical samples early in the infection process, and the type of sample collected is somewhat dependent on the clinical symptoms. In this study, we succeeded in isolating SAFV from the throat swabs of two URI patients, thus we suppose that the fever and URI were associated with SAFV. Using the tissue culture method, we isolated 67 viruses from 124 samples taken during 2008 in Gunma Prefecture for the surveillance of viral diseases. Although 39 strains of influenza virus were isolated, only two of these were SAFV. This could suggest that SAFV is seldom the cause of URI, although much larger numbers of strains will be required to address its epidemiology more comprehensively.

In conclusion, SAFV might be a causative agent of acute respiratory infection. A previous report has suggested that SAFVs are ubiquitous human viruses with a global prevalence, therefore SAFV might be the cause in samples with enterovirus-like CPEs (6). The growth characteristics of our strains are similar to those seen

previously in other slow-growing SAFVs (5), and the HEp-2 cell line has been shown to be an effective cell line for further research into SAFV, including clarification of its epidemiology and etiology, although blind passages may be necessary to isolate the virus (15). A more detailed analysis, including serological surveys of SAFV, is needed to determine its exact role in human disease.

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Conflict of interest None to declare.

REFERENCES

1. Jones, M.S., Lukashov, V.V., Ganac, R.D., et al. (2007): Discovery of a novel human picornavirus in a stool sample from a pediatric patient presenting with fever of unknown origin. J. Clin. Microbiol., 45, 2144-2150.
2. Xu, Z.Q., Cheng, W.X., Qi, H.M., et al. (2009): New 655 Saffold cardiovirus in children, China. Emerg. Infect. Dis., 15, 993-994.
3. Drexler, J.F., Luna, L.K., Stöcker, A., et al. (2008): Circulation of three lineages of a novel Saffold cardiovirus in humans. Emerg. Infect. Dis., 14, 1398-1405.
4. Chiu, C.Y., Greninger, A.L., Kanada, K., et al. (2008): Identification of cardioviruses related to Theiler's murine encephalomyelitis virus in human infections. Proc. Natl. Acad. Sci. USA, 105, 14124-14129.
5. Blinkova, O., Kapoor, A., Victoria, J., et al. (2009): Cardioviruses are genetically diverse and common enteric infections in South Asian children. J. Virol., 83, 4631-4641.
6. Zoll, J., Erkens Hulshof, S., Lanke, K., et al. (2009): Saffold virus, a human Theiler's-like cardiovirus, is ubiquitous and causes infection early in life. PLoS Pathog. 5, e1000416.
7. Itagaki, T., Abiko, C., Ikeda, T., et al.: Sequence and phylogenetic analyses of Saffold cardiovirus from children with exudative tonsillitis in Yamagata, Japan. Scand. J. Infect. Dis. (in press).
8. Cherry, J.D. (2003): The common cold. p. 140-146. *In* Feigin, R.D., Cherry, J.D., Demmler, G.J., et al. (eds.), Textbook of Pediatric Infectious Diseases. 5th ed. Philadelphia, Saunders.
9. Oberste, M.S., Maher, K., Flemister, M.R., et al. (2000): Comparison of classic and molecular approaches for the identification of untypeable enteroviruses. J. Clin. Microbiol., 38, 1170-1174.
10. Olive, D.M., Al-Mufti, S., Al-Mulla, W., et al. (1990): Detection and differentiation of picornaviruses in clinical samples following genomic amplification. J. Gen. Virol., 71, 2141-2147.
11. Mizutani, T., Endoh, D., Okamoto, M., et al. (2007): Rapid genome sequencing of RNA viruses. Emerg. Infect. Dis., 13, 322-324.
12. Saitou, N. and Nei, M. (1987): The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol., 4, 406-425.
13. Freymuth, F., Quibriac, M., Petitjean, J., et al. (1987): Viruses responsible for respiratory infections in pediatrics. Evaluation of 3,480 nasal aspirates performed in children over a 6-year period. Ann. Pediatr., 34, 493-501.
14. Finkbeiner, S.R., Allred, A.F., Tarr, P.I., et al. (2008): Metagenomic analysis of human diarrhea: viral detection and discovery. PLoS Pathog., 4, e1000011.
15. Chiu, C.Y., Greninger, A.L., Chen, E.C., et al. (2010): Cultivation and serological characterization of a human Theiler's-like cardiovirus associated with diarrheal disease. J. Virol., 84, 4407-4414.

