Isolation, Identification and Genetic Characteristics of an Uncommon Norovirus Genotype, GIX.1[GII.P15], From Kunming, China

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Abstract

Human norovirus, an RNA virus of the family *Caliciviridae*, is a common viral pathogen causing acute gastroenteritis of all age groups worldwide. To date, tens of thousands genome sequences of norovirus have been uploaded to NCBI database, more than half of them were epidemic strains of GII.4 or GII.17 genotype. However, sequence information on the non-epidemic norovirus strains remains poorly studied. In this study, an uncommon norovirus genotype, GIX.1[GII.P15], was isolated using Raji cells and the full-genome sequence of the strain was extensively characterized. The norovirus virus particles with a diameter of approximately 30 nm and a morphology of spherical and lace-like appearance were observed by electron microscopy. Viral genome replication in Raji cells were confirmed by real-time quantitative reverse transcription-PCR from viral replication kinetics and passaging experiments of the primary virus. Phylogenetic analysis showed that the strain (KMN1) belonged to the GIX.1[GII.P15] genotype and indicated that no recombination has occurred in this strain thus far. Further compared analysis of the full genome sequence with the consensus sequence of GIX.1[GII.P15] genomes revealed a total of 81 nucleotide substitutions (53 in ORF1, 20 in ORF2, and 8 in ORF3) across the genome, but only 6 substitutions resulted in amino acid changes (3 in ORF1, 1 in ORF2, and 2 in ORF3). Moreover, one amino acid substitution at the 302 amino acid site (P302S) was observed in the P2 domain of the capsid protein, and the site was around one of the predicted conformational epitopes on the VP1 protein structure. The genomic information obtained from the novel strain may extend the understanding of the non-epidemic GIX.1[GII.P15] strains.

Background

Human norovirus (HuNoV) is a member of genus norovirus in the family *Caliciviridae*, and it is one of the most common enteric pathogens causing epidemic gastroenteritis in humans of all ages [23, 32]. The genome sequence of norovirus is ~7.6 kb in length, which comprised of three open reading frames (ORFs). ORF1 encodes six nonstructural (NS) proteins including p48, NTPase, P22, VPg, Pro and Pol protein and they play critical roles in virus replication [29]; ORF2 encodes the major capsid protein (VP1) which consists of a protruding (P) domain and a shell (S) domain. [10, 30]. The P domain can be divided into P1 and P2 subdomain, and P2 is the most important factor in determining the diversity, antigenicity, and glycan binding patterns of different types of norovirus. ORF3 encodes the minor capsid protein (VP2) which responsible for capsid assembly and genome encapsidation [37]. It has been demonstrated that the types of norovirus was expanded to ten genogroups and more than forty different genotypes based on the diversity of VP1 and RdRp regions [5].

The GII.4 genotype has been the most prevalent strain in the world for over two decades [26, 36]. However, in recent years some non-GII.4 genotype strains, such as GII.17, GII.2, GI.3, GII.3, GII.6, GII.12 [1, 4, 15, 17], have also been reported to cause the outbreak of norovirus illness. Notably, an un-epidemic genotype, GIX.1[GII.P15] strains that was reported in several countries in recent years [22, 25, 33]. Currently, there are only a dozen genome sequences of GIX.1[GII.P15] strain are available in the NCBI database, and there are no studies that address the genetic characteristics of the GIX.1[GII.P15] strains in the whole-genome setting. Although this strain might be circulating with a low prevalence in the population at present, one important aspect that merits further attention is that non-GII.4 strains can also cause large outbreaks and even prevail over GII.4 strains from the perspective of the evolution and epidemiology of norovirus [7, 8, 39]. Therefore, more datasets of viral genomes of non-epidemic strains are urgently needed to facilitate comparisons with epidemic strains, which will increasing our knowledge of the emerging mechanisms of novel strains and will also provide insights for the prevention and control of pandemic noroviruses.

Here, we report a novel GIX.1[GII.P15] strain collected from a gastroenteritis surveillance program performed by the Kunming City Center for Disease Control and Prevention. To better understand the genetic characteristics of this virus,

we first isolated and cultured this strain in a human B cell culture system and then carried out a comprehensive analysis of the full genome sequence.

