Early Evolution of Human Respiratory Syncytial Virus ON1 Strains: Analysis of the Diversity in the C-Terminal Hypervariable Region of Glycoprotein Gene within the First 3.5 Years since Their Detection

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Key Words

Human respiratory syncytial virus · Molecular evolution · ON1 genotype · Glycoprotein gene · Variability

Abstract

Objective: Characterization of the phylogeny and diversity of human respiratory syncytial virus (HRSV) genotype ON1 that occurred during its early evolution (within the first 3.5 years since the detection of the first ON1 strains). ON1 strains have a 72-nucleotide-long in-frame duplication within the second hypervariable domain of the glycoprotein gene (HVR2). **Methods:** All available HVR2 sequences of strains belonging to the ON1 genotype published prior to June 20, 2014 were collected. Multiple sequence alignments, phylogeny, phylogeography, sequence clustering and putative protein analyses were performed. **Results:** The worldwide spread and diversification of ON1 strains are presented. Only in a minority of ON1 strains do the two replicas remain identical, and various ON1 strains possess common differences between the first and the second copy (segments A and B). Mutations of the progenitor sequence were more frequent in segment B, a higher overall diversity on the protein level and more putative glycosylation sites exist in segment B,

and, unlike in segment A, positive selection acts on that protein region. **Conclusions:** The fast spread of the novel HRSV genotype ON1 has been accompanied by its rapid concurrent diversification. Differences in variability of the two replicas within HVR2 were detected, with C-terminal replica being more variable.

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Introduction

Human respiratory syncytial virus (HRSV) is the most important viral agent causing severe lower respiratory tract infection in infants. Due to limited immune protection from earlier exposure, HRSV causes frequent reinfections, especially during early childhood [1].

HRSV belongs to the Pneumovirus genus of the Paramyxoviridae family. Strains are classified into two antigenic groups, A and B [2], representing separate genetic lineages whose divergence was calculated to have occurred 350 years ago [3]. The two groups co-circulate and are both responsible for epidemics, although group A usually dominates during epidemics [4].

HRSV phylogenetic studies are commonly based on sequences of the second hypervariable region of the glycoprotein (G) gene (HVR2), located at the protein's C-terminus. The G protein ectodomain consists of two large variable regions which have an extended, unfolded secondary structure [5] and contain several N- and O-linked sugar moieties [6]. The carbohydrate content of the G protein augments its molecular weight several times and possibly leads to its shielding from immune recognition [7].

To date, 15 HRSV-A (GA1–GA7, SAA1, SAA2, NA1–NA4, ON1 and CB-A) genotypes have been established, 4 of them in the last 4 years: ON1 [8], CB-A [9], NA3 [10] and NA4 [10]. The specificity of the ON1 genotype is that it has a 72-nucleotide (nt)-long in-frame duplication within the HVR2 domain. The first report about the appearance of ON1 strains was from Canada [8], where it was detected in December 2010. Shortly afterwards, its presence was reported in a number of Asian countries [11–15], in South Africa [16], Kenya [17] and in Europe [18–21]. A similar major genetic incident has previously happened in group B, where a 60-nt in-frame duplication has occurred also within HVR2 [22]. HRSV-B strains possessing this duplication, now classified as genotype BA strains, were first isolated in 1999 and within 4 years they had spread worldwide [23]. The BA genotype has increased in prevalence in every country analyzed so far. Almost 100% of the B strains identified after 2005–2006 are BA strains [24]. The cause of the remarkable BA genotype epidemiological success remains unknown. Similarly, the first epidemiological reports regarding ON1 (from Italy [25] and Korea [26]) show that its prevalence has increased dramatically within just 3 years since its first detection.

The detection of 4 different ON1 strains among clinical samples in Croatia between late 2012 and early 2013 prompted us to analyze sequences of all strains belonging to this genotype which were available in open public databases prior to June 20, 2014 (prior to a new winter HRSV season in Europe). It was our goal to investigate the versatility of this genotype in its early evolution within the first 3.5 years of its first detection.

Materials and Methods

Patients and Clinical Samples

The clinical samples from 1,102 inpatients (aged 0–5 years) with acute respiratory tract infections were collected from October 1, 2012 to April 1, 2013 in the Zagreb region. The study was approved by the Ethics Committees of the Croatian National Insti-

tute of Public Health. Children were included into the study after written consent had been obtained from their parents or caretakers. HRSV was confirmed in 318 inpatients' nasopharyngeal secretions using a direct fluorescence assay (DFA Light Diagnostics, Chemicon International, Temecula, Calif., USA). Sixteen HRSV samples were chosen for sequencing, based on the availability of nasopharyngeal secretions for RNA isolation.

