Functional evaluation of bacterial surface displayed P domain from human norovirus capsid proteins

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Abstract. Human noroviruses (HuNoVs) are the major cause to acute nonbacterial gastroenteritis outbreaks. The receptors of HuNoVs are difficult to identify because HuNoVs cannot be easily cultivated *in vitro* and they lack effective animal models. To develop an effective method for mining functional viral receptors, engineering *Escherichia coli* were constructed to display P domain of capsid protein of HuNoVs (GI.1 or GII.4) on the cell surface, using the N-terminal of the ice nucleation protein (InaQN). In this study, membrane protein-porcine gastric mucin binding ELISA methods were employed to confirm the surface display efficiency. The receptor binding capacity of InaQN-P (GI.1 or GII.4) fusion proteins on the bacterial surface was evaluated through whole-cell fluorescence immunoassay with saliva-based histo-blood group antigens. Results revealed remarkable characteristic of antigenicity and receptor binding properties. Therefore, this bacterial surface display system should be considered as a candidate to mine and investigate HuNoV receptors *in vivo* or *in vitro*.
Keywords: human norovirus; P domain; cell surface display; receptor binding properties.

1 Introduction

Noroviruses are a group of single-stranded, positive sense RNA virus from the *Caliciviridae* family [1]. The genome of norovirus includes three open reading frames (ORF1-ORF3). Based on the sequences of ORF2, noroviruses are divided into seven groups. Human noroviruses (HuNoVs) are clustered into GI, GII and GIV groups [2, 3]. The structural protein capsids of HuNoVs are composed of a major capsid protein (VP1) and a minor capsid protein (VP2) [4, 5]. VP1 binds to the functional ligand or receptors, such as human histo-blood group antigens (HBGAs) [6]. VP1 consists of two domains, the shell (S) and the protruding (P) domains, linked by a short hinge [5]. The P domain forms the arch-like structure and is responsible for the specificity of receptor binding [7-9]. The expression of the P domain *in vitro* can form a large complex as P particles [8]. A saliva-based HBGAs-binding assay demonstrated that the P particles retained the ability to bind to human HBGAs and resembled virus-like particles (VLPs) or norovirus particles [10].

To date, HBGAs are the only known ligands or receptors of HuNoVs. HBGAs are complex carbohydrates, which present on red blood cells and mucosal epithelia, or as free antigens in biological fluids, such as saliva and milk. Especially, the type III porcine gastric mucin (PGM)

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contains the HBGAs and is often used to evaluate the binding efficiency of HuNoVs [11]. HBGAs are highly polymorphic related to the ABO, secretor, and Lewis families [12]. GII HuNoVs have about 8-10 amino acids residues which interact with the A, B, O or Lewis antigens of HBGAs. While, in GI.1 HuNoVs (Norwalk virus), the amino acids residues interact with A or Lewis antigens of HBGAs. Although the types of the interaction between HBGA side chains and individual HuNoVs are variable, the involved amino acids residues are highly conserved in each HuNoVs group [13, 14].

HuNoVs are the most common causes of acute gastroenteritis in all age groups. Less than 100 virus particles can cause illness *via* the fecal-oral route of transmission [15]. Thus far, HuNoVs are the major foodborne viruses that significantly influence food safety [4]. HuNoVs are difficult to be cultivated *in vitro* due to the lack of effective cell culture and animal models. Although a promising method to culture HuNoVs in B cells was reported [16, 17], there was still a limitation on the source of viruses. Therefore, to explore viral receptors is the first step for studying the interaction mechanism between HuNoVs and their host cells. In our previous study, a novel approach for mining viral receptor was developed by targeting the P domain of VP1 from HuNoVs onto the surface of *Escherichia coli via* the N-terminal domain of the ice nucleation protein InaQ (InaQN) [18]. This system can be a good candidate for mining viral receptors (Fig. 1), although the receptor-binding function of this system still needs further determinations. This study aims to: 1) get the direct evidences for the surface localization of InaQN-P fusion proteins; 2) confirm the receptor-binding function of this cell surface display system.