Methods

Sample information and collection

Vomit samples from a 70-year-old female patient with diarrhea were collected by the Kunming City Center for Disease Control and Prevention in a norovirus surveillance program during winter in Kunming of China in 2017. Informed consent for these studies was obtained from the patient, and the protocol was approved by the Ethics Committee of the Institute of Medical Biology, Chinese Academy of Medical Sciences in accordance with the Declaration of Helsinki. Following dissolution the sample in 2ml phosphate-buffered saline solution with antibiotics, the vomit supernatant was collected after centrifugation at 12000 xg for 10 min at 4 °C and then kept at -80 °C.

Viral RNA extraction and primary identification

TRNzol Universal Reagent (TianGen Biotech, Co., Ltd., Beijing, China) was used to extract the total RNA from 100 ul of the vomit supernatant, according to the instructions of the manufacturer. The norovirus type I and II amplification kits (MABSKY, China) was used to primarily identify the genogroup of this strain. For further quantification, real-time TaqMan RT-PCR assay was performed using the one-step PrimeScript™ RT-PCR kit (Takara, Code No. RR064A) in the CFX96 Touch™ Real-Time PCR Detection system (Bio-Rad, Laboratories, Hercules, CA, USA). The PCR reactions were performed by using the forward primer, 5'-CARGARBCNATGTTYAGRTGGATGAG-3'; reverse primer 5'-TCGACGCCATCTTCATTCACA-3' and the probe 5'-FAM-TGGGAGGGCGATCGCAATCT-TAMRA-3'. The PCR reaction conditions were as following: 42 °C for 5 min and 95 °C for 10 s, followed by 40 cycles at 95 °C for 5 s, and 60 °C for 20 s. Then a standard curve for cycle thresholds (Cts) was established by measuring the serially diluted the norovirus RNA standards. Viral copy number for each sample was calculated based on the standard curve and Ct values of the samples.

Cell culture, virus isolation and transmission electron microscope observation of viral particles

Raji cells (Human B-lymphocytes cells) were stored in the laboratory and cultured in RPMI-1640 culture medium (Opti-MEM, Thermo Fisher, USA) supplemented with 10% fetal bovine serum (Worthington Biochemical Corporation, USA) and 1% penicillin/streptomycin (pen/strep) in an incubator containing 5% CO₂ at 37°C. To isolate the norovirus strain, the vomit supernatant was inoculated in Raji cells in accordance with previously described methods [14]. Briefly, 10⁴ viral genome copies of the vomit sample was added into 2×10⁵ Raji cells; then complete RPMI640 was added to top up the mixture to 100 ul and the mixture was incubated for 2h at 5% CO₂ at 37°C; The samples were then centrifuged and the pellet was resuspended using 100 µl of complete RPMI; 50 ul of each sample was added to the 48-well plate, and complete RPMI640 was added to top up each well to 1 ml, then the plate was incubated in a 37 °C incubator with 5% CO₂. At 0-3 days post infection, each well at the indicated times post-infection (6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 72 hours post-infection) was collected to detect the virus titers via qRT-PCR.

To facilitate the detection of viral particles, transmission electron microscope was employed to identify the size and morphology of the norovirus strain. After purification by iodixanol super-centrifugation, the sampled layers containing nanoparticles were applied to formvar-carbon-coated 400-mesh copper grids using glass microspray device. The grids were stained with 2% aqueous uranylacetate at pH 4.5 for 5 min and viewed under a Hitachi TEM at 10,000–30,000× magnification.

Full-length genome sequencing and sequence analysis

The full-length genome of the KMN1 strain was sequenced via next-generation sequencing (NGS) technology. Briefly, 1 ng of input cDNA was used for library construction with A NEBNext Ultra II RNA Library Prep Kit (NEB, USA). The Illumina MiSeq sequencer (NovaSeq 6000, USA) was used to generate paired-end 150bp reads. After removing adapters and trimming from the 3' end, sequencing reads were de novo assembled into contigs with SPAdes 3.9.0 [2].

Geneious software package was used to align the nucleotide sequence of KMN1 with other reference strains downloaded from NCBI. Then phyML 3.0 [9] was used to construct the phylogenetic trees respectively based on full-length genome sequences, RdRp and VP1 sequences by using the general time reversible nucleotide substitution model of the maximum-likelihood method.

