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# cDNA Cloning of Korean Human Norovirus and Nucleotidylylation of VPg by Norovirus RNA-Dependent RNA Polymerase

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**Norovirus, a member of the *Caliciviridae* family, is a major causative agent of gastroenteritis worldwide. The cDNA of the entire genome of human norovirus (HuNV) was cloned using the RNA extracted from the stool sample of a Korean patient. The RNA genome consists of 7,559 nucleotides, carries 3 open reading frames (ORFs), 5′ and 3′ noncoding regions, and a poly(A) tail at the 3′ end. Phylogenic analysis of the nucleotide sequence indicated that it belongs to GII.4, the most dominant genogroup. To analyze RNA synthesis and nucleotidylylation of VPg by RNA-dependent RNA polymerase (RdRp), recombinant RdRp and VPg were expressed in *Escherichia coli* as His-tagged forms. The HuNV RdRp exhibited template and divalent cation-dependent RNA synthesis *in vitro*. The HuNV RdRp nucleotidylylated HuNV VPg but not murine norovirus (MNV) VPg, whereas MNV RdRp nucleotidylylated both MNV and HuNV VPg more efficiently than HuNV RdRp.**

***Keywords***: norovirus, cDNA cloning, RNA-dependent RNA polymerase, VPg

## Introduction

Viruses in the *Caliciviridae* family are non-enveloped, have an icosahedral shape, and possess a linear, positive-sense, single-stranded RNA genome. Four genera are assigned to the family: Norovirus, Sapovirus, Vesivirus, and Lagovirus (David and Peter, 2007). Norovirus is the major causative agent of non-bacterial acute gastroenteritis in humans of all ages in Korea as well as worldwide (Lindell *et al*., 2005; Koh *et al*., 2008; Patel *et al*., 2009). Norovirus is further divided into 5 genogroups (GI, GII, GIII, GIV, and GV) based on the amino acid or nucleotide sequences in the capsid protein-coding region (Zheng *et al*., 2006). Human noroviruses (HuNVs) belong primarily to the genogroups GI, GII, and GIV (Zheng *et al*., 2006), and GII is the dominant circulating genogroup in Korea and worldwide (Dove *et al*., 2005; Yoon *et al*., 2008). The HuNV genome is ~7.7 kb in length, including the poly(A) tail at the 3′ end. The genome has 3 open reading frames (ORFs) as well as 5′ and 3′ untranslated regions. ORF1 encodes precursors of non-structural proteins that undergo autocatalytic cleavage by viral 3C-like protease ($3CL^{pro}$), generating p48, NTPase, p22, VPg, $3CL^{pro}$, and RNA-dependent RNA polymerase (RdRp) (Belliot *et al*., 2003). ORF2 and ORF3 encode major (VP1) and minor (VP2) capsid proteins, respectively (Jiang *et al*., 1993).

The RdRps of positive-strand RNA viruses mediate replication of an RNA template in a primer-independent or primer-dependent manner with the divalent metal cation $Mg^{2+}$ or $Mn^{2+}$ (van Dijk *et al*., 2004). For example, poliovirus RdRp achieves replication of the RNA template with $Mn^{2+}$ in a primer-dependent manner (Plotch *et al*., 1989; Paul *et al*., 1998; Arnold *et al*., 1999). In norovirus, RdRp has 2 active forms: the precursor protein proteinase-polymerase form (ProPol) and the proteinase-cleaved polymerase form (Pol) (Belliot *et al*., 2005). Both ProPol and Pol are known to initiate RNA replication with a poly(A) template (Belliot *et al*., 2005).

VPg is covalently linked to the 5′ end of RNA genomes in many virus families such as *Picornaviridae*, *Potyviridae*, *Luteoviridae*, and *Comoviridae* (Sadowy *et al*., 2001). Since the animal caliciviruses have VPg linked to the 5′ ends of the genomic and subgenomic RNA (Burroughs and Brown, 1978; Dunham *et al*., 1998), it has been suggested that HuNV also has VPg at the 5′ end of their genome. In poliovirus, nucleotidylylated VPg participates in the replication of the RNA genome by acting as a primer for initiation of replication. In addition, it is proposed that the birnaviruses can initiate RNA synthesis of both strands of dsRNA by using protein primers (Xu *et al*., 2004).

