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BRIEF REPORT

Molecular epidemiology of noroviruses in children and adults with acute gastroenteritis in Wuhan, China, 2007-2010

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Abstract To study epidemiological features and genetic characteristics of noroviruses in children and adults with acute gastroenteritis, fecal specimens were collected in three hospitals from Jan. 2007 to May 2010 in Wuhan, China. Noroviruses were detected in 25.9 % (286/1103) and 24.6 % (202/822) of the specimens from children and adults, respectively, with genogroup II (GII) being predominant (99.2 %). The most frequent genotype among GII strains was GII.4 (2006b variant) (77.3 %) (72.0 % in children and 87.9 % in adults), followed by GII.3 (15.0 %) and GII.6 (3.4 %). Potential recombinant genotypes (polymerase/capsid) were detected in 51 GII strains (15.9 %), including the most frequent type, GII.12/GII.3 (28 strains), and GII.16/GII.2, detected for the first time in China, which were found in only children. The results indicated that genetically similar noroviruses were circulating among children and adults as a cause of gastroenteritis, except for some recombinant genotypes.

Keywords Norovirus · Children · Adults · Genotype · Recombination

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Norovirus is one of the important causes of acute gastroenteritis in humans worldwide [18, 27]. Noroviruses, are members of the family *Caliciviridae* and possess a linear, positive-sense, single-stranded RNA genome [11]. The genome is organized into three open reading frames (ORFs), ORF1, 2, and 3, which encode a polyprotein including RNA-dependent RNA polymerase (RdRp), a major capsid protein, and a small capsid protein, respectively [11]. Noroviruses are classified into six genogroups, of which genogroups I, II and IV are found in humans, with genogroup II (GII) being predominant. Genogroups are further subdivided into genotypes. To date, 31 genotypes of RdRp and 21 genotypes of the capsid gene of GII noroviruses have been determined [19]. Recombination of norovirus genomes is believed to occur in nature at high frequency, mostly in the junction region of ORF1 and ORF2, which causes more genetic divergence of noroviruses [4]. Therefore, it is possible that the genotype of the RdRp and capsid genes are different due to recombination.

Although molecular epidemiological studies have been conducted in many locations throughout the world, data from of norovirus infections in all age groups in the same study setting are limited [16, 26, 27]. Understanding the differences in epidemiological features in different age groups, i.e., prevalence, seasonality, susceptibility to different genotypes of norovirus, will be helpful for us for

Table 1 Primers used for RT-PCR and sequencing in this study

Primer	Target gene	Genogroup	Polarity	Sequence (5'-3')	Nucleotide position[b]	Purpose	Reference
JV12	RdRp[a]	GI&GII	+	ATACCACTATGATGCAGATTA	4278	RT-PCR & sequencing	[35]
JV13	RdRp	GI&GII	−	TCATCATCACCATAGAAAGAG	4605	RT-PCR & sequencing	[35]
GII.7JV13	RdRp	GII	−	TCATCGTCCCCATAAAATGAA	4605	sequencing	this study
GII.16JV13	RdRp	GII	−	TCGTCATCACCGTAAAATGAG	4605	sequencing	this study
GII.17JV12	RdRp	GII	+	CTACCATTATGATGCAGACTA	4278	sequencing	this study
G1SKF	Capsid	GI	+	CTGCCCGAATTYGTAAATGA	5342[c]	RT-PCR & sequencing	[17]
G1SKR	Capsid	GI	−	CCAACCCARCCATTRTACA	5671[c]	RT-PCR & sequencing	[17]
G2SKF	Capsid	GII	+	CNTGGGAGGGCGATCGCAA	5046	RT-PCR & sequencing	[17]
G2SKR	Capsid	GII	−	CCRCCNGCATRHCCRTTRTACAT	5389	RT-PCR & sequencing	[17]
G2B	Capsid	GII	+	TGGAGGGCGATCGCAATCT	5048	RT-PCR & sequencing	this study

[a] RNA-dependent RNA polymerase

[b] Location of the 5' end of the primer in the nucleotide sequence of Lordsdale virus (X86557)

[c] Location of the 5' end of the primer in the nucleotide sequence of Norwalk virus (M87661)

providing efficient infection control measures for the population. In central China, few studies have analyzed the epidemiology of norovirus infections [15, 37]. To compare the epidemiological and genetic characteristics of noroviruses in children and adults with acute gastroenteritis, we conducted a hospital-based study of norovirus in Wuhan, a metropolitan city located in central China with a population of ten million, from January 2007 until May 2010.

