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Genetic analysis of human rhinovirus species A to C detected in patients with acute respiratory infection in Kumamoto prefecture, Japan 2011–2012

Naoko Kiyota [a], Miho Kobayashi [b], Hiroyuki Tsukagoshi [b], Akihide Ryo [c], Seiya Harada [a], Takashi Kusaka [d], Masatsugu Obuchi [e], Naoki Shimojo [f], Masahiro Noda [g], Hirokazu Kimura [g,*]

[a] Kumamoto Prefectural Institute of Public Health and Environmental Sciences, 1240-1 Uto-shi, Kumamoto 869-0425, Japan
[b] Gunma Prefectural Institute of Public Health and Environmental Sciences, 378 Kamioki-machi, Maebashi-shi, Gunma 371-0052, Japan
[c] Department of Molecular Biodefence Research, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama-shi, Kanagawa 236-0004, Japan
[d] Maternal Perinatal Center, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan
[e] Toyama Institute of Health, 17-1 Nakataikoyama, Imizu-shi, Toyama 939-0363, Japan
[f] Department of Pediatrics, Graduate School of Medicine, Chiba University, Chiba-shi, Chiba 260-8670, Japan
[g] Infectious Disease Surveillance Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan

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ABSTRACT

We performed detailed genetic analysis of the *VP4*/*VP2* coding region in human rhinovirus species A to C (HRV-ABC) strains detected in patients with a variety of acute respiratory infections in Kumamoto, Japan in the period 2011–12. The phylogenetic tree and evolutionary timescale were obtained by the Bayesian Markov chain Monte Carlo method. Phylogenetic analyses showed that the present HRV-A, -B, and -C strains belonged to 25, 4, and 18 genotypes, respectively. Some new genotypes were confirmed as prevalent strains of HRV-C. An ancestor of the present HRV-ABCs could be dated back to about 20,000 years ago. The present HRV-A and -C strains have wide genetic divergence (pairwise distance >0.2) with rapid evolutionary rates (around 7×10^{-4} to 4×10^{-3} substitutions/site/year). Over 100 sites were found to be under negative selection, while no positively selected sites were found in the analyzed region. No evidence of recombination events was found in this region of the present strains. Our results indicate that the present HRV strains have rapidly evolved and subsequently diverged over a long period into multiple genotypes.

1. Introduction

Human rhinovirus (HRV) belongs to the genus *Enterovirus* and family *Picornaviridae*. HRV species are typically causative agents of mild acute respiratory infection (ARI), such as the common cold. However, HRV has been implicated as an agent of more severe ARI such as bronchiolitis and pneumonia (Arakawa et al., 2012; Smuts et al., 2011; Turner and Couch, 2007; Watanabe et al., 2010) and may be associated with virus-induced asthma (Fujitsuka et al., 2011; Johnston et al., 1995; Linsuwanon et al., 2009; Smuts et al., 2011). In addition, HRV may be a common agent of acute sinusitis (Pitkäranta et al., 1997, 2001) and an exacerbator of chronic obstructive pulmonary disease (Mallia et al., 2011; Seemungal et al., 2001). HRV might therefore be involved in various ARIs and additional respiratory complications.

HRV is classified into three species: HRV-A, HRV-B, and HRV-C (Lau et al., 2007). HRV-A and -B species comprise over 100 serotypes and HRV-C is the most recently discovered (Lamson et al., 2006; McErlean et al., 2007; Turner and Couch, 2007). All HRV species can be detected throughout the year in many areas and countries, and HRV-A and -C appear to be the predominant species detected in patients with various ARI (Arakawa et al., 2012; Briese et al., 2008; Jin et al., 2009; Lau et al., 2009; McErlean et al., 2008). Previous reports suggest that various genotypes of HRV-ABCs are associated with a variety of ARIs (Arakawa et al., 2012; Simmonds