Nucleotidylylation of HuNV VPg can be mediated by both forms of RdRps. The ProPol form is able to nucleotidylylate VPg without a poly(A) template, but the Pol form is template-dependent (Rohayem *et al*., 2006; Belliot *et al*., 2008). With nucleotidylylated VPg, RdRp was able to initiate RNA replication of a poly(A) template, thus producing VPg-poly(U) (Rohayem *et al*., 2006). Further, RNA replication using VPg as a protein primer was more efficient than that using an oligo(N) primer (Rohayem *et al*., 2006).

Apart from protein priming, the VPgs of many viruses are involved in translation and transportation (Sadowy *et al*., 2001). VPg participates in the initiation of translation in the virus by interacting with different host proteins. Potato virus VPg interacts with viral RNA polymerase and host eIF4E. Moreover, in calicivirus, VPg interacts with various

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**Table 1.** Primers used in PCR for amplifying human norovirus cDNA

| | Name | Orientation | Sequence | Position |
|---|---|---|---|---|
| Clone #1 | HuNV-1-F | + | GTG AAT GAA GAT GGC GTC TAA CGA CGC T | 1–28 |
| | HuNV-1-R | - | CCT TTT CGA CTT CAG GGG CTT CTG CAT A | 1845–1872 |
| Clone #2 | HuNV-2-F | + | AGG TTG CAA GAA CTC GCT GAC ACT TGC | 1679–1705 |
| | HuNV-2-R | - | CCT TGC CAC CAA GAT AGG CTG GTT CAT A | 3692–3719 |
| Clone #3 | HuNV-3-F | + | TCG TGG GGG AAA CAC TGT CAT ATG TGC | 3511–3537 |
| | HuNV-3-R | - | GGA CTC GAC AAG AGA CTG TGA AGA CAT C | 5279–5306 |
| Clone #4 | HuNV-D-F | + | CAR GAR BCN ATG TTY AGR TGG ATG AG[a] | 5029–5054 |
| | HuNV-4-R | - | AAA AGA CAC TAA AGA AAG GAA AGA TAA TC | 7531–7559 |

[a] N, A+T+G+T; D, A+T+G; Y, C+T; S+C+G; R, A+G; I, inosine

host proteins, including eIF3 and eIF4E (Daughenbaugh *et al.*, 2003; Goodfellow *et al.*, 2005; Chaudhry *et al.*, 2006).

Because of the lack of an efficient tissue culture system, the molecular mechanisms of HuNV replication remain poorly understood. Since murine norovirus (MNV) isolated from immunocompromised mice (Karst *et al.*, 2003) was successfully incorporated into the macrophage-derived cell line RAW264.7 (Wobus *et al.*, 2004), MNV has been used as a surrogate system to study HuNV replication (Han *et al.*, 2010).

In this study, the full-length cDNA sequence of Korean-type HuNV was cloned. Phylogenic analysis indicated that it belongs to GII.4, the most dominant genogroup. cDNAs encoding RdRp and VPg were subcloned and expressed in *Escherichia coli*. With these recombinant proteins, the nucleotidylylation of VPg by RdRp was examined.

## Materials and Methods

### RNA extraction

A stool sample was collected from a Korean patient with symptoms of gastroenteritis. About 200 µl of stool was dissolved in phosphate-buffered saline (PBS), followed by addition of 750 µl of Trizol (Invitrogen, USA). After 5-min incubation at room temperature, 250 µl of chloroform was added, mixed, and centrifuged at 15,700×*g* for 15 min. The RNA in the aqueous phase was precipitated by adding 500 µl of isopropanol and recovered by centrifugation at 15,700×*g*. The pellet was washed with 800 µl of 70% ethanol, dried, and dissolved in 20 µl of RNase-free DI water.

### cDNA synthesis

cDNA was synthesized with M-MLV Reverse Transcriptase (Bioneer, Korea), according to the manufacturer's instruction. A 10-µl mixture including 1 µg of RNA, 20 pmol random 9-mer, and oligo(dT) was incubated at 65°C for 5 min in a thermocycler (Eppendorf, Germany) for RNA denaturation and primer annealing, and then chilled on ice. For reverse transcription, 20 µl of the reaction mixture including 1× M-MLV RTase reaction buffer, 10 µl of RNA-primer mixture, 10 mM DTT, 5 mM dNTPs, 20 units RNase inhibitor (TaKaRa, Japan), and 200 units M-MLV reverse transcriptase (Bioneer) was incubated at 42°C for 1 h, followed by enzyme inactivation at 70°C for 10 min. Norovirus dsDNA was amplified by PCR with 4 sets of primers (Table 1). After denaturation at 95°C for 5 min, 35 cycles of PCR were performed at 95°C for 30 sec, at 55°C for 30 sec, and at 72°C for 2 min 30 sec. Final extension was carried out at 72°C for 5 min. The PCR products were cloned into the T-blunt vector (Solgent, Korea).

