Infection, Genetics and Evolution 59 (2018) 155–162

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Detailed genetic analyses of the *HN* gene in human respirovirus 3 detected in children with acute respiratory illness in the Iwate Prefecture, Japan

Masaki Takahashi[a,g], Koo Nagasawa[b,c], Koichi Saito[a], Shun-ichi Maisawa[d], Kiyotaka Fujita[e], Koichi Murakami[c], Makoto Kuroda[f], Akihide Ryo[g], Hirokazu Kimura[c,e,g,*]

[a] Iwate Prefectural Research Institute for Environmental Sciences and Public Health, 1-11-16 Kitaiioka, Morioka-shi, Iwate 020-0857, Japan
[b] Department of Pediatrics, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba-shi, Chiba 260-8677, Japan
[c] Infectious Disease Surveillance Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan
[d] Department of Pediatrics, Morioka Children's Hospital, 11-14 Matsuyashiki, Ueda, Morioka-shi, Iwate 020-0102, Japan
[e] School of Medical Technology, Faculty of Health Science, Gunma Paz University, 1-7-1 Tonyamachi, Takasaki-shi, Gunma 370-0006, Japan
[f] Pathogen Genomics Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
[g] Department of Microbiology, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanagawa-ku, Yokohama-shi, Kanagawa 236-0004, Japan

ARTICLE INFO

Keywords:
Human respirovirus 3
Hemagglutinin-neuraminidase (HN) gene
Molecular evolution

ABSTRACT

We performed detailed genetic analyses of the partial hemagglutinin–neuraminidase (*HN*) gene in 34 human respirovirus 3 (HRV3) strains from children with acute respiratory illness during 2013–2015 in Iwate Prefecture, Japan. In addition, we performed analyses of the evolutionary timescale of the gene using the Bayesian Markov chain Monte Carlo (MCMC) method. Furthermore, we analyzed pairwise distances and performed selective pressure analyses followed by linear B-cell epitope mapping and N-glycosylation and phylodynamic analyses. A phylogenetic tree showed that the strains diversified at around 1939, and the rate of molecular evolution was 7.6×10^{-4} substitutions/site/year. Although the pairwise distances were relatively short (0.03 ± 0.018 [mean ± standard deviation, SD]), two positive selection sites (Cys544Trp and Leu555Ser) and no amino acid substitutions were found in the active/catalytic sites. Six epitopes were estimated in this study, and three mouse monoclonal antibody binding sites (amino acid positions 278, 281, and 461) overlapped with two epitopes belonging to subcluster C3 strains. Bayesian skyline plot analyses indicated that subcluster C3 strains have been increasing from 2004, whereas subcluster C1 strains have declined from 2004. Based on these results, Iwate strains were divided into two subclusters and each subcluster evolved independently. Moreover, our results suggested that some predicted linear epitopes (epitopes 3 and 5) are candidates for an HRV3 vaccine motif. To better understand the details of the molecular evolution of HRV, further studies are needed.

1. Introduction

Human respirovirus 3 (HRV3) is a member of the genus *Respirovirus* and family *Paramyxoviridae*. Together with respiratory syncytial virus (RSV), it is the major causative agent of respiratory disease in children and aged humans (Collins and Karron, 2013; Loubet et al., 2016). HRV3 is responsible for various acute respiratory infections (ARI) such as the common cold, croup, bronchiolitis, and pneumonia (Karron and Collins, 2013). A previous epidemiological study showed that at least 60% children had been infected with HRV3 by the age of 2 and over 80% by the age of 4 (Parrott et al., 1962). Furthermore, HRV may be responsible for approximately 10% of acute lower respiratory tract reinfections in adults (Azevedo et al., 2003; Matsuse et al., 2005).