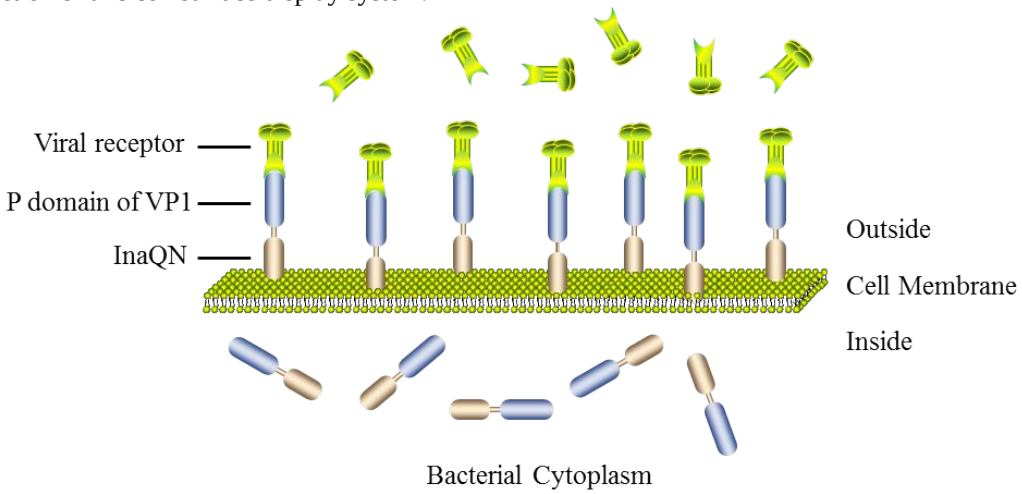

Figure 1. Schematic diagram of the interaction between receptors of HuNoVs and surface displayed InaQN-P fusion proteins.

2 Materials and methods

2.1 Bacterial Strains and Culture Conditions

E. coli BL21 (DE3) was grown in Luria-Bertani (LB) medium at 37 ºC with shaking of 200 rpm.

Recombinant *E. coli* BL21 (DE3) strains harboring recombinant plasmids pET28a-inaQN-P (GI.1), pET28a-P (GI.1), pET28a-inaQN-P (GII.4), or pET28a-P (GII.4) were constructed in the previous study [18] according to the standard procedure [19]. Cells were grown in LB medium containing 100 μg mL^{-1} of kanamycin (Kan) at 37 ºC. To express target proteins, when the OD_{600nm} (optical density) of the cell suspensions reached 0.6, isopro-pyl-β-D-thiogalactoside (IPTG) was added at a final concentration of 0.1 mmol L^{-1}. The cells were further incubated at 20 ºC for 24 h with shaking of 200 rpm.

2.2 Membrane protein extraction and Western blot analysis

Cells with different recombinant plasmids were induced with IPTG as described above. The cells were harvested and resuspended in PBS (pH 7.2) buffer. After washing thrice with PBS, cells were subjected to 90 cycles of 6 s of ultrasonication with 4 s intervals on ice. The cell lysate was centrifuged at 18000×g for 30 min at 4 °C. The membrane proteins were extracted according to the instruction manual of the FOCUS™ Global Fractionation Kit (G-Biosciences, USA).

InaQN-P (GI.1) and InaQN-P (GII.4) fusion proteins were separated by electrophoresis in 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels, respectively [19]. Proteins were then transferred to Hybond-polyvinylidene fluoride membranes (Amersham). Anti-VP1 (GI.1) or anti-VP1 (GII.4) polyclonal antisera (Friendbio Science and Technology (Wuhan) Co., Ltd., Hubei, China) were used as primary antibodies. The horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) was used as secondary antibodies. Bound antibodies were detected with 3,3'-diaminobenzidine (Chemicon). Western blot was performed in accordance with standard procedures [19]. *E. coli* BL21 (DE3) harbored plasmid pET28a was used as negative control. PageRuler Prestained Protein Ladder (SM0671) was obtained from ThermoFisher, USA.

2.3 Membrane protein-porcine gastric mucin binding ELISA

For ELISA, PGM (Sigma, USA) was used as a receptor. Membrane proteins were tenfold diluted by PBS. The wells of NuncImmuno Modules (ThermoFisher, USA) were coated with PGM (1.0 mg mL^{-1}) in 0.5 mol L^{-1} carbonate-bicarbonate coating buffer (pH 9.6) at 4 °C overnight. The wells were washed thrice with PBS and blocked with BSA (1.0% in PBS) at 37 °C for 1 h. After washing thrice with PBS again, 100 μL of the tenfold diluted membrane protein was added into each well and incubated at 37 °C for 30 min. Each well was washed thrice with PBS before 100 μL of anti-VP1 (GI.1) or anti-VP1 (GII.4) polyclonal antisera (1:10000 diluted with 1% BSA) was added. Wells were incubated at 37 °C for 60 min and washed thrice with Tris-buffered saline and Tween-20 (TBST). Subsequently, 100 μL of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (ThermoFisher, USA) with a dilution of 1:10000 was added to each well and incubated at 37 °C for 30 min. After washing thrice with TBST, 100 μL of 3,3',5,5'-tetramethylbenzidine (Qianchen Biotech, China) was mixed to each well and incubated in the dark for 10 min. Finally, 100 μL of 2 mol L^{-1} H_2SO_4 was added to each well to stop the reaction. The OD_{450nm} was immediately measured with Multimode plate readers (Tecan, Switzerland). In addition, the recombinant *E. coli* BL21 (DE3) harboring plasmid pET28a was used as the negative control.