RNA Isolation, RT-PCR and Sequencing

RNA isolation, RT-PCR and sequencing were performed as previously described by Forcic et al. [27]. Twelve different sequences were obtained and deposited in GenBank under accession Nos. KF057865–KF057868 (ON1 strains), KJ173827–KJ173833 (NA1 strains) and KJ173834 (BA9 strain).

Collections of ON1 and NA1 Sequences

Up until June 20, 2014, 248 sequences of HVR2 belonging to the ON1 genotype were identified in the PubMed database. Strains' names, sequences and all accession numbers are listed in online supplementary file 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000382018). The collection included strains detected in 13 different countries: Canada, Thailand, Malaysia, India, Japan, China, South Korea, South Africa, Kenya, Germany, Italy, Latvia and Croatia.

Similarly, a large compilation of sequences of HVR2 of strains belonging to the NA1 genotype was established. It contained sequences downloaded from the PubMed database, as well as 15 NA1 strains we identified in clinical samples collected from Croatian acute respiratory tract infection patients from 2006 to 2013. Out of this pool, 57 different sequences were chosen for analyses, based on the criterion that they were placed on different branches in phylogenetic analyses previously published by others. Strains' names, sequences and accession numbers are listed in online supplementary file 2.

Multiple Sequence Alignments, Phylogeny and Sequence Clustering

The genomic region used in analyses spanned bases 5,274 to 5,543 of the HRSV-A prototype strain A2 (GenBank accession No. M11486). The duplicated region in the ON1 strains corresponds to nts 5419–5490 of A2. The length of the analyzed fragment was 270 nts for non-ON1 strains and 342 nts for ON1 strains, with the exception of strain RSV-A/GG818/12 which has a 1-nt deletion within a second copy of the duplicated region (presented in 'Worldwide Spread and Diversification of ON1 Strains', Discussion and in article by Kim et al. [26]; it is the sole member of ON1 OS subgenotype).

In ON1 strains, the 72-nt-long duplicated segment is inserted after nt 865 of the G gene (the numbering corresponds to strain A2). It disrupts codons for amino acids (aas) 260 and 284, leads to the addition of 24 aas and creates a duplication of the 23 aa region. The total length of putative G protein in ON1 strains is 321 aas, as the triplet coding for the aa 322 is changed into a stop codon. Exceptions are strains RSV-A/GG818/12 and HD12248 (presented in 'Worldwide Spread and Diversification of ON1 Strains', 'N- and O-Glycosylation Sites Prediction' and in Discussion).

Alignments were performed using MEGA 6.06 and BioEdit 7.2.5 software. Phylogenetic trees were constructed using sequences of HRSV-A strains whose genotypes were previously determined and described. Phylogenetic trees were generated using the

Fig. 1. Phylogenetic analysis of HRSV-A strains constructed using the neighbor-joining method. The scale bar indicates the branch length equivalent to 0.01 base substitutions per site; the numbers on the branches are percentages of bootstrap values determined for 1,000 iterations (only values of at least 50% are shown). Strain designations are composed of GenBank accession No., name and genotype; strains belonging to genotype ON1 are shown in bold.

neighbor-joining method, p-distance model with MEGA 6.06 software. Bootstrap probabilities for 1,000 iterations were calculated to evaluate confidence estimates.

For sequence clustering, the CD-HIT Suite web server was used (<http://cd-hit.org>). The WebLogo program (<http://weblogo.berkeley.edu>) was used to generate graphical presentation of multiple sequence alignment (frequency pictogram). Ka/Ks [the ratio of the number of nonsynonymous substitutions per nonsynonymous site (Ka) to the number of synonymous substitutions per synonymous site (Ks)] was calculated using MEGA 5.10 software.

Analysis of Deduced Amino Acid Sequences and Potential N- and O-Glycosylation Site Prediction

Deduced aa sequences were generated by translating nt sequences with the standard genetic code using MEGA 6.06 software. Potential N-glycosylation sites were predicted by the NXT or NXS motif, where X is not a proline. Potential O-glycosylation sites were predicted using the NetOGlyc 4.0 program (<http://www.cbs.dtu.dk/services/NetOGlyc/>).

Results

As a starting point of our analysis, 342-nt-long sequences of all 248 available ON1 records were compared. Ninety-two different sequences were identified: some were found as unique submissions, while others were submitted multiple times. In analyses when just a single strain from each set of identical sequences was included, we refer to those representatives as entries.