Fecal specimens were collected from children (<15 years old, the age limit in the pediatric clinic in Wuhan) and adults (≥15 years old) with acute diarrheal disease in Wuhan Children's Hospital, Wuhan Commercial Staff Hospital and Wuhan the Sixth Hospital from January 2007 through May 2010. Fecal specimens were analyzed as part of routine surveillance that had been approved by the ethics committee of Wuhan Centers for Disease Prevention and Control. Oral informed consent was obtained from the patients or guardians for all samples collected.

Viral RNA was extracted from 250 μl of 10 % stool suspension using a Total RNA Isolation Kit (SBS Genetech Co., Ltd). Noroviruses were screened by RT-PCR using a QIAGEN One Step RT-PCR Kit and primers G1SKF, G1SKR, G2SKF and G2SKR (Table 1) [17] to amplify a portion of the capsid genes (330 bp and 343 bp for GI and GII, respectively). For the norovirus-positive specimens, a partial RdRp gene sequence (328 bp) was amplified by RT-PCR with JV12 and JV13 primers (Table 1) [35]. The nucleotide sequences of partial capsid and RdRp genes were determined directly from PCR products. Genotypes of noroviruses were preliminarily assigned using BLAST and then confirmed using a web-based genotyping tool (http://www.rivm.nl/mpf/norovirus/typingtoolversion1.0) [19], together with phylogenetic analysis with reference strains (Supplementary Table S1). Phylogenetic and molecular evolutionary analyses were conducted using the MEGA program, version 5 [34]. A phylogenetic tree was generated by the neighbor-joining algorithm using the Kimura two-parameter model. The phylogenetic trees were supported statistically by bootstrapping with 1000 replicates.

For potential recombinant genomes of noroviruses, to exclude cases of mixed infection, the nucleotide sequence covering the junction between RdRp and capsid was amplified by RT-PCR using primers JV12 and G2SKR (Table 1), and the PCR products were sequenced directly. Recombination analysis was conducted using the SimPlot program (v 3.5) [24]. Sequences of the RdRp gene (22 strains), capsid genes (17 strains), and RdRp and capsid genes with junction sequences (42 strains) determined in the present study were deposited in the GenBank database under accession numbers JQ750975-JQ751055.

Norovirus was detected in 25.4 % of the specimens (488/1925): 25.9 % in children (286/1103) and 24.6 % in adults (202/822). Detection rates for norovirus were higher in the 7- to 24-month-old (30-34 %) and 50- to 59-year-old (33 %) age groups than in the other age groups (Table 2(A)). Incidence of norovirus in young children not exceeding 24 months accounted for 58 % of noroviruses

Table 2A Prevalence of norovirus in patients with acute gastroenteritis in Wuhan, China: Epidemiological profile of norovirus diarrhea

Parameter	No. of specimens[a]	Norovirus-positive specimens (detection rate [%])
Gender[b]		
Male (≤5y)	677	185 (27.3)
Female (≤ 5y)	357	89 (24.9)
Male (all ages)	1043	278 (26.7)
Female (all ages)	752	180 (23.9)
Age group[c]		
≤6 m	445	88 (19.8)
7-12 m	397	136 (34.3)
13-24 m	152	46 (30.3)
25 m-5y	48	7 (14.6)
6-14y	38	5 (13.2)
15-19y	15	2 (13.3)
20-29y	102	24 (23.5)
30-39y	75	19 (25.3)
40-49y	140	30 (21.4)
50-59y	173	57 (32.9)
60-69y	86	21 (24.4)
70y <	118	31 (26.3)