In addition, simPlot program (3.5.1) [21] was used to estimate the similarities between the nucleotide sequences and the consensus sequence of the GIX.1[GII.P15] strains. The Kimura 2-parameter method with a window size of 200 nucleotides and a step size of 20 nucleotides in the full-length genome sequence was used to examine the similarity among GIX.1[GII.P15] strains.

Creation of the capsid protein structure and prediction of conformational epitopes for the B-cell in the VP1 protein

VP1 dimer structural models of each GIX.1[GII.P15] strains were constructed using SWISS-MODEL Server (The NoV GIX.1[GII.P15] VP1 dimer structural model of KMN1 strain was constructed by SWISS-MODEL Server). The templates for homology modeling were based on the crystal structures of four strains (PDB ID: 1IHM, 4X07, 4OP7, and 4OPS). Protein structure was visualized and analyzed using the online tool provided by the Swiss Model server. Four bioinformatics tools DiscoTope 2.0 [16], BEPro [34], EPCES [18], and EPSVR [19] were used to predict the conformational epitopes on the capsid VP1 protein of GIX.1[GII.P15] strains. The thresholds for the epitopes were 1.3 for BEPro, -3.7 for DiscoTope 2.0 and 70 for EPCES and EPSVR. Conformational epitopes were determined by the Consensus sites according to all four tools and regions with similar residues across two of the sites in the VP1 dimer structures.

Results

Virus isolation from Raji cells

The norovirus from the sample was isolated via the inoculation of Raji cells cultured in RPMI-1640 culture medium and passaged successively 8-10 times. Electron microscopy yielded images of virus particles with a diameter of approximately 20-40 nm and a morphology of spherical and lace-like appearance in the supernatant of infected Raji cells, as shown in Figure 1A and 1B, indicating successful infection of the KMN1 strain in Raji cells. Norovirus genome replication was detected by the growth kinetics of the primary virus in Raji cell cultures, the number of virus copies was increased from $4 \times 10^{3.5}$ copies/well to $5 \times 10^{4.3}$ copies/well between 1 to 48 hpi. At 72 hours post-infection, approximately fourfold increases in genome copy numbers was detected by qRT-PCR in comparison with that at 0 dpi (Figure 1C). Of note, the inoculated cells at 72h post-infection (hpi) were harvested and subjected to next-generation sequencing (NGS) to provide full-length genomic sequence. To further confirm whether viral genome replication in Raji cells represented productive infection, lysates of the primary virus from Raji cultures at 3 dpi were serially passaged onto Raji cells. Results revealed a time-dependent increase in HuNoV genomes from P1 to P4 after inoculation with primary virus (Figure 1D), indicating that primary Raji infection results in the production of new infectious virus particles.

The complete genome of the KMN1 strain

The full genome sequence of KMN1 strain was submitted to GenBank with an accession number of MT707683.1. Similar with other types of norovirus, the genome sequence length of the KMN1 strain is 7594 bp and consisted of three

ORFs. The GIX.1[GII.P15] variant identified recently as a new norovirus group caused sporadic infections in the USA and China during 2017-2019. To determine the genetic diversity of KMN1 strain, similarity analysis based on the full-length genome sequences of all GIX.1[GII.P15] norovirus strains available in the NCBI database was performed. As illustrated in Figure 2, the genome of the KMN1 strain shared 94–100% nucleotide sequence identity with other strains identified in China and the USA during 2017-2018 but showed lower similarities (84-96%) with the strain identified in Japan in 2007, suggesting that the nucleotide sequences of the 2017-2019 GIX.1[GII.P15] strains presented more divergence after ten years of circulation in the human population.