The HRV3 genome encodes six genes and translates into two major structural proteins: hemagglutinin–neuraminidase (HN) and fusion (F) glycoproteins (Karron and Collins, 2013). The HN protein displays two distinct biological functions: hemagglutination (binding sialic acid-containing receptors) and neuraminidase activity (cleaving sialic acid-containing receptors) (Henrickson, 2003; Moscona, 2005). In addition, the HN protein is recognized as an antigen in the host immune systems (Henrickson, 2003; Karron and Collins, 2013). The active/catalytic sites in HN protein act not only as a cellular receptor binding site but are also involved in neuraminidase activities (Lawrence et al., 2004; Palermo et al., 2007; Porotto et al., 2007). These functions of the HN protein

Abbreviations: AICM, Akaike's information criterion through MCMC; ARI, acute respiratory infection; BSP, Bayesian skyline plot; ESS, effective sample size; FEL, fixed effects likelihood; GTR, general time reversible; HN, hemagglutinin-neuraminidase; HPD, highest posterior density; HRV, human respirovirus; MCMC, Markov chain Monte Carlo; MAb, mouse monoclonal antibody; MEME, mixed effects model of evolution; REL, random effects likelihood; RSV, respiratory syncytial virus; SD, standard deviation; SLAC, Single likelihood ancestor counting

* Corresponding author at: School of Medical Technology, Faculty of Health Science, Gunma Paz University, 1-7-1 Tonyamachi, Takasaki-shi, Gunma 370-0006, Japan.
E-mail address: h-kimura@paz.ac.jp (H. Kimura).

https://doi.org/10.1016/j.meegid.2018.01.021
Received 28 October 2017; Received in revised form 15 January 2018; Accepted 24 January 2018
Available online 14 February 2018

may reflect viral adsorption, entry, and budding of the progenies of HRV3. Thus, amino acid substitutions in the HN protein may be associated with both molecular functions and molecular evolution (Goya et al., 2016; Moscona, 2005). However, using many modern bioinformatics methods, the molecular evolution of the HN protein in HRV3, as well as detailed local epidemiology with regard to HRV3 infection, has yet to be elucidated.

Recent advances in bioinformatics technology enable us to analyze various aspects of the evolution of viral genomes and antigenic proteins (Kimura et al., 2016; Kobayashi et al., 2016). Very recently, a study using various bioinformatics methods was reported concerning the molecular evolution of the HRV3 *F* gene detected in Japan (Tsutsui et al., 2017). This report revealed that the HRV3 *F* gene is relatively highly conserved and that one viral strain has an amino acid substitution that may affect its resistance to neutralization by antibodies. However, with regard to the molecular evolution of the HRV3 *HN* gene detected in Japan, no previous reports utilizing various bioinformatics methods are available, including epitope mapping and/or Bayesian skyline plot analyses. In the present study, to reveal a detailed picture of the molecular evolution of the HRV3 *HN* gene, including phylodynamics and the relationship between amino acid substitution and the predicted B-cell epitope, we improved upon these bioinformatics methods and performed detailed molecular evolutionary analyses of the partial *HN* gene sequence of HRV3 detected in Iwate Prefecture, Japan.

2. Materials and methods

2.1. Subjects

Throat swabs were collected from 405 hospitalized patients in the Morioka Children's Hospital in Iwate Prefecture, who were mainly diagnosed with a lower respiratory infection (bronchitis, pneumonia). Samples of patients diagnosed with influenza using immunochromatography kits and those of patients diagnosed with bacterial infection using bacterial cultural tests were excluded. These specimens were obtained by the local health authorities of Iwate Prefecture for the 2013–2015 surveillance of viral diseases in Japan. The 61 patients in whom HRV3 was detected by RT-PCR were enrolled in this study. Patients were aged 0–15 years (mean ± SD, 28.5 ± 34.0 months) (Table 1).

