Emergence in Taiwan of novel norovirus GII.4 variants causing acute gastroenteritis and intestinal haemorrhage in children

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Norovirus is the leading cause of viral gastroenteritis globally. Norovirus genotype GII.4 is responsible for the majority of outbreaks, but new variants are continuously emerging. The objective of the study was to delineate the clinical manifestations and complications associated with these new norovirus GII.4 variants in children. We investigated norovirus infections from the community outbreak in October 2011–September 2012 and an earlier outbreak in 2006–2007, in northern Taiwan. Norovirus genotypes and their variants were validated using molecular methods. A norovirus outbreak started in mid-2011 and continued through 2012 in northern Taiwan. Hospitalized children infected by norovirus in 2012 showed a significantly higher incidence of intestinal haemorrhage, as indicated by grossly bloody faeces \( P = 0.012 \) and occult blood in faeces \( P < 0.001 \), and also presented with more high fever \( > 39 \, \text{°C} \) \( P < 0.001 \), fever \( > 38.5 \, \text{°C} \) \( P < 0.001 \) and fever of any temperature \( > 38 \, \text{°C} \) \( P < 0.001 \), compared with children hospitalized in 2006–2007. Analysis of 20 near-full-length genome sequences indicated an emergence of GII.4 2012 variants in 2011–2012. Circulating noroviruses can be divided into two clusters: GII.4 2012a, which is identical to the newly reported strain GII.4 Sydney 2012, and GII.4 2012b, which is close to GII.4 2006b, the earlier predominant strain. The emerging new variants of norovirus GII.4 caused a distinct clinical syndrome of acute gastroenteritis with severe fever and a high rate of intestinal haemorrhage in children. The genetic diversity associated with changing clinical manifestations poses major obstacles to norovirus control.

INTRODUCTION

Norovirus, a genus within the Caliciviridae family, is the leading cause of sporadic and epidemic acute gastroenteritis worldwide (Glass et al., 2009; Lopman et al., 2004). Our understanding of norovirus epidemiology has significantly progressed in recent years due to the development of sensitive molecular diagnostic techniques. We now understand that human noroviruses are extremely diverse, with three

Abbreviations: CGCH, Chang Gung Children’s Hospital; NCBI, National Center for Biotechnology Information; RdRp, RNA-dependent RNA polymerase; RT-PCR, reverse-transcription PCR.

The GenBank/EMBL/DDBJ accession numbers for the 20 near-full-length genome sequences of norovirus are KC517359–KC517378.

Three tables are available with the online Supplementary Material.
genogroups (GI, GII and GIV), at least 25 genotypes and numerous subgenotypes or variants identified in the past two decades (Glass et al., 2009). In recent years only a few strains, primarily those of genogroup II, genotype 4 (GII.4), have been responsible for the majority of outbreaks (Glass et al., 2009). The norovirus GII.4 variants differ from each other by more than 5% at the amino acid level within the capsid protein and have been identified as the aetiologic agent in large epidemics of gastroenteritis in many countries, accounting for >90% of the outbreaks worldwide (Bull & White, 2011; Gallimore et al., 2007, 2004). Other than gastrointestinal symptoms, norovirus infection in humans is associated with several atypical complications, such as benign infantile convulsion, necrotizing enterocolitis and exacerbations of inflammatory bowel disease, as well as chronic, serious outcomes in immunocompromised patients (Bok & White, 2011; Gallimore et al., 2007, 2004). Other than gastrointestinal symptoms, norovirus infection in humans is associated with several atypical complications, such as benign infantile convulsion, necrotizing enterocolitis and exacerbations of inflammatory bowel disease, as well as chronic, serious outcomes in immunocompromised patients (Bok & White, 2011; Gallimore et al., 2007, 2004).