Phylogenetic analysis of the KMN1 strain

To understand the genetic characterization of the KMN1 strain, we first performed the phylogenetic analysis based on the complete genome sequences. Figure 2 showed that the KMN1 strain clustered with 11 other GIX.1[GII.P15] strains identified in the US and China during 2017-2019. The KMN1 strain was most closely related to xinghuang/GII.P15/China/2018 (accession number: [MN473468.1](#), identity=99.20%) and YIYANG/HUNAN/CHINA/GII.P15-GIX.1/2018 isolates (accession number: [MN462922.1](#), identity=99.18%), both of which were collected in China in early 2018. When these three GIX.1[GII.P15] norovirus were analyzed by collection date, the KMN1 strain appeared to represent the ancestor of GIX.1[GII.P15] sequences reported in China since 2017. Moreover, the tree based on the nucleotide sequences of VP1 and RdRp showed 99.46% and 99.54% identities respectively, with YIYANG/HUNAN/CHINA/GII.P15-GIX.1/2018 strain (Figure 3 and 4), which consistent with the result based on the full-length genome phylogenetic tree and suggested that no evidence of recombination is found in this novel strain.

Alignment analysis of full amino acid sequences of KMN1

To further determine the sequence diversity of KMN1 with other GIX.1[GII.P15] strains, the complete deduced amino acid sequences of the KMN1 strain were compared with other completely sequenced GIX.1[GII.P15] strains available from NCBI. BLAST analysis showed that KMN1 exhibited $\geq 99\%$ amino acid identity across structural and nonstructural proteins with the consensus sequence of GIX.1[GII.P15]. The consensus sequence of the GIX.1[GII.P15] genotype was constructed from the most frequent nucleotides or residues at each nucleotide site of 14 other completed sequenced GIX.1[GII.P15] genotype strains available from NCBI. A total of 81 nucleotide substitutions were observed across the complete genome sequence (53 in ORF1, 20 in ORF2 and 8 in ORF3 respectively). The nucleotides differences between KMN1 and the consensus sequence are shown in detail in Table 1. Most of them (75/81, 92.6%) were synonymous mutations that do not cause amino acid substitutions. And the remaining 6 were the non-synonymous mutations, 3 of which were in ORF1 (H84R in p48; V93I in VPg; M212V in Pol), while 1 was in ORF2 (P302S in VP1), and 2 were in ORF3 (T163I and H215Y in VP2) (Table 2). Of note, two amino acid sites (M212V in Pol and T163I in VP2) were found to be specific to the KMN1 strain. Among these variations, only one substitution (P302S in VP1) was found in the P2 domain of the VP1 region, but it was not located in the histo-blood group antigen (HBGA)-binding sites.

Prediction of conformational epitopes on the VP1 structures of GIX.1[GII.P15] strain

Since the main neutralizing antibody epitopes of norovirus are located on the VP1 protein, and the antigenicity of the novel strain may be changed due to the mutations occurred on the VP1 protein, it is important to estimate the conformations epitopes and amino acid substitutions on the VP1 protein of the GIX.1[GII.P15] strain. Here, we identified five regions as conformations epitopes by using computational methods [3, 41], four of which were located on the P2 domain and one was on the P1 domain. Of note, the amino acid substitution in VP1 (P302S) was estimated around one of the conformational epitopes.

Discussion

Norovirus is one of the most common enteric pathogens that cause viral-related gastroenteritis of all age groups worldwide. Most previous studies of norovirus have focused on epidemic strains such as GII.4 and GII.17 [11, 28, 38, 40], which were the important causative agents causing the outbreak of norovirus illness all over the world in the past years. However, it should be noted that non-epidemic strains can also cause outbreaks or even epidemics, especially when some specific mutations arise in the RdRp or VP1 proteins [6, 24, 35]. Thus, it is also important to study the genomic characteristics and monitor the mutations of the novel GIX.1[GII.P15] strain. Here, we isolated one GIX.1[GII.P15] strain using Raji cells from a female patient in Kunming, China and performed a comparative genomic analysis for the first time.

The KMN1 strain is able to proliferate in the Raji cell culture system, and infection of Raji cells results in a fourfold increase in genome copy number from 0 dpi to 3dpi.

These results are consistent with previous reports of HuNoV cultivation in BJAB and Raji cell lines, where Raji cells support ~6 fold increase of the genome copy numbers from 0 to 3 dpi of the GII.4-Sydney infection [14]. Although the fold increase in genome number in present study was lower than that in previous studies [13, 14], of note, unfiltered vomitus sample used in the present study might also contain cofactors as previously reported unfiltered stool. Moreover, we confirmed that the KMN1 strain could be passaged in Raji cells on the basis of previous studies, which will facilitate future research on HuNoV in vitro cultivation system.