ON1 Phylogeny

Phylogeny of the ON1 strains shows that they evolved from genotype NA1, the same as the strains belonging to CB-A and NA3. All of these genotypes group within a common cluster supported with a bootstrap value of 81% (fig. 1). In order to rule out possible disturbances introduced by genetic duplication, we performed the analysis using sequences of all 92 entries in which the first or the second copy of duplicated segment was excluded (online suppl. fig. 1 and data not shown, respectively). The clustering and positioning of ON1 strains remained unchanged, confirming that placement of ON1 strains within a phylogenetic tree is not primarily based on the duplication event.

The borders of the duplicated segments are the same in all ON1 strains, suggesting that they share a common ancestor and originate from a single duplication event. In phylogenetic analysis that included solely NA1 and ON1 entries, NA1 strains ON201-0111A detected in January 2011 in Canada and CU2011-182 detected in October 2011 in Thailand were found to be the most similar to genotype ON1 (fig. 2). The NA1 strains included in analysis were detected worldwide in the years 2004–2013.

Fig. 2. Phylogenetic analysis of 57 differing NA1 strains and 92 differing ON1 strains constructed using the maximum likelihood method. The scale bar indicates the branch length equivalent to 0.005 base substitutions per site. Only the positions of NA1 strains ON201-0111A and CU2011-182 are indicated.

ON201-0111A and CU2011-182 differ only in one nt, nt 836 of the G gene (T in ON201-0111A and C in CU2011-182), which is in a region that is duplicated in ON1 strains (at position 43 in the C-terminal replica). This T↔C transition in ON1 strains is further analyzed in ‘Analysis of the Duplicated Segment’.

Worldwide Spread and Diversification of ON1 Strains

Similar to other HRSV genotypes [3, 28], the appearance of ON1 strains has been followed by their fast global dissemination – viruses with identical sequence were detected in geographically distant places (table 1), confirming high virulence potential of the new genotype. The most widely distributed are strains that have the same sequence as the first reported Canadian strain ON67-1210A. Such strains were identified in Malaysia, Thailand, Japan, Germany, Italy, Latvia, South Africa and Kenya. Detection of the same strain in 2 or more different countries has been observed for 6 other ON1 sequences (table 1).

Table 1. ON1 strains with identical sequences in the second variable region of G gene, detected in different countries

Strain name	GenBank accession No.	Country	Group
ON67-1210A*	JN257693	Canada	1
RXH/ON1/004*	JX885733	South Africa	1
WUE/13373/12*	JX912356	Germany	1
LV/029/12	KF030154	Latvia	1
CU2011/118*	KC342415	Thailand	1
Yamaguchi/Yu270/2012	AB808779	Japan	1
MY-2444006-11	JX256871	Malaysia	1
13212AN/2012*	JX988440	Italy	1
KEN/Kilifi/114165/23-Jul-2012*	KF587987	Kenya	1
WUE/10293/12	JX912355	Germany	2
HRSV-A/US469/11*	AB860237	South Korea	2
RSV/Yokohama.JPN/P6587/2012	AB761611	Japan	2
RSV-A/NIV1114046/11	KC731482	India	3
Chiba-C/24226*	AB808757	Japan	3
KEN/Kilifi/115590/27-Oct-2012*	KF587964	Kenya	3
RXH/ON1/002*	JX885731	South Africa	4
HD12244	KJ710374	Germany	4
1251-96RM	KC858246	Italy	5
HD11076	KJ710402	Germany	5
1307-593AN*	KC858239	Italy	6
HD11065	KJ710403	Germany	6
Yamaguchi/TK128/2012	AB808778	Japan	7
HD12214	KJ710376.1	Germany	7

Seven groups of identical strains were identified, marked as groups 1–7. In cases where multiple identical sequences from the same country were deposited in GenBank, a single strain was chosen as a representative (marked with asterisk).

The fast spread of the ON1 genotype has been accompanied by its rapid concurrent diversification – 92 different ON1 nt entries (resulting in 80 different putative aa entries) were identified within the first 3.5 years of their detection. The overall diversity of the analyzed fragment in ON1 entries (calculated as the average number of base or aa differences per site) and its Ka/Ks value are only slightly lower than numbers obtained for the NA1 genotype (table 2), although 57 NA1 entries included in our analysis were representatives from a period that was more than twice as long (2004–2013).