The rapid emergence and persistence of GII.4 noroviruses in the past decade may be explained by their rapid genetic evolutionary pattern. GII.4 noroviruses have displayed swift evolution, similar to influenza, and the emergence of the GII.4 2002 variant in 2002 marked the beginning of a period of increasing GII.4 variation, with new pandemic strains appearing every 2–3 years (Boon et al., 2011; Motomura et al., 2010; Shanker et al., 2011). In 2005, a highly successful variant, GII.4 2006b, displacing other previously circulating variants, emerged; since 2006, it has become the predominant circulating variant causing illness in many countries, including Taiwan (Hoa Tran et al., 2013). We previously reported on an epidemic in 2006–2007 in Taiwan of acute gastroenteritis caused by norovirus GII.4 2006b and its association with a high frequency of infantile convulsion (Chen et al., 2009). Our continued surveillance of the molecular epidemiology of noroviruses in Taiwan confirmed that new norovirus GII.4 variants have been emerging and causing a large community outbreak in northern Taiwan since mid-2011. In this study, we describe our investigation of these emerging GII.4 variants. Using sensitive reverse-transcription PCR (RT-PCR) techniques, we detected multiple variants closely related to the novel GII.4 Sydney 2012 variant circulating in northern Taiwan [Centers for Disease Control and Prevention (CDC), 2013]. A hospital-based case-control study was conducted to delineate the clinical manifestations and complications associated with these new norovirus GII.4 variants in children.

**METHODS**

**Hospital-based surveillance.** This study was approved by the Institutional Review Board (98-3759B and 100-4283A3) of Chang Gung Children’s Hospital (CGCH), a medical centre and teaching hospital with 400 beds. The records of all children with acute gastroenteritis treated at CGCH from 2010 to 2012, who had stool specimens sent for norovirus detection using RT-PCR methods in the Department of Laboratory Medicine, were reviewed. From October 2011 to September 2012, 101 children hospitalized in the Division of Pediatric Gastroenterology, Department of Pediatrics for treatment with confirmed norovirus infection were enrolled. We also analysed stool samples of 114 patients hospitalized in the same department during October 2006–September 2007 with norovirus infection for comparison. Clinical information of these paediatric patients was retrospectively collected and analysed. All patients with positive bacterial cultures for Salmonella, Shigella and Campylobacter were excluded from the study.

Patients with confirmed norovirus infection were surveyed for indicators of disease severity, including diarrhoeal course, vomiting, fever, dehydration status and the occurrence of complications. Other clinical information, including demographic data, physical examination findings and laboratory testing results, were also collected and analysed. Complications were defined as the occurrence of extra-intestinal or unusual presentations of acute gastroenteritis, such as hypoglycaemia (serum sugar level <80 mg dl⁻¹), electrolyte imbalance (hyponatraemia, serum sodium <135 mmol l⁻¹; hypokalaemia, serum potassium <3.5 mmol l⁻¹; hypochloraemia, serum chloride <98 mmol l⁻¹). The reference data were also applied in our previous studies (Chen et al., 2007, 2009), hypotension (systolic pressure <70 mmHg) or severe hyperthermia (body temperature ≥41°C). A total leukocyte count >10,000 mm⁻³ was deemed leukocytosis. The severity of illness was evaluated using a previously described scoring system (Vesikari et al., 1990).

**Nucleic acid isolation and RT-PCR.** Viral nucleic acid extraction from faecal samples was performed using a kit according to the manufacturer’s recommendations (QiAamp Viral RNA Mini kit; Qiagen). The concentration of viral nucleic acids was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). First-strand cDNA synthesis and PCR were performed according to the manufacturer’s recommendations (SuperScript III First-Strand Synthesis System; Invitrogen). The PCR primers and conditions used for determining norovirus genotypes were described previously (Bok et al., 2009).

**Norovirus genome sequencing.** To sequence the norovirus genomes, primers specific for GII.4 were designed (for a list of the primers, see Table S1, available with the online Supplementary Material). The norovirus genomic DNA was amplified as previously described (Bu et al., 2008). The cDNA products were cloned into a plasmid (pCR-XL-TOPO vector; Invitrogen) and the recombinant plasmid was transferred into competent Escherichia coli (Topo XL PCR Cloning kit; Invitrogen). The plasmids were purified and sequenced for the DNA insert sequence by using an ABI 3730 DNA analyser (Applied Biosystems). The sequences of different PCR products from the same specimen were used to reconstruct the near-full-length norovirus genome using the Vector NTI software package (Invitrogen). All reference sequences of noroviruses used for comparison were all obtained from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov).