### Nucleotide sequence analysis

The nucleotide sequence of the cloned Korean-type human norovirus ORF2 region was compared with the published norovirus sequences present in GenBank by a MegAlign software

### Expression of RdRp and VPg in *E. coli*

cDNAs encoding RdRp and VPg were amplified by PCR using the primers listed in Table 2 (Fukushi *et al.*, 2004; Belliot *et al.*, 2005), and the PCR products were cloned into the *Nde*I and *Bam*HI sites of the pET-14b expression vector (Novagen, USA). The *E. coli* BL21 Rosetta strain, previously transformed by the expression vectors, was grown at 37°C in 500 ml of Luria-Bertani medium with ampicillin. The cells were grown until the optical density reached 0.6, and then, protein expression was induced with 1 mM IPTG at 30°C for 12 h. The cells were harvested by centrifugation at 4,000×*g* for 30 min, were resuspended with 10 ml of lysis buffer (50 mM $NaH_2PO_4$; pH 8.0, 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, 0.5% Tween-20, and 10% glyc-

**Table 2.** Primers used in PCR for constructing RdRp and VPg expression vectors

| | Name | Orientation | Sequence | Position |
|---|---|---|---|---|
| RdRp[a] | RdRp-F | + | cat atg GGT GGT GAC AGT AAG GGA ACA TAC | 3572–3595 |
| | RdRp-R | - | gga tcc TCA TTC GAC GCC ATC TTC ATT Ca | 5104–5081 |
| VPg[a] | VPg-F | + | cat atg GGT AAG AAA GGG AAG AAC AAG ACT GG | 2630–2655 |
| | VPg-R | - | gga tcc TTA[b] CTC AAA GCT GAG TTT TTC ATT GTA GTC C | 3028–3001 |

[a] cat atg, *Nde*I; gga tcc, *Bam*HI
[b] TAA, Stop codon

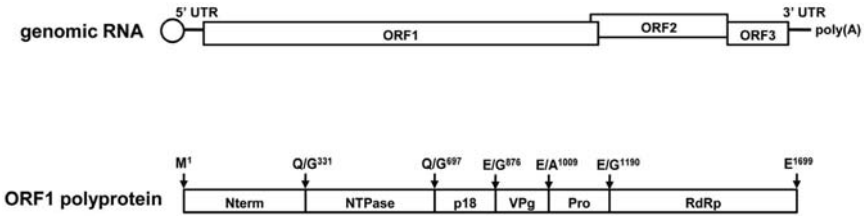


**Fig. 1. Schematic representation of the genome organization of Korean human norovirus.** The genome contains 3 ORFs, 5′ and 3′ untranslated regions, and a poly(A) tail. VPg, depicted as a circle, is presumptively linked covalently at the 5′ end of the genome (top). ORFs 2 and 3 encode the major and minor capsid proteins, respectively. ORF1 encodes a non-structural polyprotein and is cleaved by protease. The putative protease cleavage sites are indicated with arrowheads at the positions of the amino acids (bottom).

erol), and treated with lysozyme (final concentration of 1 μg/ml) on ice for 30 min. The resuspended cells were lysed by sonication on ice and centrifuged at 10,000×*g* for 30 min at 4°C. We added 500 μl of 50% Ni-NTA slurry (QIAGEN, Germany) to the supernatant and gently mixed it by shaking for 1 h on ice. The resin was washed twice with 40 ml of wash buffer (50 mM $NaH_2PO_4$; pH 8.0, 300 mM NaCl, 20 mM imidazole, 10 mM β-mercaptoethanol, 0.5% Tween-20, and 10% glycerol), and eluted with 5 ml of elution buffer (50 mM $NaH_2PO_4$; pH 8.0, 300 mM NaCl) with 40 mM, 50 mM, 70 mM, 80 mM, or 100 mM imidazole. The buffer was then changed to storage buffer (50 mM Tris; pH 8.0, 150 mM KCl), the purified proteins were quantified by BCA protein assay (Pierce, USA), glycerol concentration was made up to 20%, and the samples were stored at -80°C. Proteins were analyzed by 15% SDS-PAGE and Coomassie blue staining.