[a] Specimens for which age and/or gender data were not obtained were excluded

[b] $P > 0.05$

[c] $P < 0.01$

detected in all of the age groups. Almost equal detection rates were found between male patients and female patients, in all age groups as well as in young children (<5 years). During the study period, norovirus was detected throughout the year in both children and adults (Supplementary Fig.S1). Although a higher incidence of norovirus was observed mostly in winter (November to February) in adults, no apparent seasonality was seen in children, with peaks in July, September, and February, depending on the year. In some studies in China, an increase of norovirus gastroenteritis in children as well as in adults has been described to be associated with cold and dry weather [7, 10, 22]. Our present study showed that seasonality of norovirus prevalence is not necessarily observed concurrently between children and adults, even at the same study site. It is suggested that the year-round prevalence of norovirus may facilitate viral transmission among the population, causing variable incidence in children and adults depending on the season. This observation contrasts with what was observed for rotavirus infections in Wuhan in our previous study, in which high detection rates of rotaviruses in winter were observed in both children and adults [36].

Higher incidence of norovirus gastroenteritis in children than in adults has been described in the Netherlands [1], while the opposite finding was reported in Sweden [21]. Although the reason of such inconsistency in incidence of norovirus in children and adults is not definite, it is possible that the incidence in adults may be affected by dominant age groups in the study population, because the norovirus-positive rate in adults varies considerably depending on age, as observed in the present study. Furthermore, the difference in distribution of histo-blood group antigens in different age groups may be related to the age dependence of norovirus incidence, which remains to be investigated.

Among the norovirus detected, GII and GI were identified in 99.2 % (484/488) and 0.8 % (4/488) of the specimens, respectively. Further genetic analysis of partial RdRp and capsid gene sequences was performed for 321 specimens (214 from children, 107 from adults) that were randomly selected from each year (Table 2(B)). Three out of the four GI strains was assigned to GI.4/GI.4. The most frequent genotype (capsid) among GII strains was GII.4 (77.3 %) (72.0 % in children and 87.9 % in adults), followed by GII.3 (14.6 %) and GII.6 (3.4 %). A potential recombinant genotype (polymerase/capsid) was detected in 51 GII strains (15.9%), including the most frequent type GII.12/GII.3 (28 strains). GII.4 (RdRp)/GII.4 (capsid) was predominant in both children and adults in individual years during the study period. Most of genotype GII.3 (capsid) (46/47), including the recombinant genotype GII.12/GII.3, were detected in children (Fig. 1A). GII.b/GII.3 and GII.16/GII.2 were identified only in children, while GII.4/GII.3 and GII.4/GII.6 were found in both children and adults.

Partial RdRp and capsid gene sequences along with the junction between RdRp and capsid (approx. 1100 bp) were determined for GII.12/GII.3 (n = 22), GII.4/GII.4 (n = 11), GII.b/GII.3 (n = 3), GII.16/GII.2 (n = 3), GII.7/GII.14 (n = 1), GII.7/GII.7 (n = 1) and GII.17/GII.17 (n = 1) strains. Within a single genotype, the nucleotide sequence identity of these sequences was 97-100 % (data not shown). Using BLAST and construction of phylogenetic trees of partial RdRp (328 bp) and capsid (324 bp) gene sequences, the GII.4/GII.4, GII.12/GII.3, GII.7/GII.14 and GII.7/GII.7 strains detected in Wuhan clustered with strains detected in Japan, Korea, Australia and China during 2006-2010, showing 97-99 % nucleotide sequence identity (Fig. 1B and C). Both the RdRp and capsid genes of the GII.4/GII.4 strains in Wuhan clustered with GII.4 variant 2006b strains. Among GII.4/GII.4 noroviruses from children and adults, sequence identities of the partial RdRp gene as well as partial capsid gene were 96-100 %. The GII.3 (capsid) strains clustered with those found in India and Australia and had nucleotide identities of 94-99% to those detected in Asia, Europe and America. The RdRp and