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NOTE

Endemicity of human metapneumovirus subgenogroups A2 and B2 in Yamagata, Japan, between 2004 and 2009

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ABSTRACT

To clarify a longitudinal epidemiology, we isolated 280 hMPV strains from patients with acute respiratory infections in Yamagata, Japan, between 2004 and 2009. We observed that the high season for hMPV was from winter to spring (between January and May) and the low season was in the fall (around September and October). A further molecular analysis revealed that subgenogroup A2 (A2) strains were the most commonly isolated (151/280; 53.9%), followed by B2 (108/280; 38.6%) and B1 (19/280; 6.8%). Our results suggested that A2 and B2 have been endemically in circulation as the major types almost every year, whereas other subgenogroups have appeared less frequently.

Key words acute respiratory infection, endemicity, human metapneumovirus, subgenogroup.

Human metapneumovirus (hMPV) was first described in 2001 following its isolation from infants and children with ARI (1). Based on genetic analyses, hMPV has been categorized as a member of the genus *Metapneumovirus* of the subfamily *Pneumovirinae* of the family *Paramyxoviridae* (2, 3). hMPV has been recognized as an important causative agent of respiratory tract disease worldwide, especially in the pediatric and elderly populations (2). hMPV is associated with the common cold and with lower respiratory tract illnesses, such as pneumonia, bronchiolitis, bronchitis, croup, and the exacerbation of reactive airway disease (2, 3). Serological studies have indicated that, by the age of 5 years, almost all children show evidence of hMPV infection (3–5). Based on genomic sequencing and phylogenetic analysis, hMPV appears to have a single serotype with two subgenogroups, A and B, which have extensive cross-reactivity and cross-protection (2, 3). Further, each genotype appears to have at least two distinct subgenogroups; A1 and A2, and B1 and B2 (3, 6). hMPV outbreaks occur in annual epidemics during late winter and early spring in temperate climates, although sporadic hMPV infection also occurs year round in temperate areas (2, 3, 7, 8). Although a longitudinal study is necessary to clarify the epidemiology of hMPV, including its seasonality, only a limited number of studies of more than 3 years in duration have been reported (9–11). Thus, we aimed to clarify the longitudinal epidemiology of hMPV based on virus isolation in a community such as Yamagata, Japan. Although we previously reported hMPV isolation using Vero E6 cell lines based on data obtained between 2004 and 2005 (12, 13), here we present the results for the entire study period, between 2004 and 2009, as a longitudinal study.

Between January 2004 and December 2009, 12 504 nasopharyngeal swab specimens were obtained from patients with ARI at pediatric clinics collaborating with the local

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List of Abbreviations: ARI, acute respiratory infection; CPE, cytopathic effect; hMPV, human metapneumovirus; RT-PCR, reverse-transcription PCR.

health authority of Yamagata Prefecture for the surveillance of viral diseases in Japan. Among them, 8112 (64.9%) were from patients <5 years old, 2918 (23.3%) were from patients between 5 and 9 years, 1064 (8.5%) were from patients between 10 and 14 years, 357 (2.9%) were from patients >14 years, and 53 (0.4%) were from patients of unknown age. The specimens were collected and placed immediately in tubes containing a transport medium, and then transported to the Department of Microbiology, Yamagata Prefectural Institute of Public Health for virus isolation (12).

Virus isolation was carried out using a modified microplate method (i.e. the HHVe6MRG plate) (12, 13). Briefly, human embryonic lung fibroblast (HEF), human laryngeal carcinoma (HEp-2), Vero E6, Madin-Darby canine kidney cells (MDCK), rhabdomyosarcoma (RD-18S) and green monkey kidney (GMK) cell lines were prepared on the wells of a 96-well microplate (Greiner Bio-One, Frickenhausen, Germany) (12, 13). After medium change, each specimen was centrifuged at 450 *g* for 20 min and 75 μL of the supernatant was inoculated onto two wells of each cell line. We observed the plates two to three times per week for CPE for approximately 1 month without passage or medium change. When a suspected hMPV CPE was observed, viral identification and genotyping were carried out by RT-PCR and sequence analysis (13). For RT-PCR and sequencing analysis, primers MPVF1f, MPVF1r, BF101, BF104 and HMPV-F1 were used (14–16). Sequence data for the isolates from Yamagata between 2004 and 2009 were registered under accession numbers AB251496-AB251574, AB518311-AB518476 and AB548217-AB548249 at GenBank. Sequence data were analyzed with CLUSTAL W version 1.83, and a phylogenetic tree was constructed by the neighbor-joining method (17) using the same software.