Phylogenetic analysis based on the full-length genome sequence indicated that KMN1 belonged to GIX.1[GII.P15] genotype and it clustered closely with two GIX.1[GII.P15] strains, both of which were collected in China in early 2018. Further phylogenetic analysis based on VP1 and RdRp sequences showed that no recombination was found of this strain. Previous studies suggested that recombination was the most important driving force of genetic diversity of non-GII.4 noroviruses [29]. However, recombination was not observed in the KMN1 strain, which might be related to limited variants of GIX.1[GII.P15] co-circulation over short periods. The GIX.1[GII.P15] strains isolated in recent years exhibited a large diversity compared with the strain isolated ten years ago, which might facilitate the virus escape human immune pressure. Further analysis of the full nucleotide sequences revealed a total of 81 nucleotide substitutions identified in the full-length genome sequence compared with the consensus sequence of GIX.1[GII.P15], but only 6 of these substitutions resulted in amino acid sequence changes. Of which, two amino acid sites (M212V in RdRp and T163I in VP2) were found to be specific to the KMN1 strain. Moreover, one substitution (P302S in VP1) was found in the P2 domain of the VP1 region, but it was not located within the amino acid sequences of the HBGA-binding sites. The GIX.1[GII.P15] genotype has seven conserved residues that form the major components of the HBGA-binding sites [20]. The alignment of HBGA-binding pocket amino acid sequences in KMN1 strain and other GIX.1[GII.P15] strains showed very high identity and none of these residues were mutated in the GIX.1[GII.P15] strains in this study, indicating the HBGA-binding pocket is conserved in GIX.1[GII.P15] strains. However, it should be noted that the HBGA binding profiles within a genotype were likely to evolve over time, which might result in the changes in the epidemic. This was exemplified by the fact that the previously rare genotype GII.17 became prevalent during 2014-2015 [4, 12] due to minor mutations arose at the glycan binding sites which extended the HBGA binding spectrum [6, 12, 31]. Therefore, further epidemiological and virological surveillance are needed to monitor the GIX.1[GII.P15]-related mutations and outbreaks.

Last but not the least, the predicted conformational epitopes were analyzed using

computational methods and then mapped to the VP1 protein structure of the GIX.1[GII.P15] strain. Previous studies on other genotypes indicate that most epitopes have been predicted within the P2 domain and amino acid substitutions arose in the epitopes might change the antigenicity of these genotypes [26, 27]. Likewise, our results showed that the

four of five predicted epitopes were located on the P2 domain, the remaining one epitope was located on the P1 domain. Note that the P302S mutation in the P2 domain was predicted around one of the epitopes. However, whether this substitution will affect the antigenicity of this epitope from structure is unknown, and requires further investigation.

In summary, the full-length genome sequence of a novel GIX.1[GII.P15] strain was extensively studied in this study. The analysis of its replication features in Raji cells system indicated the need to improve the current norovirus culture systems. The genome information obtained from the KMN1 strain is important for us to understand the genetics and evolution of GIX.1[GII.P15] strains and will provide critical information for prevention and control the GIX.1[GII.P15]-related outbreaks. Future research should focus on the specific roles of each mutation on the biology of norovirus.

Abbreviations

HuNoV: Human norovirus; RdRp: [RNA-dependent RNA polymerase](#)

qRT-PCR: Real-time reverse transcription-PCR; ORFs: open reading frames

HBGA: Histo-blood group antigens; TEM: Transmission electron microscope

Declarations

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Availability of data and materials

All data presented in this manuscript is included in the text.

