The G428A Nonsense Mutation in FUT2 Provides Strong but Not Absolute Protection against Symptomatic GII.4 Norovirus Infection

Beatrice Carlsson1,2, Elin Kindberg1,2, Javier Buesa3, Gustaf E. Rydell4, Marta Fos Lidón3, Rebeca Montava3, Reem Abu Mallouh3, Ammi Grahn4, Jesús Rodríguez-Díaz3, Juan Bellido5, Alberto Arnedo5, Göran Larson4, Lennart Svensson1,2

1 Division of Molecular Virology, University of Linköping, Linköping, Sweden, 2 Department of Forensic Genetics and Forensic Toxicology, National Board of Forensic Medicine, Linköping, Sweden, 3 Department of Microbiology, School of Medicine and Hospital Clínico Universitario, University of Valencia, Valencia, Spain, 4 Department of Clinical Chemistry and Transfusion Medicine, Sahlgrenska University Hospital, Göteborg, Sweden, 5 Sección de Epidemiología, Centro de Salud Pública, and CIBER-ESP, Castellón, Spain

Abstract

In November 2004, 116 individuals in an elderly nursing home in El Grao de Castellón, Spain were symptomatically infected with genogroup II.4 (GII.4) norovirus. The global attack rate was 54.2%. Genotyping of 34 symptomatic individuals regarding the FUT2 gene revealed that one patient was, surprisingly, a non-secretor, hence indicating secretor-independent infection. Lewis genotyping revealed that Lewis-positive and negative individuals were susceptible to symptomatic norovirus infection indicating that Lewis status did not predict susceptibility. Saliva based ELISA assays were used to determine binding of the outbreak virus to saliva samples. Saliva from a secretor-negative individual bound the authentic outbreak GII.4 Valencia/2004/Es virus, but did not in contrast to secretor-positive saliva bind VLP of other strains including the GII.4 Dijon strain. Amino acid comparison of antigenic A and B sites located on the external loops of the P2 domain revealed distinct differences between the Valencia/2004/Es and Dijon strains. All three aa in each antigenic site as well as 10/11 recently identified evolutionary hot spots, were unique in the Valencia/2004/Es strain compared to the Dijon strain. To the best of our knowledge, this is the first example of symptomatic GII.4 norovirus infection of a Lewisb- individual homozygous for the G428A nonsense mutation in FUT2. Taken together, our study provides new insights into the host genetic susceptibility to norovirus infections and evolution of the globally dominating GII.4 viruses.

Introduction

Noroviruses (NoV) have emerged as an important cause of gastroenteritis outbreaks in institutions such as elderly nursing homes, hotels, hospitals and schools [1,2]. NoV contains a linear positive-sense single stranded RNA genome of ~7.7 kb in length, surrounded by a 530 amino acid (aa) long capsid protein (Norwalk strain), which is folded into two major domains; the conserved S (shell) domain and a more variable P (protruding) domain [3]. Recent studies have suggested that the protruding NoV capsid domain, subdivided into P1-1, P1-2 and P2, bear antigenic determinants affecting the immunological response and host specificity [4–8]. Transmission of NoV occurs predominantly through contaminated food, water, fomites, and by person-to-person through the fecal-oral route [9]. Evidence of protective immunity to NoV is controversial; short-term to no immunity has been reported [10–12] and antibodies do not seem to provide protection, at least not against the genogroup I Norwalk virus [13]. Furthermore, volunteer studies have shown that a subset of individuals remain uninfected even after repeated challenges [10,12,14]. This information, together with the fact that only low infectious doses are required for infection, and that attack rates seldom exceed 70% [15], suggest that some type of inherited factors act to prevent certain individuals from symptomatic NoV infection. Recent studies have shown that secretor status; the ability to express histo-blood group antigen (HBGA) on mucosa and in secretions may affect the risk of being symptomatically infected by NoV [16–22].

Non-secretors (ses), who do not express the Fuc-TI α1,2-fucosyltransferase and consequently do not express H type 1 or Lewis b (Leb) antigens, have been shown to be less susceptible or even resistant to authentic NoV infections [16,19,21,22]. Approximately 20% of Northern Europeans and Caucasian Americans are secretor-negative [23]. Furthermore, sero-epidemiology studies have shown that secretors have significantly higher antibody titers and prevalence against NoV than non-secretors [24]. However,
the fact that certain non-secretors are NoV antibody-positive, suggests that secretor-independent infections do occur [25,26], maybe with distinct virus strains.

Several mutations are known in the FUT2 gene [23], and some of them show high ethnic specificity [27,28]. The G428A nonsense mutation is typically found in the Caucasian population [23,27] whereas the nonsense C571T mutation is found mainly in Pacific Islanders [29]. Both these mutations give rise to an early stop codon, giving a truncated non-functional protein. Homozygous carriers of any nonsense mutation in the FUT2 gene are called non-secretors. Homozygous carriers of a missense mutation at position 385 (A→T) are so called “weak secretors”, expressing lower levels of ABH antigen in saliva and, if Lewis positive, a Lewis (a+b+) phenotype on erythrocytes [30].

In vitro binding studies have suggested that not only secretor status but also Lewis status may affect susceptibility to NoV [31,32]. However, different strains show different binding patterns, with the worldwide dominating genogroup II.4 strains expressing the broadest histo-blood group-binding pattern and thought to be able to infect secretor-positive individuals of all ABO blood group types irrespective of Le status [31].