### RNA-dependent RNA polymerase assay

The RNA-dependent RNA polymerase assays were performed as described by Belliot *et al.* (2005) with minor modifications. Briefly, 20 μl of the reaction mixture containing 50 mM HEPES (pH 7.4), 2 mM $MnCl_2$, 50 mM DTT, 10 μM UTP, 0.2 μg of poly(A) template, 3 μM oligo(U) primer, 10 units RNase inhibitor, 15 pmol RdRp, and 2 μCi [α-$^{32}$P]UTP was incubated at 30°C for 30 min; then, the reaction was stopped by adding an equivalent volume of 0.5 mM EDTA. The reaction mixture was spotted on DE81 membrane (Whatman, UK), dried at room temperature for 30 min, and washed 3 times with 2 ml of 2× SSC for 10 min. After dehydration with 2 ml of absolute ethanol, the membranes were dried at 85°C for 30 min, and the incorporated radioactivity was quantified by liquid scintillation counting (Wallac 1407).

### VPg nucleotidylylation assay

The nucleotidylylation assay was performed as described by Han *et al.* (2010) with some modification. Twenty microliters of the reaction mixture containing 50 mM HEPES (pH 8.0), 1.5 mM $MnCl_2$, 5 mM DTT, 10 μM GTP, 2 μCi [α-$^{32}$P]GTP, 15 pmol RdRp, and 55 pmol VPg was incubated at 30°C for 2 h. The reaction was stopped by adding 5 μl of 5× protein sample buffer, and then heated at 98°C for 10 min. The reaction products were separated by 15% SDS-PAGE, dried, and the [α-$^{32}$P]NMP-incorporated VPg bands were visualized by phosphorimaging (BAS-1000).

## Results and Discussion

### cDNA cloning of HuNV genomic RNA

Full-length cDNA of HuNV was synthesized from the RNA extracted from the stool specimen of a Korean patient showing symptoms of gastroenteritis. The entire genome was 7,559 nucleotides long, excluding the poly(A) tail. Computer-based prediction of the amino acid sequence indicated the presence of 3 ORFs and 5′ and 3′ noncoding regions, as observed in other HuNVs (Fig. 1) (Hardy, 2005). HuNV ORF1 encodes a polyprotein that is eventually cleaved by a virally encoded protease into 6 individual proteins, including RdRp and VPg. ORF2 and ORF3 encode the major and minor capsid proteins, respectively (Liu *et al.*, 1996; Prasad *et al.*, 1996; Glass *et al.*, 2000).

Nucleotide sequence homology studied using BLAST with other isolates showed that the virus belongs to GII.4 strain, one of the dominant strains causing gastroenteritis in Korea as well as worldwide (Dove *et al.*, 2005; Yoon *et al.*, 2008). Phylogenic tree analysis with the ORF2 region of norovirus

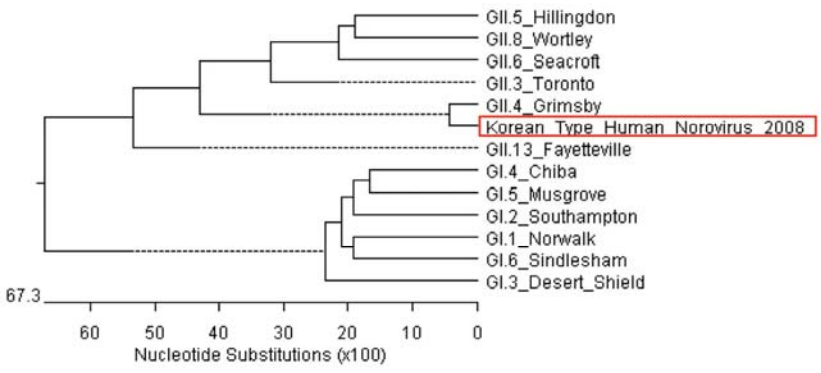


**Fig. 2. Phylogenic tree of Korean-type human norovirus.** The tree was constructed by MegAlign. Within each pair at the branch, the length indicates the relative distance of the sequences between the pair. A dotted line indicates a negative branch length. The numbers at the bottom represent the number of nucleotide substitutions for both DNA and protein sequences.