Table 2B Prevalence of norovirus in patients with acute gastroenteritis in Wuhan, China: Incidence of genotypes (polymerase and capsid) of noroviruses in children and adults from 2007 to 2010

Genotypes based on RdRp / capsid sequences	2007		2008		2009		2010		Total (%)		Total (%)
	Ch	Ad	Ch	Ad	Ch	Ad	Ch	Ad	Ch	Ad	
GII.4 2006b / GII.4 2006b	23	30	56	34	53	26	22	4	154(72.0)	94(87.9)	248(77.3)
GII.12 / GII.3	4	0	8	0	15	0	1	0	28(13.1)	0(0.0)	28(8.7)
GII.4 2006b / GII.3	0	0	2	0	3	0	2	1	7(3.3)	1(0.9)	8(2.5)
ND / GII.6	1	2	0	1	2	0	1	0	4(1.9)	3(2.8)	7(2.2)
ND/ GII.3	0	0	2	0	3	0	1	0	6(2.8)	0(0.0)	6(1.9)
GII.b / GII.3	0	0	0	0	1	0	4	0	5(2.3)	0(0.0)	5(1.6)
GII.4 2006b / GII.6	1	2	0	0	0	0	0	0	1(0.5)	2(1.9)	3(0.9)
GII.16 / GII.2	0	0	0	0	0	0	3	0	3(1.4)	0(0.0)	3(0.9)
GII.17 / GII.17	0	1	0	0	0	0	0	0	0(0.0)	1(0.9)	1(0.3)
GII.b / GII.4 2006b	0	0	0	1	0	0	0	0	0(0.0)	1(0.9)	1(0.3)
GII.6 / GII.6	0	0	0	1	0	0	0	0	0(0.0)	1(0.9)	1(0.3)
GII.4 2006b / GII.12	0	0	0	0	0	1	0	0	0(0.0)	1(0.9)	1(0.3)
GII.12 / GII.12	0	0	0	0	0	1	0	0	0(0.0)	1(0.9)	1(0.3)
GII.7 / GII.14	0	0	1	0	0	0	0	0	1(0.5)	0(0.0)	1(0.3)
ND / GII.14	0	0	0	0	1	0	0	0	1(0.5)	0(0.0)	1(0.3)
GII.4 2006b / GII.7	0	0	0	0	0	0	1	0	1(0.5)	0(0.0)	1(0.3)
GII.7 / GII.7	0	0	0	0	0	0	1	0	1(0.5)	0(0.0)	1(0.3)
GI.4 / GI.4	0	1	1	1	0	0	0	0	1(0.5)	2(1.9)	3(0.9)
ND/ GI.3	0	0	0	0	1	0	0	0	1(0.5)	0(0.0)	1(0.3)
Total no. genotyped	29	36	70	38	79	28	36	5	214(100)	107(100)	321(100)
Total no.of norovirus-positive samples	51	93	98	68	95	36	42	5	286	202	488

RdRp=RNA-dependent RNA polymerase, Ch=children, Ad=adults, ND=not determined

capsid genes of those GII.12/GII.3 strains also clustered with the noroviruses detected in oysters and ark clams in Guangdong, Shandong and Taiwan. The GII.16/GII.2 strains clustered with GII.16/GII.2 strains found in Japan in 2008-2011, with sequence 93-99% identity. Representative strains with potential recombinant genotypes of GII.b/GII.3, GII.16/GII.2 and GII.12/GII.3 (E1779, E2116 and Z791, respectively) were analyzed using Simplot software. These strains were confirmed to have resulted from recombination of different genotypes at the junction site of ORF1 and ORF2 (Fig. 1D, Supplementary Fig. S2).