As a result, we succeeded in isolating a total of 280 hMPV strains, which included 79 strains reported in previous studies (12, 13), with a total isolation rate of 2.2% (280/12 504). Among 13 of these 280 samples, other respiratory viruses were co-isolated with hMPV by using the HHVe6MRG plate: adenovirus 1, echovirus 18, coxsackievirus A4, coxsackievirus B3 (three cases), coxsackievirus B4, parainfluenza 1, parainfluenza 2, parainfluenza 3, influenza B, and cytomegalovirus (two cases). The age distribution of the cases where hMPV was isolated was as follows: 208 (74.3%) were from patients <5 years old, 52 (18.6%) were from patients between 5 and 9 years, 14 (5.0%) were from patients between 10 and 14 years, and six (2.1%) were from patients >14 years. The results shown in Figure 1 are based on all 280 hMPV genotyped isolates, and those shown in Figure 2 are based on the four reference strains and 63 representative Yamagata isolates, most of which were analyzed in this study.

As shown in Figure 1, hMPV were only isolated over a 3-month period in 2004 and over a 6-month period in 2008. However, hMPV were isolated throughout most of the year between 2005 and 2007 and in 2009, although only a small number of hMPV were isolated in September and October.

As shown in Figures 1 and 2, phylogenetic analysis revealed that three hMPV subgenogroups (A2, B1 and B2) were isolated in each of the 6 years. Subgenogroup A2 (A2) strains were the most commonly isolated (151/280; 53.9%), followed by B2 (108/280; 38.6%) and B1 (19/280; 6.8%). A mixture of the three subgenogroups was observed only in 2005 and two subgenogroup strains co-circulated

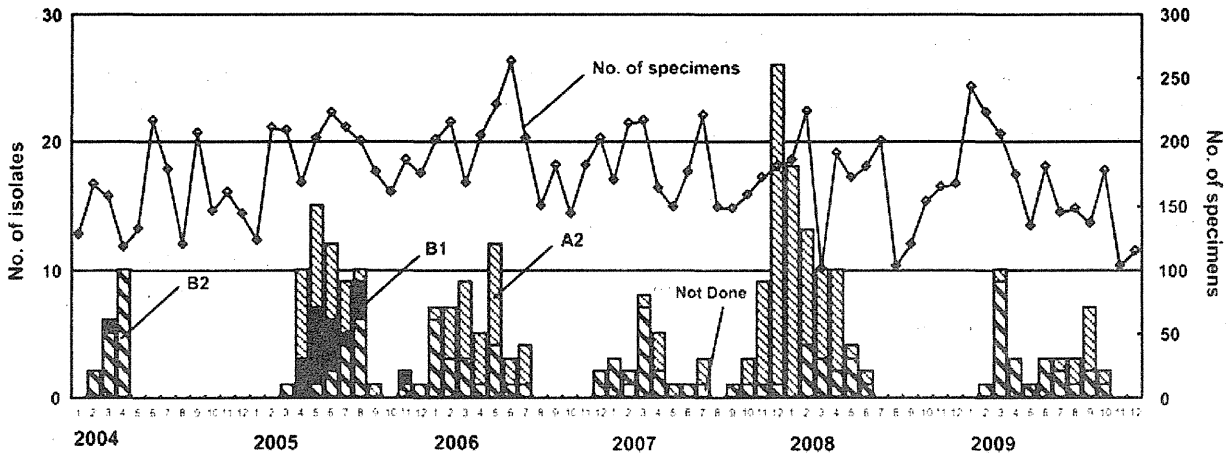

Fig. 1. Monthly distribution and subgenogroups of hMPV strains isolated in Yamagata, Japan, between 2004 and 2009. Subgenogroups, A2, B1, and B2, were grouped according to the phylogenetic analysis shown in Figure 2 and are based on reference 13.