Out of all the ON1 strains published so far, mutations causing differing G protein length have only been detected in two strains. In strain RSV-A/GG818/12, a deletion of the 49th nt occurred in the second copy of the duplicated segment. This deletion caused a frameshift from aa 300 and elongation of G protein for 3 aas. The other case

is strain HD12248 in which G protein was shortened for 10 aas. This corresponds to the stop codon at aa position 288 in non-ON1 strains.

Analysis of the Duplicated Segment

In 92 differing entries, we split the region containing the 72-nt duplication into A and B segments (A is closer to the 5' end and B is closer to the 3' end of viral cDNA) and analyzed whether some specificities for each replica can be found. The consensus sequence for both segments was the same (fig. 3). When the Ka/Ks value was used as an indicator of selective pressure, contrasting results were obtained (table 2): segment B's Ka/Ks was 4.8-fold higher than the ratio obtained for segment A, indicating that different selections act on the two replicas.

Identical sequences of segments A and B were detected in 6 entries (represented by strains ON67-1210A from

Fig. 3. Frequency pictogram of a 72-nt-long duplicated region in the ON1 strains, based on 92 differing ON1 strains. The upper panel shows a sequence of segment A and the lower panel shows a sequence of segment B (A is closer to the 5' end and B is closer to the 3' end of viral cDNA). The asterisks mark the positions that are 100% conserved in both segments, horizontal brackets show codon

positions, white triangles show positions of amino acids that are conserved in all sequences (T264/288, T269/293 and S270/294), and the black triangle shows the position of Q278/302 which is conserved in all strains except in segment B of strain RSV-A/GG818/12.

Table 2. The overall diversity calculated as average number of nt or aa differences per site and Ka/Ks values determined for 57 different NA1 strains and 92 different ON1 strains

	Overall diversity, %		Ka/Ks
	nt	aa	
NA1	2.8	4.6	0.66
NA1 segment	2.5	6.0	1.41
ON1	2.2	4.2	0.81
ON1 segment A	3.0	4.6	0.38
ON1 segment B	2.8	6.5	1.83

Analyzed fragments spanned the following regions: 5274–5543 for NA1, 5419–5490 for NA1 segment (both numberings correspond to strain A2, GenBank accession No. M11486), 5284–5625 for ON1, 5429–5500 for ON1 segment A, and 5501–5572 for ON1 segment B (the last 3 numberings correspond to strain RSV-A/NIV1114046/11, GenBank accession No. KC731482).

Canada, Wue/5577/12, HD12206 and HD12307 from Germany, and CU2011/91 and CU2011/208 from Thailand). All 6 possess identical sequences of the duplicated segment, termed progenitor sequence, identical to the segments' consensus. The progenitor sequence was detected in 28 other entries in one of the segments (either A or B). The A and B segments in other entries differed from the progenitor sequence in 1–5 nts, and in 1–8 nts mutually (i.e. within the same entry). Overall, 60 different 72-

nt sequences were detected with 28 nt positions fully conserved in all sequences in both the A and B segments. In a 23-aa-long duplicated segment, only 3 aas were found to be 100% conserved in all strains in both replicas: aas T264/288, T269/293 and S270/294. Amino acid Q278/302 was conserved in all strains except in segment B of strain RSV-A/GG818/12 (fig. 3).

In 46 entries (50.0%), a T43C mutation was found in both segments. The same mutation was present in 3 additional A segments and 6 B segments. This mutation leads to L274P (or L298P in case the mutation occurred in segment B).

In 47 entries (51.1%), T60C mutation was found in a B segment, leading to Y304H. The same mutation was found only once in the A segments.

N- and O-Glycosylation Site Prediction

Within the 110-aa-long segments in the ON1 strains which we analyzed, the glycosylation sites located outside of the duplicated region were highly conserved. Seven sites were detected and each of them was present in over 87% of the entries. Namely, out of 80 different protein entries, the N-glycosylation site at N237 was present in 79 and at N318 in 77. O-glycosylation sites at T219, T220, T227, T228 and T231 were present in 72, 70, 72, 72 and 70 protein entries, respectively.

Within duplicated segments, additional glycosylation sites were identified (fig. 4), but only in 19 strains (out of 248; 7.7%): a single putative N-glycosylation site (present

Fig. 4. Alignment of deduced sequences of HVR2. Alignment is shown relative to the sequence of the ON67-1210A strain. Besides ON67-1210A, sequences of 19 strains possessing putative glycosylation sites in the duplicated region are shown. Identical residues

are indicated by dots; stop codons are indicated by asterisks. Potential N- and O-glycosylation sites are indicated by dotted lines and gray rectangles, respectively. The two copies of the duplicated 23-aa region are indicated by full-line rectangles.

in 2 strains) and 5 putative O-glycosylation sites whose positions differed between the 2 replicas. The progenitor sequence does not possess any glycosylation sites within the 2 replicas.