All of the GII.4/GII.4 strains detected in Wuhan from 2007 through 2010 belonged to the GII4-2006b variant. This variant strain, known also as "Lincoln House/2006/UK", was first detected in Europe as a causative agent of gastroenteritis outbreaks on cruise ships in February 2006 [33] and thereafter became associated with global epidemics [9, 22]. In China, since its first identification in July 2006, GII.4-2006b has been reported to be a predominant genotype in outbreaks and sporadic cases [7, 12, 15, 23]. The present study revealed the GII.4-2006b variant to be the predominant strain in both children and adults in Wuhan from 2007 until 2010, suggesting the rapid spread and persistence of this virus in the population.

Of the recombinant genotypes of noroviruses identified in the present study, GII.16/GII.2 has never been reported in China. All of the GII.16/GII.2 strains in Wuhan were detected in 2010 and were closely related to those that were highly prevalent in Japan during 2009-2010 [14]. Therefore, it is possible that the GII.16/GII.2 strains in Wuhan were derived from the Japanese epidemic strains, and it will be of significance to survey the distribution and spread of this recombinant strain in China.

GII.3 (capsid) norovirus infection can be traced back to 1975 [2]. GII.3 (capsid) was reported as a dominant genotype in Malawi [8], Japan [28], Australia [25] and Canada [20]. It was associated with nosocomial spread more often in children [32]. GII.12/GII.3 norovirus infections have been reported mainly in China, Japan, Korea and Australia [5, 12, 13, 25, 30]. In the present study, GII.12/GII.3 was found as the most frequently detected recombinant type. Thus, it is suggested that the GII.12/GII.3 strains may be primarily distributed to the Asia-Pacific region. Another recombinant type, GII.b/GII.3 (C14/02/AU-like recombinant), has been also reported as the pediatric genotype in several countries [3, 6, 29]. Similarly, in the present study, GII.b/GII.3 noroviruses as well as GII.12/GII.3 were found only in children, suggesting that children,