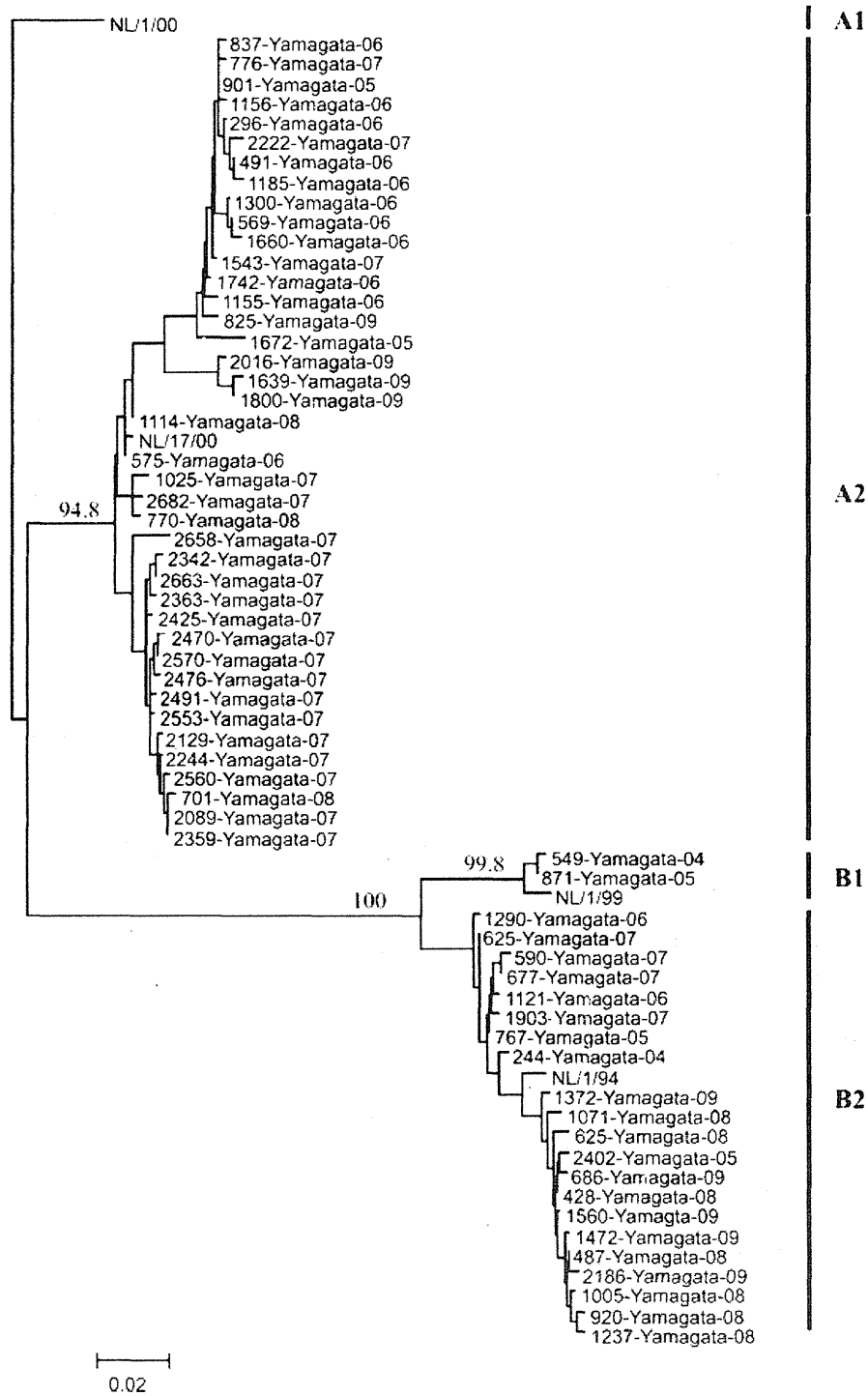

Fig. 2. Phylogenetic tree for the partial (441 bp) sequence of the fusion region of representative hMPV strains isolated in Yamagata, Japan between 2004 and 2009 as well as for reference strains. Details for the 2004–2005 Yamagata isolates are given in reference 13 and reference strains (NL/1/00, NL/17/00, NL/1/99 and NL/1/94) were based on reference 15. Branch lengths are proportional to the number of nucleotide differences. Numbers are the bootstrap probabilities (%). The marker denotes the measurement of relative phylogenetic distance.