Previously, only secretor-positive individuals have been symptomatically infected with the globally dominating GI.4 virus [16,19,21]. However, Lindesmith and co-workers have shown that a GI.4 strain detected in 2002 (2002a), bound not only secretor-positive but also to secretor-negative saliva under certain conditions [33], hence indicating infection with GI.4 virus also in non-secretors. In this study we report for the first time of symptomatic GI.4 NoV infection of an individual homozygous for the G428A nonsense mutation, a mutation that previously has been shown to provide complete protection from authentic GI.4 NoV disease [19,21]. Furthermore, we show that antigenic regions A and B in the P2 domain as well as 10/11 recently identified evolutionary hot spots proposed to be associated with molecular evolution [34] are distinct in the outbreak virus.

Results

Description of the outbreak

During November 6th to November 20th 2004 an outbreak of acute gastroenteritis consisting of 116 cases occurred in an elderly nursing home in El Grao de Castellón, Spain. The facility consists of a building exclusively dedicated to this purpose and includes 65 double-rooms in two floors occupied by 130 residents. In addition, 30 old persons visited the residence daily, which serves as a daycare center. The nursing home employs 90 healthcare workers and other staff members, 56 of whom were interviewed.

Out of the 130 residents in the facility, 75 (57.7%) persons suffered acute gastroenteritis. Sixteen (61.5%) of 26 out-patients were interviewed and 25 (43.1%) of the staff members experienced an episode of acute gastroenteritis, with a total of 116 persons affected.

The first three cases were reported on the 6th of November 2004 and since then other residents developed symptoms of acute gastroenteritis with progression towards the peak of the outbreak on November 12th 2004, with 44 cases on that date. The global attack rate was 54.2%. The most common symptoms were diarrhea (79%) and vomiting (66%), with fever (≥37.3°C) recorded in 13% of the patients. The average duration of symptoms was less than two days. Five patients were hospitalized, but no casualties were observed.

The outbreak was caused by a GI.4 strain

NoV were detected by RT-PCR in 27 out of 33 (81.8%) fecal samples tested from symptomatic patients, both residents and healthcare workers. As no other enteric virus (rotavirus and enteric adenovirus) or bacteria were detected from the patients it was concluded that NoV was the etiological agent of this outbreak. Sequencing of a portion of the RNA polymerase gene as well as the P2 region of the capsid gene, from four and three different specimens respectively, confirmed that the outbreak was caused by GI.4a-2004 variant virus (Figure 1A and 1B).

The G428A nonsense mutation in FUT2 provides strong but not absolute protection against symptomatic GI.4 NoV infection

To investigate any association between mutation in the FUT2 gene and resistance to symptomatic infection, genotyping was performed to identify individuals as secretor-negative, heterozygous secretors or homozygous secretors (Table 1). None of the individuals were carriers of the mutations at nt 385 (weak secretor) or at nt 571 in the FUT2 gene. Of the symptomatic individuals 38.2% (13/34) were homozygous secretors and 38.8% (20/34) were heterozygous secretors. Most interesting was that one (2.9%) symptomatic female patient (patient A) was found to be homozygous carrier of the G428A mutation and hence a non-secretor. Among the asymptomatic/non-exposed individuals, 15.4% (4/26) were homozygous secretors, 23.1% (6/26) were heterozygous and 61.5% (16/26) were non-secretors. Thus significant difference (P<0.001) in susceptibility to symptomatic NoV infection was found between secretors and non-secretors.

Lewis status was not identified as a susceptibility marker for symptomatic NoV GI.4 infection

While Le<sup>a+b</sup> but not Le<sup>a-b</sup> individuals are highly susceptible for symptomatic NoV infections, little information is available regarding Le<sup>a-b</sup> individuals in authentic NoV outbreaks. To determine whether Lewis status affected the susceptibility of infection, FUT3 genotyping was performed using PCR with sequence specific primers (PCR-SSP) [35]. Forty four of 60 individuals (73.3%) were genotyped and the results showed that six individuals were Lewis-negative due to being homozygous carriers of inactivating mutations at nt 202 and 314 (two individuals), at nt 59 and 1067 (one individual) and the remaining three being compound heterozygous at nt 202, 314, 59, 508 and 1067. Two Le<sup>a-b</sup> individuals were secretor-positive and four were non-secretors (Table 1). The two secretor-positive Lewis-negative individuals were both symptomatically infected, whereas none of the four Lewis-negative non-secretors became ill.

Saliva from secretors and a non-secretor as well as Lewis-positive and Lewis-negative individuals bound the outbreak virus strain

Several previous studies have shown that saliva of secretor-positive but not of secretor-negative individuals can bind NoV VLP [17] and authentic virus [19]. To investigate the property of our outbreak virus, the virus was incubated with saliva from symptomatic and asymptomatic/non-exposed individuals in an ELISA assay. Figure 2 shows that saliva from secretors bound the outbreak virus, but interestingly also saliva from one asymptomatic non-secretor (Le<sup>a-b</sup>), referred to as patient B. Because of the restricted amount of saliva (at the time for first sample collection), patient A (symptomatically infected non-secretor, Le<sup>a-b</sup>) could not be tested. Figure 2 shows that except for the binding of patient B, saliva of Le<sup>a+b</sup> but not of Le<sup>a-b</sup> individuals bound the virus (P<0.001) but more interestingly that Lewis status (positive vs negative) could not predict binding. The virus bound to saliva from two of six Le<sup>a-b</sup> individuals both of which were secretors.
Figure 1. Phylogenetic analysis of the outbreak Valencia/2004/Es strain. A) Phylogenetic tree of the NoV RNA polymerase gene (region A in ORF1) from the outbreak (Valencia/2004/Es) and reference strains, obtained from the European Food-borne viruses database [2]. The phylogenetic tree was constructed using the UPGMA clustering method with distance calculation using the Jukes-Cantor correction for evolutionary rate by Molecular Evolutionaty Genetics Analysis (MEGA version 2.1). B) Phylogenetic tree of the outbreak (Valencia/2004/Es) capsid P2 domain (aa 279 to 405) and selected reference strains. The phylogenetic tree was constructed using the UPcMA clustering method with distance calculation using the Poisson correction for evolutionary rate by Molecular Evolutionaty Genetics Analysis (MEGA version 4.1).

doi:10.1371/journal.pone.0005593.g001
The four Le^a+b^- individuals whose saliva could not bind the virus were all non-secretors.