Abbreviations: AdV, adenovirus; ARI, acute respiratory infection; ESS, effective sample size; EV, enterovirus; FEL, fixed effects likelihood; GTR, general time reversible; HA, hemagglutinin; HBoV, human bocavirus; HMPV, human metapneumovirus; HPD, highest posterior density; HPIV, human parainfluenza virus; HRV, human rhinovirus; IFEL, internal fixed effects likelihood; LRI, lower respiratory infection; MCMC, Markov chain Monte Carlo; NJ, neighbor joining; NPS, nasopharyngeal swabs; PCR, polymerase chain reaction; *p*-distance, pairwise distance; RSV, respiratory syncytial virus; RT, reverse transcription; SD, standard deviation; SLAC, single likelihood ancestor counting; tMRCA, time of the most recent common ancestor; URI, upper respiratory infection.

* Corresponding author. Tel.: +81 42 561 0771; fax: +81 42 565 3315.
E-mail address: kimhiro@nih.go.jp (H. Kimura).

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et al., 2010). Recent studies suggest that HRV-A and -C, but not HRV-B, are frequently detected in patients with various ARI (Arakawa et al., 2012; Linsuwanon et al., 2009; Smuts et al., 2011). However, the epidemiology of HRV-ABC detected in patients with ARI is unclear in Asian areas, including Japan.

HRV shows wide genetic divergence (Arakawa et al., 2012; Kiyota et al., 2013; Mizuta et al., 2010; Wisdom et al., 2009). Indeed, our previous research has suggested that HRV-A and -C detected in samples from Japanese patients have over 30% divergence based on sequences in the *VP4*/*VP2* coding region (Arakawa et al., 2012; Fujitsuka et al., 2011; Mizuta et al., 2010).These strains can be classified into many genotypes by phylogenetic analysis (Arakawa et al., 2012). However, evolution of the coding region is not fully understood.

The neighbor joining (NJ) method is frequently used in phylogenetic analysis to examine the molecular epidemiology of various viral genes (Kimura, 1980; Saitou and Nei, 1987). This method is based on a classification algorithm that allows for cluster analysis and reveals the viral evolutionary rate. Another technique, the Bayesian Markov chain Monte Carlo (MCMC) method, enables the time-scaled phylogeny of viral genes to be estimated (Lepage et al., 2007; Thorne et al., 1998). Many reports show that *VP4*/*VP2* in addition to *VP1* sequences are useful for the detection and genetic analysis of HRV (McIntyre et al., 2010; Simmonds et al., 2010; Wisdom et al., 2009). Thus, sequences from the *VP4*/*VP2* coding region can be used to identify HRV-C types among large datasets of *VP4*/*VP2* sequences (Simmonds et al., 2010).

Viral evolution depends on the biological features of the host cells and organisms (Domingo, 2006). Previous reports suggest that a representative event in the evolution of a virus may be due to positive selection pressure in the host (Domingo, 2006). Furthermore, a previous report showed that selective pressure changes of HRV-A and -B occur in both the structural (1B–D) and nonstructural (3C and D) genes (Kistler et al., 2007). Analysis of full-genome sequences shows that HRV-A and -C share a common ancestor, which is a sister group of HRV-B (Palmenberg et al., 2009). HRV-C is consistently more diverse than the other species but has a similar genetic distribution. The potential for recombination in HRVs has been evaluated and it is suggested that recombination events are largely located in the 5′UTR or adjacent capsid genes (Palmenberg et al., 2009). It is therefore important to analyze selective pressure regions and recombination events to gain a better understanding of the evolution of HRV.

Thus, here we conducted detailed genetic analyses of the time-scaled phylogenetics by MCMC method, genetic distances, selective pressures, and predicted recombination events in the *VP4*/*VP2* coding region of HRV-ABC strains in samples obtained from patients with ARI in Kumamoto prefecture, Japan.