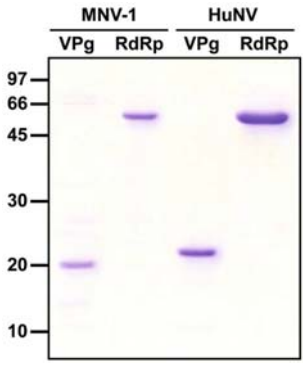


**Fig. 3. SDS-PAGE analysis of purified murine and human norovirus RdRp and VPg.** Murine norovirus-1 RdRp (59.8 kDa), VPg (16.5 kDa), human norovirus RdRp (58.8 kDa), and human norovirus VPg (18.1 kDa) were expressed in *E. coli* with a His-tag, and purified by affinity chromatography using Ni-NTA resin. Proteins were separated by 12% SDS-PAGE and stained with Coomassie brilliant blue. Molecular weights of standard proteins are shown in kDa (left).

GI.1 to GII.4 also showed that the cloned Korean-type norovirus was classified as the GII.4 strain (Fig. 2).

### Expression of RdRp and VPg in *E. coli*

RdRp plays a critical role during RNA virus replication by both synthesizing positive- and negative-sense RNA. Because poliovirus uses uridylylated VPg as a protein primer (Kok and McMinn, 2009), it has been suggested that other viruses with VPg, including calicivirus, might utilize NMP-linked VPg as a primer for virus genome replication (Daughenbaugh *et al.*, 2003; Goodfellow *et al.*, 2005; Chaudhry *et al.*, 2006).

To study whether the Korean HuNV RdRp and VPg retain their biochemical properties, these proteins were expressed in and purified from *E. coli* with an N-terminal His tag. SDS-PAGE analysis of the recombinant proteins indicated that both RdRp and VPg were purified to >90% homogeneity (Fig. 3). The molecular masses of RdRp and VPg estimated from gel electrophoresis were within the range of the calculated masses of the fusion proteins.

### RNA synthesis activity of recombinant RdRp

To examine the biochemical activity of HuNV RdRp, RNA synthesis assays were performed under different conditions in the presence of poly(A) as a template and [α-$^{32}$P]UTP as a substrate. The His-tagged recombinant RdRp was able to catalyze RNA synthesis by using oligo(U) as a primer in the presence of divalent cations (Fig. 4). RdRp activity was minimal in the absence of any one of the following components: poly(A) template, $MnCl_2$, or RdRp (Fig. 4). RdRp activity was not decreased to the minimal level in the absence of oligo(U) as a primer, suggesting that *de novo* initiation was carried out, although with poor efficiency. Addition of rifampicin, an inhibitor of bacterial DNA-dependent RNA polymerase, did not interfere with RNA synthesis (Fig. 4). These results indicated that the recombinant HuNV RdRp retained RNA synthesis activity *in vitro,* and that no co-purified cellular enzymes were involved in RNA synthesis.

### Nucleotidylylation of VPg by HuNV and MNV RdRp

Studies on HuNV replication have been hampered because the virus has not been adapted for replication in tissue culture cells, and because of the lack of a suitable small animal model. However, murine norovirus (MNV), initially isolated from immunocompromised mice, has been successfully cultivated in tissue culture cells (Wobus *et al.*, 2004). Since then, MNV has been used widely as a surrogate model system for investigating HuNV replication (Han *et al.*, 2010).

Nucleotidylylation of VPg is critical and essential for viral replication in FCV (Mitra *et al.*, 2004) and evidences that norovirus VPg may act as a protein primer were reported