To confirm the histo-blood group phenotype of the saliva samples from the two non-secretors, ABO and Lewis phenotyping was performed on these saliva samples. Consistent with the FUT3 Lewis-positive genotyping, the two saliva samples from the secretor-negative individuals (patient A and B) were identified as Le^a+b^- by phenotyping (Figure 3A). Furthermore, neither patient A nor B expressed A or B antigen in saliva, which is, indirectly, further support that they indeed were non-secretors.

To determine if saliva (collected at a second time point) of patient A (symptomatically infected non-secretor) and patient B (asymptomatic non-secretor whose saliva bound the outbreak virus) would bind NoV from different genogroups and genotypes, a saliva VLP assay was established. Figure 3B shows that saliva from patients A and B, in contrast to secretor-positive controls

![Figure 2. Binding of authentic Valencia/2004/Es virus to saliva.](image-url)

**Table 1.** Strong but not absolute correlation between the G428A FUT2 nonsense mutation and symptomatic NoV infection.

| No (%) of patients | SeSe | Sese | LeLe/Lele | SeSe/Sese/LeLe/Lele |
|-------------------|------|------|-----------|---------------------|
| Symptomatic       | 34 (56.7) | 13^* (38.2) | 20^* (58.8) | 1^* (2.9) |
| Asymptomatic/Nonexposed | 26 (43.3) | 4^* (15.4) | 6^* (23.1) | 16 (61.5) |
| Total             | 60 | 17 (28.3) | 26 (43.3) | 17 (28.3) |

^*SeSe and Sese^428 vs. se^428 se^428 p<0.001.

^*Le^a+b^- vs. Le^a+b+ P<0.001.

The Lewis-genotype could be determined in 44 of 60 individuals.

SeSe and LeLe: homozygous wildtype for FUT2 and FUT3.

Sese and Lele: heterozygous for the inactivating mutations of FUT2 and FUT3.

Le^a+b+: Secretor-negative Lewis-positive phenotype.

Le^a+b+: Secretor-positive Lewis-positive phenotype.

lele: Secretor-negative phenotype.

doi:10.1371/journal.pone.0005593.t001

**Figure 2.** Binding of authentic Valencia/2004/Es virus to saliva. Binding of saliva from secretors and non-secretors to the GII.4 outbreak virus. Dotted line represents cut-off value. Cut-off value (0.380) was three times the mean OD450 of three known negative control samples. The box shows interquartile range; the range between the first and third quartiles. Median is marked as a horizontal line within the box. Whiskers represent samples not more than 1.5 times the box width away from the box. The ring represents a sample within 1.5–3 box lengths from upper or lower edge of the box and the asterisk marks an extreme case (a value more than 3 box lengths from the edge of the box), here representing patient B (asymptomatic non-secretor).

doi:10.1371/journal.pone.0005593.g002
(ABLeSe and ALeSe individuals) did not bind VLP from GI (Norwalk strain), GII.3 (Chron1 strain) or GII.4 (Dijon strain), the latter belonging to the same genotype as the outbreak strain. Figure 3B also shows that saliva of secretor-negative controls (ALese and Olese individuals) did not bind any of the VLPs. The outbreak Valencia strain have distinct amino acids in antigenic A and B regions of the P2 domain compared to the Dijon strain. The fact that saliva from a non-secretor (patient B) recognized the outbreak virus strain and one non-secretor become ill (patient A) raised the question if the virus had an unique aa sequence in the P2 domain of the capsid protein. To address this question the P2 domain (nt 667 to nt 1382) of three random samples (no 207, 208, 225) was sequenced and a BLAST search on the NCBI server was performed. This revealed not only that the Valencian isolates were identical in the P2 domain but also that the outbreak strain was most similar to the GII.4 strain Monastir/2003/Tun [EU916960] isolated in Tunisia 2003 [36]. The Valencia/2004/Es and the Monastir/2003/Tun strains were identical in every position (aa 248 to 420) except for a conserved substitution at residue 356, where a valine in the Monastir strain was changed for an isoleucine in the Valencia strain.

Despite that saliva from patient B bound the outbreak virus, it did not bind the Dijon VLP used in the binding assay, even though both strains belong to GII.4. This observation suggested that the Valencia strain might have HBGA-receptor specific domains different from the Dijon strain. To investigate this, the aa of the P2 domain of Valencia and Dijon were aligned and compared (Figure 4). Particular interest was given to antigenic region A and B of the P2 domain that previously have been associated with molecular evolution [34]. As illustrated in Figure 4 antigenic region A and B of the Valencia strain are distinct from the Dijon strain, all 3 aa in each antigenic site are different. The Valencia strain have a TQN and a STT motif in the A and B site respectively, while the Dijon strain have a SHD motif in site A and a –NN motif in site B. Besides the A and B sites, 11 additional substitutions (marked number 1–11, Figure 4) were found when comparing the capsid P2 domain of Valencia and Dijon, 10 (number 2 to 11, figure 4) out of these 11 correlated with evolutional hot spots identified by Allen and coworkers [34].