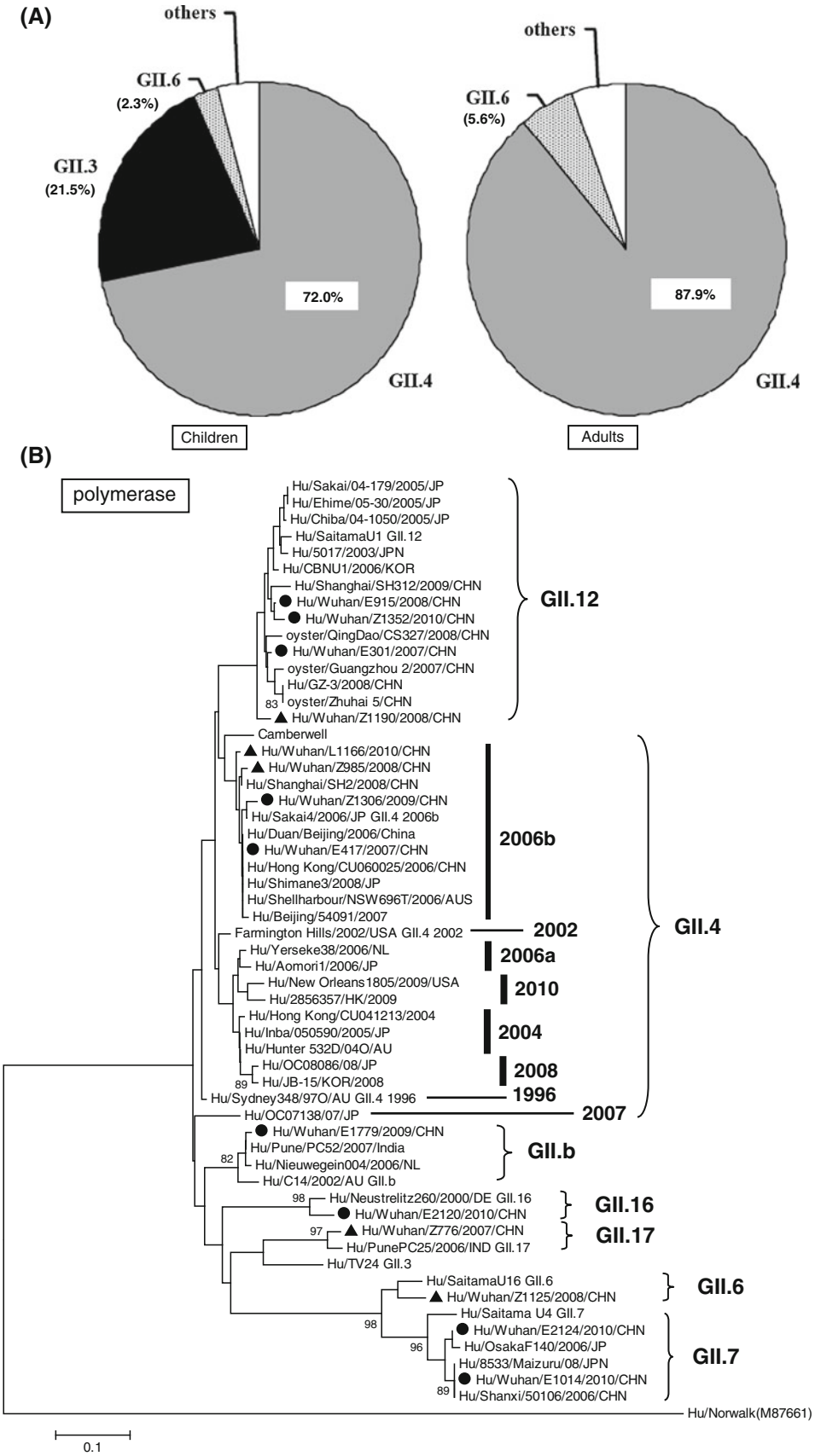
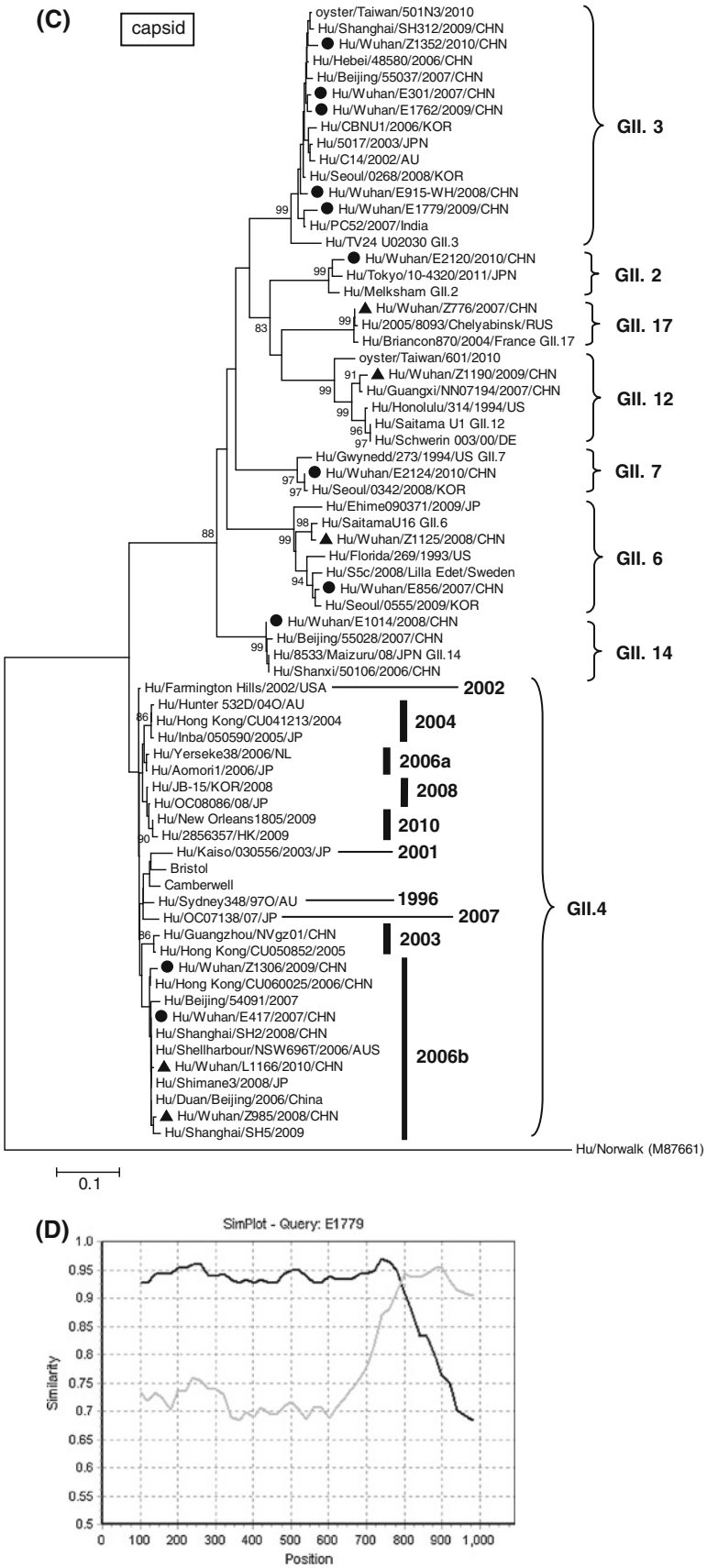