In strain HD12248, in which the putative G protein was shortened by 10 aas, this truncation also influenced its predicted glycosylation status – the serines at positions 299 and 307 were not predicted to be used for glycosylation, as they would be if the length of G protein was 321 aas. ON1 strains both with and without predicted sugar moieties attached to these serines have been detected in the area around Heidelberg, Germany in 2012–2013.

In strain RSV-A/GG818/12, the mutation that caused the change in the protein's C-terminus did not change its predicted glycosylation status.

Discussion

HVR2 is commonly sequenced and has been used in investigations of genetic diversity of HRSV strains since 1998 [29]. Although it accounts for approximately only 2% of the whole HRSV genome, it has been identified as the most reliable to describe the evolutionary changes of HRSV [29, 30]. Genotype ON1 was shown to have evolved from NA1, the dominant HRSV A genotype in a number of molecular epidemiologic studies from 2007 to 2012 [8, 15, 18, 19, 27, 31, 32].

The capacity of the G protein to accommodate large insertions within HVR2 and epidemiological dominance

of strains possessing such an insertion was previously demonstrated with the HRSV-B genotype BA. BA strains do not cause more serious clinical cases and the reasons for selective advantage of the BA genotype still remain elusive. Similar to data presented by Tabatabai et al. [33], our patients' records indicate that ON1 strains do not lead to more severe illness. Panayiotou et al. [20] concluded that young children infected with ON1 suffered a milder disease compared to infections with genotypes BA or GA2, and a similar observation was also made by Balmaks et al. [18]. Based on the first epidemiological studies after ON1 appearance [25, 26], it is likely that epidemiological dominance of ON1 among HRSV-A strains will also occur.

Although all of the NA1 strains reported so far possess a 297-aa-long G protein, 2 strains with differing G protein length have already appeared within the ON1 pool (similar situation was observed within BA genotype, which is also characterized by G protein length polymorphism [24]). The mutation observed in strain RSV-A/GG818/12 [26], whose G protein is elongated for 3 aas, has not been described for other non-ON1 HRSV-A strains. On the contrary, strain HD12248 possesses shorter G protein. Non-ON1 strains possessing a stop codon at the corresponding position have been observed sporadically during certain epidemics [28, 34, 35].

The two 72-nt replicas remained identical (i.e. likely unmutated) in only a few of the ON1 entries. The evolutionary rate for the ON1 genotype based on HVR2 sequences was calculated to be 7.2×10^{-3} nt/site/year by Ren

et al. [36] and 7.92×10^{-3} nt/site/year by Balmaks et al. [18], which is higher than the value previously reported for the HVR2 segment of BA strains (4.7×10^{-3} nt/site/year [24]). We found that there are considerable differences in the variability of the two replicas: mutations of the progenitor sequence were more frequent in segment B, a higher overall diversity on the protein level exists in segment B, and, unlike in segment A, positive selection acts on that protein region. Whether there are structural reasons that restrict variability in segment A remains to be determined.

Within the duplicated segment, two mutations are present in very high percentages: T43C and T60C. Both of them are within codons for aa positions previously identified to be under positive selection in HRSV-A strains [34]. The mutation T43C leads to aa change L274P (or L298P if it occurred in segment B). This aa change exhibits a flip-flop pattern (tends to reverse over time [34]), probably reflecting the changes in the immune status of the human population.

Generally, N- and O-glycosylation of the G protein differs in number and location among HRSV strains. Our analysis has shown that locations of putative glycosylation sites within HVR2 of ON1 strains are quite uniform,

being very conserved outside of the duplicated region. Within duplicated segments, only a minority of strains are predicted to be glycosylated. More putative glycosylation sites have been recognized in segment B, adding to previously pointed conclusion that the second replica of the duplicated segment is more variable.

In conclusion, in this article we used HVR2, and especially the duplicated segment, as a natural tag for investigation of ON1 early molecular evolution in its natural host. We presented data regarding phylogeny and fast global dissemination of this genotype that has been accompanied by its rapid concurrent diversification. In particular, we found that there are differences in evolution of the two replicas of the 72-nt segment, C-terminal replica being substantially more variable.

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