**Discussion**

In this study we describe an outbreak with a GII.4 NoV affecting both secretors and a non-secretor, but with significantly higher susceptibility among secretors (76.7%, 33/43) compared to non-secretors (5.9%, 1/17) (P<0.001). One female non-secretor (patient A) was symptomatically infected and virus was found in her stool. FUT2 genotyping identified 38.2% (13/34) of the infected individuals as homozygous wild types, 58.8% (20/34) were heterozygous secretors and one (2.9%) was a non-secretor. Among asymptomatic/non-exposed individuals, 15.4% (4/26) were homozygous secretors, 23.1% (6/26) were heterozygous and 61.5% (16/26) were non-secretors. The total frequency of
non-secretors was 28.3% (17/60) and was thus higher than the frequency of the Northern European population, which is approximately 20% [19,21,23,37]. Saliva from a non-infected non-secretor (patient B) recognized the outbreak virus in a saliva-based ELISA, suggesting that the virus may have different binding properties from what is generally observed among GII.4 NoV strains. A similar finding has previously been reported by Lindesmith and co-workers [33] who demonstrated binding of a GII.4 virus to saliva from both secretors and non-secretors.

Most surprisingly, we found that saliva from the non-secretor did not only fail to recognize the Chron GII.3, Norwalk G1.1 but also the GII.4 Dijon VLP, even though both Valencia/2004/Es outbreak virus and the Dijon strains belong to GII.4. This observation implied that the Valencia strain might have HBGA-receptor specific domains different from the Dijon strain, suggesting that association of histo-blood group antigens with susceptibility to NoV infection may be strain-specific rather than genogroup dependent [38]. Indeed, recent data suggest variations in HBGA recognition within the GII.4 genotype [33].

To investigate the possibility that the outbreak virus had unique aa sequence and structural properties in the capsid protein, the P2 domain (aa 248 to 420), of 3 isolates (patients no 207, 208 and 225) were sequenced and BLAST search was performed. This revealed that the outbreak strain belonged to the globally dominating GII.4 genotype and was most similar to the Monastir/2003/Tun [EU916960] strain isolated in Tunisia 2003. The Valencia and the Monastir capsid were identical in the investigated region except for a conserved substitution at residue 356, where a valine in the Monastir strain was changed for an isoleucine [4,7,33,34,40]. Recent studies have shown that a given NoV genotype predominates in a season, such as GII.4 in Europe and the United States [21,41–44]. This predominance of a given genotype or cluster is followed by a sharp drop in prevalence of the genotype in the following season [40,44], which again is followed by the emergence of a genetically distinct lineage. Thus, periods of phenotypic stasis are followed by the emergence of novel epidemic strains [33,40].

Allen and co-workers [34] have found two antigenic regions (site A and B) in the P2 domain of the VA387 crystal structure, where aa substitutions in this area have impact on the biochemical properties as well as the entire structure of the P2 domain. These positions, located to external parts in the capsid, are part of exposed loops and thus changes in site A and B may have a strong association with the emergence of novel NoV strains [34]. Since the non-secretor saliva bound the outbreak strain but not the Dijon strain, we decided to compare the antigenic site A and B of the Valencia/2004/Es strain with the Dijon strain. Alignment of the P2 domain from Valencia/2004/Es and Dijon revealed that

---

Figure 4. Comparison of antigenic sites between the outbreak Valencia/2004/Es and Dijon strain. The outbreak Valencia/2004/Es strain have distinct amino acids in antigenic A and B regions of the P2 domain compared to the Dijon strain. Amino acid alignment (Clustal W 1.8 with default parameters on the European Bioinformatics Institute server) of partial capsid protein (aa 241 to 419) of the Dijon171/96 [AF472623] and the Valencia/2004/Es strains, are obtained from three randomly chosen patients (no 207, 208 and 225). The aa constituting the P2 domain (aa 279 to 405) are shaded in light grey. Antigenic site A (aa 296–298) and site B (393–395) proposed by Allen et al. [34] are indicated. The Valencia/2004/Es has a TQN and a STT motif in the A and B site, while the Dijon strain have a SHD motif in site A and a –NN motif in site B. Besides from the A and B site, 11 additional substitutions (marked number 1 to 11) were found when comparing the capsid P2 domain of Valencia and Dijon. 10 out of these 11 (number 2 to 11) correlated with evolutionary hot spots (highlighted by dark grey) identified by Allen and coworkers [34].

---

doi:10.1371/journal.pone.0005593.g004

PLoS ONE | www.plosone.org 6 May 2009 | Volume 4 | Issue 5 | e5593

Novel GII.4 Disease Pattern
all three amino acids in both antigenic region A and B were
unique in Valencia/2004/Es as compared to the Dijon strain,
possibly causing the differences in binding properties between
the virus strains.

The Valencia/2004/Es strain have a TQN and a SIT motif in
the A and B site respectively, while the Dijon strain have a SHD
motif in site A and a NN motif in site B. According to Allen and
coworkers, the motif found in the Valencia strain characterize
GI.4 strains isolated in 2004, 2005 and 2006, while the motif from
the Dijon strain were found in strains circulating during 1997, 1998
and 1999 [34]. This pattern correlates well to the year of isolation
both for the Valencia (2004) and the Dijon strains (1996). Besides
from the A and B site, 11 additional substitutions were found when
comparing the capsid P2 domain of Valencia/2004/Es and Dijon
strains, 10 out of these 11 correlated with evolutionary hot spots
identified by Allen and coworkers [34]. We therefore speculate
that the aa differences observed in site A, B and in the evolutionary
hot spots, may affect the structural and electrostatic properties of
the capsid protein, perhaps giving a clue to the different binding
patterns observed between the Valencia/2004/Es and the Dijon
strain. However, when aligning the Dijon and the Valencia/2004/
Es strains, we also observed differences in aa surrounding the A
and B trisaccharide binding site proposed by Cao et al [6]. These
positions include e.g. residue 346, 372, 389 but also residue 393
(part of the antigenic site B identified by Allen and coworkers [34])
which all differ between the Dijon and the Valencia/2004/Es
strains. Of these positions, 393/394 are also identified by
Lindesmith and coworkers, as one site out of six in the NoV
capsid operating under positive selection [33]. Lindesmith and
coworkers suggest that the aa in position 393/394 may play an key
role in receptor binding and impact immunogenic properties of
the virus [33]. Also a study by Siebenga and coworkers describing
epochal evolution of GI.4 capsid proteins, identified position
393/394 as a hypervariable site during evolution [40]. We thereby
can not rule out that also the observed differences surrounding the
A and B trisaccharide binding site, perhaps in combination with the
changes in antigenic site A and B may contribute to the unusual
saliva binding properties observed in the outbreak virus.
Unfortunately, no host genetic susceptibility data are available for
the Monastir/2003/Tun strain. This would have been most
informative since the capsid protein of the Monastir/2003/Tun
strain are most similar to the Valencia/2004/Es strain and contain
identical aa in both antigenic site A and B.