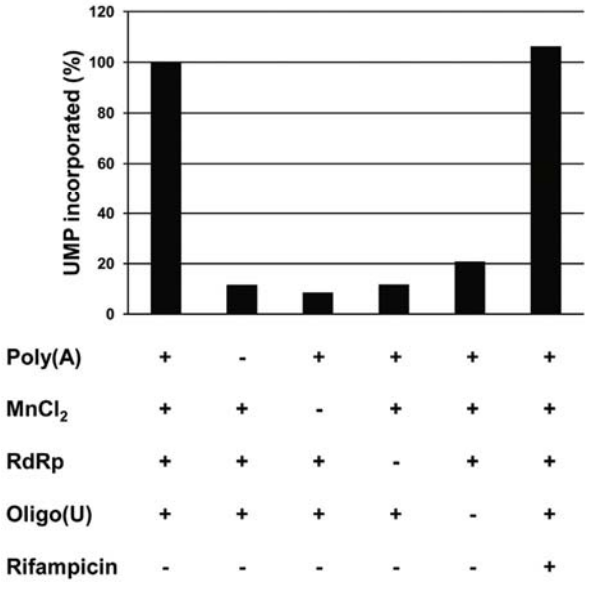


**Fig. 4. RNA synthesis activity of the recombinant human norovirus RdRp.** The reaction mixture, as described in 'Materials and Methods', with or without rifampicin, $MnCl_2$, poly(A), oligo(U), or RdRp, was incubated for 30 min at 37°C. EDTA was added to stop the reaction, and then, the incorporated [α-$^{32}$P]UMP was measured by a liquid scintillation counter.

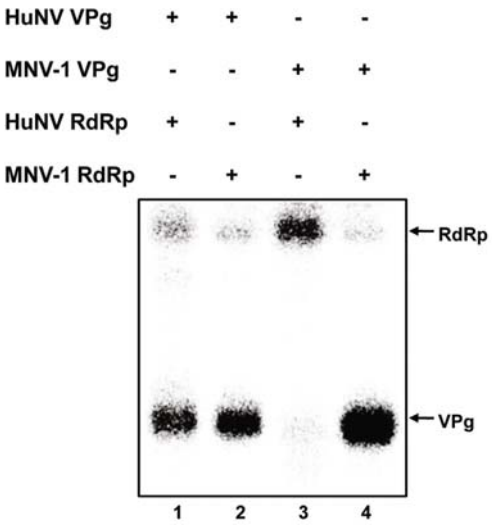


**Fig. 5. VPg nucleotidylylation cross-reactivity by recombinant RdRps.** VPg nucleotidylylation was carried out as described in 'Materials and Methods'. The reaction was stopped by adding protein sample buffer, and the proteins were denatured at 98°C for 10 min. The proteins were separated by 15% SDS-PAGE, the gel was dried, and the [α-$^{32}$P]GMP-incorporated protein bands were visualized by a phosphorimager.

(Subba-Reddy *et al*., 2011). We sought to compare the nucleotidylylation activities of HuNV and MNV RdRp on homologous and heterologous VPg. We employed GTP as a substrate for VPg nucleotidylylation because GTP is the most efficient substrate for the reaction (Han *et al*., 2010). It is highly that VPgpG, the product of VPg nucleotidylylation, acts as a primer for RNA replication; this is supported by the fact that VPg is covalently linked to the G at the 5′ end of the genomic RNA.

After the nucleotidylylation reaction, the reaction products were separated by 15% SDS-PAGE, and [α-$^{32}$P]GMP-incorporated bands were visualized by phosphorimager (Fig. 5, VPg). MNV RdRp exhibited stronger nucleotidylylation activity compared to HuNV RdRp (compare Fig. 5 lanes 1 and 4). MNV RdRp nucleotidylylated HuNV VPg, whereas HuNV RdRp failed to nucleotidylylate MNV VPg (compare Fig. 5 lanes 2 and 3). These results suggested that MNV RdRp not only retains higher nucleotidylylation activity but it is also able to nucleotidylylate HuNV VPg. Higher nucleotidylylation activity associated with MNV RdRp may be related to the ability of MNV to propagate in tissue culture cells.

In addition to VPg nucleotidylylation, human and murine norovirus RdRps were also nucleotidylylated (Fig. 5, RdRp). Recently, it was reported that norovirus RdRp was phosphorylated by host cell kinase Akt and the lack of phosphorylation altered its enzyme activity (Eden *et al*., 2011). The function and/or role of the nucleotidylylated form of RdRp remains to be determined.

The recombinant RdRp and VPg, expressed in *E. coli* by using cDNA of Korean type human norovirus, exhibited functional RNA synthesis and nucleotidylylation activities. These recombinant proteins are useful to screen RNA synthesis and nucleotidylylation reaction inhibitors that could lead to develop antiviral drug.

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