**Phylogenetic analysis.** The near-full-length norovirus genome sequences were aligned using the MAFFT program with default parameters (http://www.ncbi.nlm.nih.gov/pubmed/23329690). The alignment was put into MEGA5 software to reconstruct the phylogenetic tree (Tamura et al., 2011). The neighbour-joining algorithm was used and the Tamura-three-parameter substitution model was adopted. The substitution rates among sites were assumed to be uniform, and gaps within the alignment were completely deleted when calculating pairwise distances. The reliability of the interior branches of the phylogenetic tree was assessed by the bootstrap method with 1000 resamplings.

**Statistical analysis.** Continuous variables were analysed by the Student’s t-test and dichotomous variables by the chi-squared test. A P value of <0.05 was considered statistically significant. All the tests were analysed using SAS system software version 8 for Windows.
RESULTS

Hospital-based surveillance

A total of 5843 faecal specimens from patients with diarrhoea were sent to the Department of Laboratory Medicine, CGCH for norovirus detection by RT-PCR, from 2010 to 2012. The positive detection rate is shown in Fig. 1. A significant increase in norovirus activity was observed in mid-2011. We analysed the paediatric cases who were hospitalized at CGCH for treatment and found that cases of acute gastroenteritis due to norovirus totalled 78/306 (25.5 %) in October–December 2011 and quickly rose to 131/252 (52 %) in January–March 2012, 146/265 (55.1 %) in April–June 2012 and 173/303 (57.1 %) in July–September 2012, with an average of 46.9 % (528/1126) during the 1 year period.

Clinical investigation

The clinical and laboratory manifestations of 101 patients with norovirus infection who were admitted to the Division of Pediatric Gastroenterology, Department of Pediatrics during October 2011–September 2012 were analysed. Watery diarrhoea was the most common presentation (n=95, 94.1 %), followed in the order of frequency by vomiting (n=75, 74.3 %), fever (n=64, 63.4 %), abdominal pain (n=46, 45.5 %), positive occult blood in stools (n=23, 22.8 %), grossly bloody diarrhoea (n=11, 10.9 %; five with frank blood in stool and six with bloody and watery diarrhoea) and hypotension (n=5, 4.9 %; two with hypovolemic shock). A total of 23 of 101 hospitalized patients (22.8 %) were aged <1 year, 26 (25.7 %) were 1–2 years, 32 (31.7 %) were 2–5 years and 20 (19.8 %) were aged >5 years; the majority of patients were >2 years old. We compared clinical presentations of the 23 bloody diarrheal patients with 78 patients without bloody diarrhoea, and found significantly more diarrheal episodes per day [average (range): 6.7 (5–10) vs 4.9 (2–7)] (P=0.0417); the comparison of other manifestations between the two groups was insignificant (data not shown).

The clinical and laboratory manifestations of these patients were compared with those of 114 hospitalized cases of norovirus infection in 2006–2007 (Table 1). Hospitalized paediatric patients infected by norovirus in 2011–2012 had significantly more male gender predominance (P=0.03), high fever >39 °C (P<0.001), fever >38.5 °C (P<0.001) and fever of any temperature >38 °C (P<0.001). These patients presented a significantly higher incidence of gastrointestinal haemorrhage, as indicated by grossly bloody faeces (P=0.012) and occult blood in faeces (P<0.001), and abdominal pain (P<0.001). No significant difference was observed in other common presentations of acute gastroenteritis, such as the maximum frequency of vomiting and diarrhoea within 24 h, the duration of vomiting and diarrhoea, overall length of hospitalization and severity score of acute gastroenteritis. The only significant difference observed in laboratory findings was a higher mean C-reactive protein (CRP) level (P=0.01) in patients hospitalized in 2011–2012. More episodes of convulsion (P=0.001) and a higher incidence of hypoglycaemia (P<0.001) were recorded in patients hospitalized in 2006–2007. The 16 patients infected by non-GII.4 showed an older age [average (range): 58 (35–81) months] and the clinical presentations were the same as common viral gastroenteritis.