Another possibility that might explain the uncommon saliva
binding property observed for the Valencia/2004/Es strain could
be the unexpected appearance of type 1 chain ABH or Lewis b
antigens in patient A and B. To investigate this, Lewis genotyping
and AB(O) and Lewis phenotyping was performed on saliva from
these two patients. These investigations concluded that the patients
are indeed Le^a+b^ non-secretors. However, additional structures
used by NoV for binding, may be present in the saliva of these
individuals.

Although sero-epidemiology studies have shown that secretors
have significantly higher antibody titers and prevalence against
NoV than non-secretors [24], the fact that certain non-secretors
are NoV antibody–positive [24,26], suggests that secretor-
independent infections do occur. Also, a few documented NoV
infections of non-secretors have been reported [25,26].

Six of 44 investigated individuals were found to be Le^a+b^- by
genotyping. Four of these individuals were secretor-negative and
two were secretor-positive. Of these, only the secretor-positive
Le^a+b^- individuals were symptomatically infected. We therefore
speculate that secretor status but not Lewis status may correlate
with NoV susceptibility. Further support for this hypothesis is also
the observation that Lewis status did not predict binding of virus to
saliva (Figure 2). The conclusions are consistent with a previous
observation by Larsson and co-workers [24] who found that
antibody titers and prevalence to NoV correlate with secretor
rather than with Lewis status. This is also in agreement with the
result from Bucardo and co-workers who found NoV susceptibility
to be independent of Lewis-phenotype [45].

While the outbreak-virus infected as well as recognized saliva
from a non-secretor, current information cannot explain why not
all non-secretors were infected or why the virus did not recognize
all non-secretor saliva. Possible explanation for this might be that
the infected secretor-negative individual may express receptors
present in only a limited number of non-secretors, or an unusual
high concentration of a receptor commonly found at low
concentration in non-secretors. Rydell and co-workers recently
showed that GII.3 and GII.4 VLPs could bind Sialyl-Lewis x on
neoglycoproteins, suggesting that this may be a possible determi-
nator of NoV tropism [46]. Also, Taube and co-workers have
suggested a role of siaic acid moieties in murine NoV attachment
to murine macrophages [47]. Furthermore, Tamura and
coworkers have shown that NoV GII VLPs efficiently bind surface
heparin sulfate on the surface of different cell lines [48]. Together
these studies indicate a role of sialylated structures or heparin
sulfate in NoV cell tropism. Another possibility is that certain
individuals are immune. This speculation is supported with data
from other studies showing that not all secretors are infected in a
given outbreak [19]. Furthermore, short term immunity have been
demonstrated in volunteer studies [12].

In conclusion we show for the first time symptomatic NoV
infection caused by a GI.4 strain in a secretor-negative Le^a+b-
individual homozygously mutated at nt 420 of FUT2.

Materials and Methods

Subjects and samples

Fecal samples, collected from 26 symptomatic residents and
from 7 staff members, were sent to the local hospital laboratory for
bacteriology and virology investigation. Enteropathogenic bacteria
(Salmonella, Shigella, Campylobacter, Versinia and Aeromonas species)
were investigated by conventional bacterial culture procedures
[49] and rotavirus and adenovirus were analyzed by enzyme
immunoassay (Premier Rotacode and Premier Adenocline 40/
41, Meridian Bioscience Inc., Cincinnati, Oh.) and NoV by RT-
PCR. Saliva samples were collected from 39 residents, including
symptomatic patients and asymptomatic controls and from 21
health care workers of which 11 got sick and 10 remained
asymptomatic (controls).

Characterization of the nonsense FUT2 mutations G428A,
C571T and the missense mutation A385T

The secretor genotype was determined by pyrosequencing as
described [37]. Briefly, DNA was extracted from 200 μl of saliva
using QIAamp® DNA Mini kit (Qiagen, Hilden, Germany).
Extracted DNA was stored in TE-buffer in Eppendorf tubes at
−20°C until PCR amplification. For PCR amplification forward
primers 5’-BIOTIN-GAT GGA GGA GGA GGA ATC CCG CCA C-5’
(FUT2 426), 5’-CGA CTG GAT GGA GGA GGA ATA C-3’ (383)
and 5’-BIOTIN-GCA CCT TTG TAG GGG TCC A-3’ (571) were used
together with reverse primers 5’-TGG GGC TCC TCC
TCC CGC ACG T-3’ (428), 5’-GGG TGA AGC GGA CTT
ACT-BIOTIN-3’ (383) and 5’-CTT CAC TTT TGG CAT
GAC-3’ (571). For sequencing the following primers were used: 5’-
GGT GGT AGT AGG GCC-3’ (FUT2 428), 5’-GAG GAA
TAC CGC CAC-3’ (385) and 5’ TGG ACA TAG TCC CCT C-3’ (571).
FUT2 genotyping for mutations G428A, C571T and A385T was independently also performed by PCR-SSP as published [50].