2.2. Viral RNA extraction, reverse-transcription polymerase chain reaction, and sequencing

Viral nucleic acid was extracted from 200 µL of Universal Transport Medium (Copan Diagnostics Inc., Brescia, Italy) in which the throat swab was suspended using the QIAamp MinElute Virus Spin Kit (Qiagen GmbH, Hilden, Germany) on a QIAcube (Qiagen) automation device. Reverse transcription was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. The PCR procedures for amplification of the partial HN coding region were conducted as described previously (Mizuta et al., 2014). The primer sequences were as follows: first primer pair, HPIV-3USF (nt 7068–7098 for strain Washington/1957, sense: 5′-ATG ATC TAA TAC ART CAG GAG TRA ATA CAA G-3′) and HPIV-3USR (nt 7931–7956, anti-sense: 5′-ACA ACA ATR ATR GAR TTG ACC ATC CT-3′); second primer pair, HPIV-3MSF (nt 7650–7676, sense: 5′-CAY CAG GCA TAG AAG ATM TTG TAC TTG-3′) and HPIV-3MSR (nt 8432–8468, anti-sense: 5′-YGC TTT TVT GAT TTA TTT CTA CTA TRT GRA AAC AAT A-3′); and third primer pair, HPIV-3DSF (nt 8165–8190, sense: 5′-GTG YTA TCA AGA CCA GGA AAC AAT GA-3′) and HPIV-3DSR (nt 8486–8509, anti-sense: 5′-TGG AAY CTC TGK YTT RAA CAA CAT-3′). The thermal cycling conditions were as follows: 1 cycle of 95 °C for 10 min; 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and a final cycle of 72 °C for 10 min, followed by holding at 4 °C. Following amplicon purification with QIAquick PCR Purification Kit (Qiagen), cycle sequencing was performed using the BigDye Terminator™ v3.1 Cycle Sequencing Kit on a 3500 Genetic Analyzer (Applied Biosystems). Precautions were taken to prevent carry-over contamination of PCR as previously described (Lam et al., 2007). The GenBank accession numbers of all nucleotide sequences included in the present study are shown in Table S1.

Table 1
Subject data in this study.

Year	2013	2014	2015	Total
No. of samples tested	158	139	108	405
No. of detected HRV3 (%)	33(20.9)	16(11.5)	12(11.1)	61(15.1)
Clinical symptoms				
LRI[a]	157	90	60	307
URI[b]	1	20	30	51
Fever		29	18	47
Sex (male/female)	87/71	77/62	56/52	220/185
Age (month)[c]	29.1 ± 30.1	29.2 ± 36.9	26.6 ± 35.6	28.5 ± 34.0

[a] LRI: lower respiratory illness.
[b] URI: upper respiratory illness.
[c] Data are expressed as mean ± SD.

2.3. Phylogenetic analysis and estimation of the evolutionary rate by the Bayesian Markov chain Monte Carlo method

The nucleotide sequences of partial *HN* (position 7142–8491; 1350 nt for strain Washington/1957) were aligned by MEGA 6.0 (http://www.megasoftware.net/) (Tamura et al., 2013). To estimate the evolutionary rate and time-scaled phylogeny, phylogenic analyses were performed by the Bayesian Markov chain Monte Carlo (MCMC) method using BEAST 1.8.0 (Drummond et al., 2012). To construct the MCMC phylogenic tree, the best nucleotide substitution model was selected using Kakusan4 (Tanabe, 2011). Furthermore, the best clock model and demographic model were determined by calculating Akaike's information criterion (AICM) through MCMC (Table S2). The analyzed conditions of Bayesian MCMC analyses are shown in Table S3. The parameter outputs generated by the Bayesian MCMC and convergence were assessed by effective sample size (ESS) after a 10% burn-in using TRACER 1.6 (http://tree.bio.ed.ac.uk/software/tracer/). Estimate uncertainty was indicated by the 95% highest posterior density intervals. The maximum clade credibility tree was obtained after 10% burn-in using TreeAnnotator 1.8.0. The phylogenic tree was viewed in FigTree 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

In addition, we estimated the evolutionary rate of strains in each cluster using BEAST under appropriate conditions. The AICM value of each model and the analyzed conditions are shown in Tables S2 and S3.

2.4. Detection of positive selection sites in the partial HN coding region and calculation of pairwise distances

To evaluate the positive-negative selection activity on the partial *HN* gene among all HRV3 strains, we analyzed the rates of synonymous (*dS*) and non-synonymous (*dN*) substitutions at amino acid sites by random effects likelihood (REL) and mixed effects models of evolution (MEME) methods using the Datamonkey web server (http://www.