Microbial investigation

Genotypes were determined for 56 of the 114 norovirus strains in 2006–2007 and all of them were genotype GII.4. Near-full-length genome sequences were obtained for 37 of these strains, 32 (86.5 %) were GII.4 2006b (data not shown). Genotypes were determined for 52 of the 101
strains in 2011–2012, with GII.4 being the most prevalent (30, 57.6 %). Other genotypes included eight GII.2, three GII.3, three GII.6 and two GII.12, and six were undetermined. Near-full-length genome sequences were obtained for 20 of these strains and further clustered into GII.4 2012a (n=9, 45 %), GII.4 2012b (n=10, 50 %) and GII.4 2010 (n=1), with a 95 % overall prevalence of GII.4 2012 variants. Thus, we estimated that the prevalence of GII.4 2012 variants in the 2011–2012 outbreak was at least 54.7 %.

The 20 near-full-length genome sequences of noroviruses and the associated clinical information are shown in Table S2. The reference strains for comparison and their accession numbers are shown in Table S2 (Siebenga et al., 2009; Tu et al., 2008). Representative sequences of GII.4 2006b, GII.4 2012a and GII.4 2012b were aligned with other norovirus reference genomes obtained from NCBI (for a list of the reference sequences, see Table S3), and a phylogenetic tree was reconstructed based on the alignment (Fig. 2). The tree shows that two variants can be clearly distinguished: one cluster strongly resembles the noroviruses reported from Sydney, Australia in 2012 (accession numbers JX459907 and JX459908), whereas the other cluster is much closer to the strains identified in our patients in 2006, belonging to GII.4 2006b (accession numbers JN400600, JN400613 and JN400614). The two variants were therefore designated noroviruses 2012a and 2012b.

Further analysis showed that the nucleotide and deduced amino acid homology of 2012a and GII.4 New Orleans 2009 (accession number GQ845367) in viral capsid protein VP1 was 95.2 % and 97 %, respectively. The nucleotide and deduced amino acid homology between 2012b and GII.4 2006b (accession number JN400613) in VP1 was 96.9 % and 99 %, respectively. The most variable region in the GII.4 2012 variants compared with other GII.4 strains (GII.4 2012a vs GII.4 New Orleans 2009 and GII.4 2012b vs GII.4 2006b) was located in the ORF1 (data not shown). We also analysed the phylogeny of the RNA-dependent RNA polymerase (RdRp) gene of these noroviruses. GII.4 2012b RdRp gene was close to that of 2006b, whereas 2012a (GII.4 Sydney 2012) RdRp gene was close to that of GII.e (accession number AB434770) (data not shown), indicating that a recombination event happened during the evolution of the 2012a variant. By sequence analysis, we found that GII.4 2012a or Sydney 2012 comprised a partial ORF1 in relation to the GII.e strain and a partial ORF2 in relation to GII.4 New Orleans 2009 (accession number GQ845367), suggesting that the circulating GII.4 2012a or Sydney 2012 was a recombinant of GII.e and New Orleans 2009.

**DISCUSSION**
Norovirus infection in 2010–2012 showed no winter-season predominance (November to January of the following year)
in our study population in northern Taiwan. The proportion of hospitalized cases of gastroenteritis caused by norovirus increased significantly since mid-2011. The study documented that GII.4 2012 variants caused this community outbreak in northern Taiwan. Noroviruses usually cause a short-term, self-limited illness with diarrhoea and vomiting; however, the emerging new variants caused a distinct clinical syndrome of acute gastroenteritis with severe fever and a high prevalence of abdominal pain and intestinal haemorrhage mimicking bacterial enterocolitis. Although recent reports have included norovirus in the list of pathogens that could chronically infect immunocompromised individuals (Ludwig et al., 2008; Mattner et al., 2006), norovirus has never been reported as a cause of severe gastroenteritis and haemorrhage in immunocompetent children. Earlier pathological studies from ill volunteers showed that norovirus caused blunting of the intestinal villi, crypt-cell hyperplasia and infiltration of polymorphonuclear and mononuclear cells into the lamina propria, with the mucosa itself remaining intact in the proximal jejunum (Widerlite et al., 1975). Some earlier studies also reported that norovirus potentially played a role in pneumatosis intestinalis, a hallmark of necrotizing enterocolitis, in immunocompromised as well as some immunocompetent patients (Choi et al., 2011; Kim et al., 2011). Intestinal bleeding following norovirus infection in our patients suggests that norovirus, especially the recently emerged variants, holds the potential to cause more severe mucosal damage, apart from mucosal inflammation, in children. On the other hand, norovirus 2006b caused more episodes of convulsion in infants (Chen et al., 2009). This clinical difference could be due to genetic differences among norovirus variants, which lead to phenotypic changes in pathogenicity.