Determination of Lewis genotype and Lewis and AB phenotypes of saliva

Lewis genotype was determined using PCR-SSP as described elsewhere [50], detecting the mutations T59G, T202C, C314T, G508A and T1067A of the FUT3 gene. Lewis phenotypes were identified in an ELISA assay [46] using flat-bottom MaxiSorp Microtiter plates (NUNC, Roskilde, Denmark), anti blood group A (AB01 clone 9113D10) and anti B (AB02 clone 9621A0) antibodies (Diagost, Loos Cedex, France), anti Lea (Seracleone, LE1 clone 78FR 2.3) and anti Leb (Seracleone LE2 clones LM129-181 and 96 FR210) antibodies (Biotest AG, Dreieich, Germany) and finally HRP conjugated goat anti-mouse IgG (170-6516, Bio-Rad, Hercules, CA, USA) and TMB (T0440, Sigma) as substrate. The absorbance values were read at 450 nm (Labsystems iEMS Reader MF, Labsystems, Helsinki, Finland) and average values for duplicate wells calculated.

Binding of the outbreak NoV to saliva

To investigate if the outbreak strain could bind to saliva from symptomatic and asymptomatic individuals, a saliva-based ELISA was performed as described [19,51] with some modifications. Saliva samples were boiled, centrifuged at 10,000 x g for 5 min, and diluted 1:500 in coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.6). After 2 h of incubation at 37°C followed by overnight incubation at 4°C, the plates were washed three times with washing buffer (0.9% NaCl 0.05% Tween 20) and blocked (3% bovine serum albumin, BSA in PBS) for 60 min at 37°C. A NoV stool suspension in PBS (10% w/v) was diluted 1:2 in PBS with 0.05% Tween 20 and 0.5% BSA, and incubated for 2 h at 37°C followed by washing of the plates three times with washing buffer (0.9% NaCl 0.05% Tween 20) and then incubation with peroxidase-labeled genotype I and II-specific NoV polyclonal antibody (Dako, Denmark) for 1 h at 37°C. The reaction was developed using TMB (ICN Biochemicals) and the plate was read at 450 nm. The cut-off value was three times the mean OD450 value of three known negative control samples.

Binding of NoV VLP to saliva

Norwalk (GL1) and Dijon (GIL4) purified VLP was a kind gift from Jacques le Pendu and were used essentially as described above. Briefly VLP (0.16-2 μg/ml depending on VLP) was diluted in dilution buffer (0.5% BSA and 0.05% Tween 20 (Sigma) in PBS) and added to each saliva-coated well, and incubated at 37°C for 1.5 h. Following 3 × washes appropriate anti NoV antiserum was incubated for 1.5 h at 37°C. After washing HRP conjugate was added and incubated for 1.5 hr at 37°C followed by 3 × washes and development by TMB as described above. The Chron1 (GII.3) VLP is a construct from a NoV strain cloned from an patient with a chronic NoV infection [4]. Production of recombinant VLP was done in Sf9 cells. Briefly Sf9 cells were infected and harvested 5 days p.i. Cells and media were then centrifuged at 2000 rpm for 10 min and the supernatant collected and pelleted by centrifugation at 30 000 rpm, 2 h in SW41Ti and resuspended in PBS followed by purification in a sucrose gradient. Purity and integrity was determined by Coomassie staining and western blot [46].

RNA extraction and RT-PCR

Fecal suspensions (10% w/v in PBS) were clarified by low speed centrifugation. RNA was extracted by using the QiAamp viral RNA kit, according to the manufacturer’s instructions (Qiagen, Hilden, Germany). Purified RNA was then resuspended in 50 μl of RNase-free water and used as template for RT-PCR using primer pairs JV12/JV13 [52].

Virus genotyping

Genotyping was performed by nucleotide sequencing of the PCR products obtained with primers JV12/JV13 (viral RNA polymerase gene) and Mon381/Mon383 (viral capsid gene) [53]. Sequencing was carried out in both directions using the BigDye Terminator cycle sequencing kit (Perkin-Elmer) on an automated ABI PRISM model 3177 machine (Applied Biosystems). Sequence alignments were carried out by using ClustalW1.8 with reference strains obtained from the Foodborne Virus in Europe (FBVE) database [https://hycorpsates.rivm.nl/bnwww/Divine-Event/in dex.html]. A dendrogram was constructed using the UPGMA clustering method with distance calculation using the Jukes-Cantor correction for evolutionary rate by Molecular Evolutionary Genetics Analysis (MEGA version 2.1).

PCR amplification of NoV capsid P2-region

For the reverse transcriptase reaction 5 μl (0.5 μg) pe(N)6 primer (Amersham Bioscience UK Limited, Little Chalfont Buckinghamshire, UK), 28 μl extracted viral RNA and 17 μl RNase free water was added to an Illustra Ready-To-Go RT-PCR bead (GE-health care, Uppsala, Sweden) and the reaction was performed at 42°C for 60 minutes followed by inactivation of the enzymes at 95°C for 5 minutes.

In order to amplify the NoV capsid P2 domain, a PCR reaction containing 45 μl PCR SuperMix high fidelity (Invitrogen, Carlsbad, USA), 1 μl 10 μM Val fw1 (5’-GAA GTA AAC CAT TCT CTC GTC C-3’) as forward primer, 1 μl 10 μM Val rv1 (5’-AAG TGC TGC ACC CA CTG CTG-3’) as reverse primer, and 2 μl cDNA was mixed. The PCR reaction was performed at 94°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 68°C for 1 minute, before a final elongation at 68°C for 10 minutes. PCR-products were visualized on a 1% agarose gel, using EtBr staining and UV light. The final PCR amplicon had a length of ~700 bp (nt667 to nt1382).