Fig. 1 Genetic characterization of noroviruses detected in Wuhan, China. (A) Proportion of norovirus genotypes (capsid) in children and adults. Genotypes with detection rates of less than 2 % are included in "others". (B), (C); Phylogenetic dendrogram of the partial RdRp region (328 bp) (B) and capsid region (324 bp) (C) of GII noroviruses detected in Wuhan and reference strains. Norovirus strains from children and adults are denoted by closed circles and triangles, respectively. Only strains for which there was a complete sequence for the 328-bp or 324-bp region were included. The bootstrap values generated from 1000 replicates are shown at nodes, and only bootstrap values $\geq$80 % are shown. The scale bar represents 0.1 substitutions per nucleotide position. Clusters of individual genotypes are shown on the right side of the dendrograms, and GII.4 variants are indicated within the GII.4 cluster. (D) Similarity plots of GII recombinant norovirus strain E1779 (GIIb/GII.3) found in this study. Similarity plots were performed using SimPlot version 3.5 with a slide window width of 200 bp and a step size 20 bp. SimPlot analysis was conducted with 1093-bp sequences fragments including part of the RDRP and capsid genes. Black and gray lines indicate similarity to PC03/2006/IND (GIIb/GII.21, EU019230) and TV24 (GII.3, U02030), respectively

Fig. 1 continued



for unknown reasons, are susceptible to recombinant norovirus with the GII.3 capsid genotype. It may be of significance to further analyze the infectivity and transmission modes of GII.3 noroviruses.

It was unexpected that GII.12, GII.7, and GII.17 (RdRp) strains detected in the present study in Wuhan had 97 %-99 % nucleotide sequence identity in their RdRp genes to those reported previously as GII.4, GII.6, and GII.3 (RdRp), respectively [5, 12, 13, 31]. The RdRp-based genotypes in those reports are considered to be misclassified, probably due to inappropriate selection of reference strains in the phylogenetic analysis. For the exact assignment of genotypes and uniformity of genotype classification, it is recommended to use the web-based genotyping tool [19] and appropriately chosen reference strains for phylogenetic analysis.

In the present study, it was revealed that GII.4-2006b variant noroviruses were circulating among children and adults as the predominant strain in Wuhan. However, recombinant GII.12/GII.3 and GII.b/GII.3, as well as the emerging GII.16/GII.2 norovirus, are considered significant as pediatric pathogens. Since the epidemiology of norovirus strains may change rapidly, with viral genomic evolution generating new variants, continuous surveillance may be necessary for understanding the current status of norovirus infections in the population.

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