Ethics approval and consent to participate

The samples used in this study was approved by the patient.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1

Comparison of KMN1 nucleotide substitutions with the consensus sequences of GIX.1[GII.P15] strains

Gene	NTPase											P22
Position	30	63	66	69	306	345	615	660	744	924	1095	81
Consensus	C	A	G	T	C	T	T	A	G	C	G	C
KMN1	T	G	A	C	T	C	C	G	A	T	A	T
Gene	P48											VPg
Position	251	285	504	771	819	837	843	891	906	18	24	46
Consensus	A	G	A	G	G	A	C	C	C	C	G	G
KMN1	G	A	G	A	A	G	A	T	T	T	A	A
Gene	Pro											Pol
Position	108	277	330	342	72	129	252	306	483	540	174	177
Consensus	G	G	C	T	T	T	T	C	A	T	A	C
KMN1	A	A	T	C	C	C	C	T	G	C	G	T
Gene												
Position	408	414	426	504	564	634	702	807	810	948	1059	1095
Consensus	T	T	T	A	C	A	G	G	A	C	G	T
KMN1	C	C	C	G	T	G	A	A	G	T	A	C
Gene	VP1											
Position	1098	1221	1248	1284	1314	102	174	240	255	417	465	651
Consensus	A	C	T	G	C	G	G	A	A	A	C	G
KMN1	G	T	C	A	T	A	A	G	G	T	T	A
Gene												
Position	676	687	840	904	973	1035	1092	1095	1191	1287	1395	1452
Consensus	T	T	C	C	C	T	C	C	T	A	T	A
KMN1	C	C	T	T	T	C	T	T	C	G	C	G
Gene	VP2											
Position	1639	138	330	375	491	555	618	643	678			
Consensus	C	C	A	T	C	C	C	C	A			
KMN1	T	T	G	C	T	T	T	T	G			

Note: Positions in bold represent nucleotide changes that resulted in changes in the amino acid sequence.

Table 2

Differences in the deduced amino acid sequence alignment of the KMN1 strain based on alignment with other GIX.1[GII.P15] strains

Amino Acid site	84(P48)	93(VPg)	212(POL)	302(VP1)	163(VP2)	215(VP2)
Consensus Sequence	H	V	M	P	T	H
MT707683.1 China KMN1	R	I	V	S	I	Y
MT703831.1 China 07-Jun-2019	H	V	M	P	T	H
MN473468.1 China 14-May-2018	R	I	M	S	T	H
MN462922.1 China 13-Mar-2018	R	I	M	S	T	H
MT010298.1 China 18-Feb-2018	R	I	M	P	T	H
MT010299.1 China 18-Feb-2018	R	I	M	P	T	H
MN227774.1 USA 07-Jul-2018	H	V	M	P	T	Y
MN227775.1 USA 18-May-2018	H	V	M	P	T	H
MN227777.1 USA 09-May-2018	H	V	M	P	T	Y
MN227776.1 USA 19-Jan-2018	R	V	M	S	T	H
MN227771.1 USA 27-Dec-2017	H	V	M	P	T	Y
MN227773.1 USA 15-Dec-2017	H	V	M	P	T	Y
MN227772.1 USA 14-Dec-2017	H	V	M	P	T	Y
MN227770.1 USA 14-Dec-2017	R	V	M	S	T	H
NC_044045.1 Japan 2007	H	V	M	P	T	Y

Figures

Figure 1

Replication of KMN1 in Raji cells. (A-B) TEM identifies a 30-nm particle with a morphology of spherical and lace-like appearance associated with KMN1 strain infection (A, Bars =200 nm; B, Bars =100 nm). (C) Kinetics of primary virus yield at the indicated times post-infection. (D) Passaging of the KMN1 strain in Raji cells. Data represent means and standard deviations from 3 independent experiments.

Figure 2

SimPlot analysis of the representative human norovirus GIX.1 [GII.P15] strains. Each variant's similarity to the consensus sequence is represented.

Figure 3

The Phylogenetic tree based on full-genome sequences of different genotype strains of noroviruses. The GIX.1[GII.P15] strain identified in this study is indicated with red.

Figure 4

Phylogenetic tree based on full-length VP1 sequences. The GIX.1[GII.P15] strain identified in this study is indicated with red.

Figure 5

Phylogenetic analysis based on full-length RdRp sequences. The GIX.1[GII.P15] strain identified in this study is indicated with red.

Figure 6

The three-dimensional VP1 dimer structures (cartoon models) of the GIX.1[GII.P15] strain are shown. Predicted epitopes of the KMN1 strain are indicated in dark blue, and their regions are circled with black; red: P302S substitution; light blue: S domain; Green: P1 domain; Yellow: P2 domain.