Sequence analysis

Nucleotide sequencing of the P2 region was performed by Macrogen Inc. (Seoul, South Korea). The sequencing reaction was based on BigDye chemistry, using forward primer Val fw1 and reverse primer Val rv1 as sequencing primers. The amplicons were sequenced twice in each direction, and complete sequences were obtained by assembling overlapping contigs with DNASTAR (DNASTAR, Inc., Madison, Wisconsin, USA). Multiple sequence alignment of NoV capsid proteins was performed, using the ClustalW 1.8 algorithm with default parameters on the European Bioinformatics Institute server. This data was also used for constructing a phylogenetic tree of the outbreak strain capsid P2 domain and reference strains. The phylogenetic tree was constructed using the UPGMA clustering method with distance calculation using the Poisson correction for evolutionary rate by Molecular Evolutionary Genetics Analysis (MEGA version 4.1).

Statistical analysis

Fisher’s exact test (two-sided) was used to test significant differences in distribution of secretor-positive and secretor-negative individuals among symptomatic and asymptomatic/non-exposed. Mann-Whitney test was used to compare ELISA absorbance values between secretor-positive and secretor-negative
individuals as well as individuals with different Lewis phenotypes (Le$^{a+}$, Le$^{b+}$, and Le$^{a-}$). SSFS 16 for Mac was used to perform these analyses and a P-value of <0.05 was considered statistically significant.

Acknowledgments

The study was approved by the local ethical committees and included informed consent for genetic testing of saliva samples. We thank Dr. Carmina Rubert for her assistance in collecting samples from the patients and analyzing the outbreak. We also thank Jaques Le Pendu for kindly providing the Dijon and Norwalk VLP.

Author Contributions

Conceived and designed the experiments: BC EK JB JRD GL LS. Performed the experiments: BC EK GER MFI RM RAM AG JB AA. Analyzed the data: BC EK GER MFI RM RAM AG JB AA. Contributed reagents/materials/analysis tools: JB GL LS. Wrote the paper: BC EK JB GER LS.

References

1. Fankhauser RL, Noel JS, Monroe SS, Ando T, Glass RI (1998) Molecular epidemiology of “Norwalk-like viruses” in outbreaks of gastroenteritis in the United States. J Infect Dis 178: 1571–1578.
2. Lopman BA, Reacher MH, Van Duijnhoven Y, Hanson EX, Brown D, et al. (2003) Viral gastroenteritis outbreaks in Europe, 1995–2000. Emerg Infect Dis 9: 90–96.
3. Green KY, Chanock RM, Kaplakian AZ (2001) Human caliciviruses 041–074, Baltimore, Md: Lippincott, Williams & Wilkins.
4. Nilsson M, Hedlund KO, Thoelhagen M, Larson G, Johansen K, et al. (2003) Evolution of human calicivirus RNA in vivo: accumulation of mutations in the protruding P2 domain of the capsid leads to structural changes and possibly a new phenotype. J Virol 77: 13117–13124.
5. Tan M, Huang P, Meiler J, Zhong W, Farkas T, et al. (2003) Mutations within the P2 domain of norovirus capsid affect binding to human histo-blood group antigens: evidence for a binding pocket. J Virol 77: 12562–12571.
6. Cao S, Lou Z, Tan M, Chen Y, Liu Y, et al. (2007) Structural basis for the recognition of blood group trisaccharides by norovirus. J Virol 81: 5949–5957.
7. Donaldson EF, Lindesmith LC, Lobue AD, Baric RS (2008) Norovirus pathogenesis: mechanisms of persistence and immune evasion in human populations. Immunity 22: 190–211.
8. Choi JM, Hutson AM, Estes MK, crossed BV (2008) Atomic resolution structural characterization of recognition of histo-blood group antigens by norovirus. Proc Natl Acad Sci U S A 105: 9175–9180.
9. Glass RI, Noel J, Ando T, Fankhauser R, Belliot G, et al. (2000) The expression of a candidate for the human Secretor blood group alpha(1,2)fucosyl-transferase gene (FUT2). Homozygosity for an enzyme-inactivating nonsense mutation commonly correlates with the non-secretor phenotype. J Biol Chem 270: 4640–4649.
10. Wyatt RG, Dolin R, Blacklow NR, DuPont HL, Buscho RF, et al. (1974) Antibody prevalence and its relation to norovirus (genogroup II) correlate with secretor (FUT2) but not with ABO phenotype or Lewis (FUT3) genotype. J Infect Dis 194: 1422–1427.
11. Koda Y, Soejima M, Kimura H (2001) The polymorphisms of fucosyltransferases. Leg Med (Tokyo) 3: 2–14.
12. Liu Y, Koda Y, Soejima M, Pang H, Schlapfer T, et al. (1998) Extensive polymorphism of the FUT2 gene in an African (Xhosa) population of South Africa. Hum Genet 103: 298–301.
13. Henry S, Mollirone R, Lowe JB, Samuelson B, Larson G (1996) A second nonssecretor allele of the blood group alpha(1,2)fucosyl-transferase gene (FUT2). Vox Sang 70: 21–25.
14. Henry S, Mollirone R, Fernandez P, Samuelson B, Oriol R, et al. (1996) Molecular basis for erythrocyte Le(a+b+) and salivary ABH partial-secretor phenotype: expression of a FUT2 secretor allele with an A+ T mutation at nucleotide 363 correlates with reduced alpha(1,2) fucosyltransferase activity. Glycobiology 11: 803–903.
15. Huang P, Farkas T, Zhong W, Tan M, Thornton S, et al. (2005) Norovirus and histo-blood group antigen: demonstration of a wide spectrum of strain specificities and classification of two major binding groups among multiple binding patterns. J Virol 79: 6714–6722.
16. Huang P, Farkas T, Marianneau S, Zhong W, Ruvoen-Clouet N, et al. (2003) Noroviruses bind to human ABO, Lewis, and histo-blood group antigen: identification of 4 distinct strain-specific patterns. J Infect Dis 188: 19–31.
17. Lindesmith LC, Donaldson EF, Lobue AD, Cannon JL, Zheng DP, et al. (2008) Mechanisms of GI4 norovirus persistence in human populations. PLoS Med 5: e1485.
18. Allen DJ, Gray JJ, Gallimore CL, Easterday B, Brune E, et al. (2000) Analysis of amino acid variation in the P2 domain of the GI-4 norovirus VP1 protein reveals putative variant-specific epitopes. PLoS ONE 3: e1485.
19. Larson G, Svensson L, Hyrko T, Nygren M, Rydenberg J, et al. (2003) RNA expression in human lewis blood group system by quantitative (fluorescence-activated flow) cytometry: large differences in antigen presentation on erythrocytes between A(1), A(2), B, O phenotypes. J Virol 79: 227–236.
20. Sibiou-Loulis K, Ambert-Balay K, Gharbi-Klevit H, Sahly N, Hassine M, et al. (2009) Molecular epidemiology of norovirus gastroenteritis investigated using samples collected from children in Tunisia during a four-year period: detection of the norovirus variant GI4 Hunter as early as January 2003. J Clin Microbiol 47: 421–429.
21. Kindberg E, Heijlman B, Bratt G, Wahren B, Lindblom B, et al. (2006) A nonsense mutation (428G→A) in the fucosyltransferase FUT2 gene affects the progression of HIV-1 infection. AIDS 20: 685–689.
22. Tan M, Jiang X (2008) Association of histo-blood group antigen in norovirus infection with strain-specific rather than genogroup dependent. J Infect Dis 198: 940–941; author reply 942–943.
23. Chakravarty S, Hutson AM, Estes MK, Prasad BV (2005) Evolutionary trace residues in noroviruses: importance in receptor binding, antigenicity, virion assembly, and strain diversity. J Virol 79: 354–360.
24. Gallimore CL, Green J, Lewis D, Richards AF, Lopman BA, et al. (2004) Diversity of noroviruses cocirculating in the north of England from 1998 to 2001. J Clin Microbiol 42: 1396–1401.
25. Blanton LH, Adams SM, Beard RS, Wei G, Bulens SN, et al. (2006) Molecular and epidemiologic trends of caliciviruses associated with outbreaks of acute gastroenteritis in the United States, 2000-2004. J Infect Dis 193: 413–421.
26. Kroneman A, Verhoef L, Harris J, Vennema H, Duizer E, et al. (2008) Analysis of integrated virological and epidemiological reports of norovirus outbreaks
collected within the foodborne viruses in Europe Network from 1 July 2001 to 30 June 2006. J Clin Microbiol 46: 2959–2965.