2. Materials and methods

2.1. Samples and patients

Nasopharyngeal swabs (NPS; $n = 904$) and samples from tracheal aspiration ($n = 1$) and expectoration ($n = 1$) were collected from patients aged 0–91 years (3.0 ± 3.0 years; median ± the quartile deviation) with ARI. We diagnosed URI, also known as the common cold, which typically affects the upper airways, including the nose (sinusitis), throat (pharyngitis), and larynx (laryngitis) (Cherry, 2009a). Bronchitis was diagnosed in the presence of cough, rhonchi, and breath sounds (Cherry, 2009b). Wheezy bronchitis was diagnosed in the presence of wheezing alone or chest retractions in association with a URI (Welliver, 2009). Inflammation in the lung was diagnosed as pneumonia. Patients were diagnosed mainly with upper respiratory infection (URI) or lower respiratory infection (LRI; bronchitis, wheezy bronchitis, and pneumonia). Samples were obtained by local health authorities in Kumamoto prefecture for the surveillance of viral diseases in Japan (IASR 31: 69-70, March 2010, http://idsc.nih.go.jp/iasr/31/361/tpc361.html) between January 2011 and November 2012 and the detailed patient information is shown in Table 1. Informed consent was obtained from the subjects or their parents in the case of minors for the donation of samples.

Using cell culture methods (HEp-2, MDCK, Vero, and RD18S cells) and reverse transcription polymerase chain reaction (RT-PCR), we attempted to isolate and/or detect various respiratory viruses such as influenza virus, human parainfluenza virus (HPIV), adenovirus (AdV), respiratory syncytial virus (RSV), human metapneumovirus (HMPV), enterovirus (EV), and human bocavirus (HBoV), as described previously (Allander et al., 2005; Echevarría et al., 1998; Ishiko et al., 2002; Matsuzaki et al., 2009; Nakauchi et al., 2011; Sullender et al., 1993; Xu et al., 2000; Zhang and Evans, 1991).

2.2. RNA extraction, RT-PCR, and sequencing

Viral RNA was extracted from clinical samples using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). Reverse transcription was performed using a ReverTra Ace (Toyobo, Osaka, Japan), according to the manufacturer's instructions. Using cDNA, we performed the first PCR with the following primers: forward primer MD91 (5′-CCT CCG GCC CCT GAA TGC GGC TAA T-3′) and reverse primer OL68-B (5′-GGR AAY TTC CAC TAC CAN CC-3′). PCR was initiated at 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, with a final extension at 72 °C for 10 min. In addition, semi-nested PCR was carried out using the following primers: forward primer EvP4 (5′-CTA CTT TGG GTG TCC GTG TT-3′) and reverse primer OL68-B (Ishiko et al., 2002). Semi-nested PCR was initiated at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, with a final extension at 72 °C for 10 min. The semi-nested PCR products were purified with a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) and then sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), using the semi-nested primer sets above. Sequence analysis was performed on an ABI 3500 Genetic Analyzer (Applied Biosystems). The GenBank accession numbers of the nucleotide sequences obtained are AB824020 to AB824115.

2.3. Phylogenetic analysis by NJ method and genotyping of HRV-ABC

We performed phylogenetic analysis of all HRV strains detected in Kumamoto prefecture during the study period. Identical sequences similar to any other strains in this study were excluded from analysis to remove the possibility of outbreak bias. We performed alignment using MEGA 5.0 (http://www.megasoftware.net/) and phylogenetic analyses of the nucleotide sequences (623–1012 nt; 390 bp) of the *VP4*/*VP2* coding region using CLUSTAL W program available from the DNA Data Bank of Japan (http://clustalw.ddbj.nig.ac.jp/index.php?lang=ja) and MEGA 5.0. Sequences of reference strains were obtained from picornaviridae.com (URL:http://www.picornaviridae.com/; Table S1). It has been suggested that HRV-C strains can be type assigned on the basis of >10% divergence from other genotypes in the *VP4*/*VP2* coding region (Simmonds et al., 2010). Thus, in this study, available *VP4*/*VP2* sequences showing >10% divergence from other genotypes were type assigned.

2.4. Phylogenetic analysis of HRV-ABC by the Bayesian MCMC method

To estimate the evolutionary rate and phylogenetic time-scale and to genotype the present HRV-ABC strains and reference strains