The most recent reports have recognized the emergence of a new GII.4 variant, GII.4 Sydney (GII.4 late 2012), in late 2012 in the USA and some European countries [Centers for Disease Control and Prevention (CDC), 2013; van Beek et al., 2013]. We found in this study that novel GII.4 2012 variants have been emerging since mid-2011. These variants accounted for 54.7% of norovirus infection in northern Taiwan, which is similar to the high rate of GII.4 Sydney infection recently reported by the Center for Disease Control and Prevention in the USA (53%) in children and adults (Centers for Disease Control and Prevention (CDC), 2013). According to our phylogenetic analysis, multiple GII.4 variants co-circulated in northern Taiwan; one belonged to GII.4 Sydney 2012 (designated 2012a in this study) and the other was designated GII.4 2012b. GII.4 2012a was derived from GII.e–GII.4 2010 recombination, and GII.4 2012b arose from GII.4 2006b. This situation indicates that dominant norovirus strains

Fig. 2. Neighbour-joining tree for GII.4 norovirus strains. Strains are represented by their GenBank accession numbers. The place and time of isolation is listed within parentheses. Numbers at nodes indicate the reliability of interior branches by 1000 bootstrap replications. Bar, 0.02 substitutions per nucleotide position.
evolved divergently over time. Although the earlier dominant variant, GII.4 2006b, was not identified in the current outbreak, the GII.4 2012b variant, which evolved from GII.4 2006b, remains prevalent at this time. Another dominant variant, GII.4 2012a (Sydney 2012), a new variant from a GII.e–GII.4 2010 recombination event, may play an even more dominant role in the future, given many reports of the variant from North America and Europe [Centers for Disease Control and Prevention (CDC), 2013; van Beek et al., 2013; Eden et al., 2013].

A comprehensive phylogenetic investigation was undertaken based on the near-full-length norovirus genome sequences we identified. At least four conclusions can be drawn from comparison of the GII.4 strains collected from different times and places. Firstly, a single variant can spread worldwide within a certain time period. The 2012a variant circulated in Australia and Taiwan in 2012. Strains from these two places could not be separated geographically, but they interspersed together, demonstrating that they originated from the same clone. Secondly, GII.4 norovirus has many variants, and multiples variants can circulate simultaneously in a single geographical location. The reason that variant 2012a was not detected in Taiwan previously is unclear. The virulence and transmissibility of this lineage was likely enhanced through mutation, contributing to its recent dissemination together with 2012b. Thirdly, the evolution of GII.4 is rapid. Variant 2012b is not identical to its parent strain, 2006b, as evidenced in the phylogenetic tree; despite their evolution from 2006b, 2012b formed a distinct sublineage. Within 6 years, 2012b accumulated nearly 2% genetic distance. Fourthly, multiple occurrences of different norovirus lineages and their rapid evolution makes future control more difficult, because prior exposure to certain norovirus variants cannot offer complete protection from new variant infection in children.

Our study showed that more than 50% of hospitalized children were aged ≥2 years, with ages of 2–5 years holding the highest prevalence (31.7%); this distribution indeed supports the idea that previous exposure to certain norovirus cannot offer complete protection against subsequent infection by emerging norovirus variants. Genetic diversity and evolutionary plasticity are major obstacles to norovirus control. New variants are continuously emerging, and new forms of antigenic variation are a critical point of vulnerability for future norovirus vaccines. Effective tools, such as near-full-length genome sequencing applied in this study, are needed to detect the evolutionary changes in the virus population and to monitor the spread of genetic variants that could show increased virulence in humans.

In summary, in contrast to previously circulating noroviruses, the emerging new variants of norovirus GII.4 caused a distinct clinical syndrome of acute gastroenteritis with severe fever and a high rate of abdominal pain and intestinal haemorrhage in children. Host genetic factors may be one of the reasons for these unusual presentations and this is not explored in this study. The ever-changing nature of clinical manifestations and the evolution of noroviruses necessitate continuous epidemiological surveillance of the emergence and spread of new variants and associated clinical manifestations.

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