44. Siebenla JJ, Vennema H, Duizer E, Koopmans MP (2007) Gastroenteritis caused by norovirus GGII.4, The Netherlands, 1994–2005. Emerg Infect Dis 13: 144–146.

45. Bucardo F, Kindberg E, Paniagua M, Vildevall M, Svensson L (2009) Genetic susceptibility to symptomatic norovirus infection in Nicaragua. J Med Virol 81: 728–735.

46. Rydéll GE, Nilsson J, Rodríguez-Díaz J, Ruvioen-Clozet N, Svensson L, et al. (2009) Human noroviruses recognize sialyl Lewis x neoglycoprotein. Glycobiology 19: 309–320.

47. Taube S, Perry JW, Yetming K, Patel SP, Auble H, et al. (2009) Ganglioside-linked terminal sialic acid moieties on murine macrophages function as attachment receptors for Marine Noroviruses (MNV). J Virol.

48. Tanuma M, Natori K, Kobayashi M, Miyamura T, Takeda N (2004) Genogroup II noroviruses efficiently bind to heparan sulfate proteoglycan associated with the cellular membrane. J Virol 78: 3817–3826.

49. Pezzlo M (1992) Processing and Interpretation of Bacterial Fecal Cultures. In: H.D I, ed. Clinical Microbiology Procedures Handbook. Washington, D.C.: ASM. pp 1.10.11–11.10.25.

50. Grahm A, Ehngren A, Abeg L, Svensson L, Jansson PA, et al. (2001) Determination of Lewis FUT3 gene mutations by PCR using sequence-specific primers enables efficient genotyping of clinical samples. Hum Mutat 18: 350–359.

51. Harrington PR, Lindesmith L, Yount B, Mee CL, Baric RS (2002) Binding of Norwalk virus-like particles to ABO histo-blood group antigens is blocked by antibodies from infected human volunteers or experimentally vaccinated mice. J Virol 76: 12335–12343.

52. Vinje J, Koopmans MP (1996) Molecular detection and epidemiology of small round-structured viruses in outbreaks of gastroenteritis in the Netherlands. J Infect Dis 174: 610–615.

53. Noel JS, Ando T, Leite JP, Green KY, Dingle KE, et al. (1997) Correlation of patient immune responses with genetically characterized small round-structured viruses involved in outbreaks of nonbacterial acute gastroenteritis in the United States, 1990 to 1995. J Med Virol 53: 372–383.