2.4 Whole-cell fluorescence immunoassay (FIA)

A modified sandwich whole-cell FIA was developed to evaluate the P domain display efficiency. The recombinant strains were induced and prepared as described above. Cells were collected, washed with PBS, and adjusted to an OD_{600nm} of 0.8. Human saliva was collected from volunteers with blood types of A, B, and O. Saliva was treated as described in a previous report [20]. The supernatant of saliva samples was mixed with 0.5 mol L^{-1} of carbonate-bicarbonate buffer (pH 9.6) at a dilution of 1:100. Subsequently, 100 μL of diluted saliva was added to each well of the NuncImmuno Modules, coated at 4 ºC overnight, and washed thrice with PBS. Wells were further blocked with 1.0% BSA at 37 ºC for 1 h and again washed thrice with PBS. 100 μL recombinant cells of were added into each well and incubated at 37 ºC for 1 h. After washing thrice with PBS, 100 μL anti -VP1 (GI.1) or anti-VP1 (GII.4) polyclonal antisera (1:5000 diluted with 1% BSA) was added to each well and incubated at 37 ºC for 1 h. After washing thrice with PBS, 100 μL FITC-conjugated goat anti-mouse IgG (1:100 diluted) was added and incubated at 37 ºC for 1 h before each well was washed thrice with PBS. The fluorescence intensity was measured with an excitation wavelength of 492 nm and an emission wavelength of 520 nm by Multimode plate readers. In addition, the recombinant *E. coli* BL21 (DE3) strains harbouring

plasmids pET28a-P (GI.1) or pET28a-P (GII.4) were detected as controls. The fluorescence intensity of *E. coli* BL21 (DE3) was used as the blank value.

2.5 Statistical analysis

Data were analyzed by OriginPro (version 8). Each experiment was performed at least three times with independent replicates. One-way ANOVA was used and differences among means were considered significant when $p < 0.05$.

3 Results

3.1 Expression and surface localization of InaQN-P (GI.1) and InaQN-P (GII.4) fusion proteins

Membrane fractions of recombinant strains expressing InaQN-P (GI.1) or InaQN-P (GII.4) fusion proteins were extracted. SDS-PAGE (Fig. 2A) and Western blot (Fig. 2B) analysis showed that a ~53 kDa band and a ~54 kDa band were detected in the membrane of the construct cells. The molecular weights were similar to the InaQN-P (GI.1) and InaQN-P (GII.4) fusion proteins, respectively. However, the same bands were not found in the negative control. This result demonstrated, in principle, that these proteins were localized on the surface of *E. coli* BL21 (DE3) host cells.

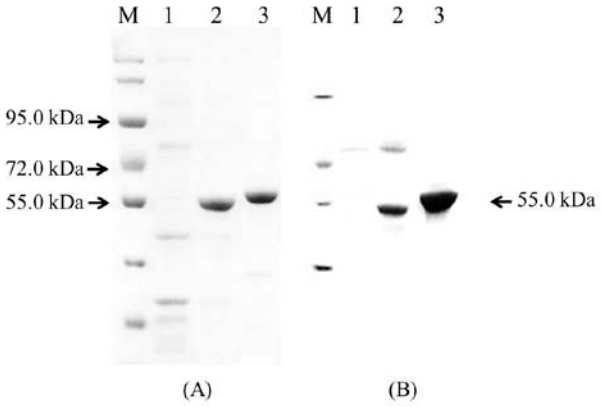

Figure 2. SDS-PAGE (A) and Western blot (B) analysis of membrane fractions. Lane M, PageRuler Prestained Protein Ladder (SM0671); Lane 1, the recombinant *E. coli* BL21 (DE3) harboring pET28a; Lane 2, the recombinant *E. coli* BL21 (DE3) expressing InaQN-P (GI.1) fusion proteins; Lane 3, the recombinant *E. coli* BL21 (DE3) expressing InaQN-P (GII.4) fusion proteins.

3.2 Membrane binding efficiency of InaQN-P (GI.1) and InaQN-P (GII.4) fusion proteins

Membrane protein-PGM binding ELISA was performed to evaluate the location and function of the displayed P domain with or without InaQN (Fig. 3). The results indicated that the InaQN-P (GI.1) and InaQN-P (GII.4) fusion proteins were both immobilized on the cell membrane, and the antigenicity of HuNoVs was maintained. The absorbance of the membrane extracted from strains expressing the InaQN-P (GI.1) or InaQN-P (GII.4) fusion protein was significantly higher than that of the membrane extracted from strains expressing the P domain without the InaQN anchor ($p < 0.01$). For membrane extraction from strains harboring pET28a-inaQN-P (GI.1) and pET28a-inaQN-P (GII.4), the ratios of the positive result to the negative result (P/N) were 9.49 and 5.11, respectively. Without the InaQN anchor, the P/N ratios of strains harboring pET28a-P (GI.1) and pET28a-P (GII.4) were 1.87 and 1.88, respectively. Therefore, the InaQN anchor promoted the transport and membrane immobilization of

the P domain from HuNoVs. In addition, the absorbance signal of InaQN-P (GII.4) fusion protein (OD_{450nm} = 0.613 ± 0.038) was significantly lower than that of InaQN-P (GI.1) fusion protein (OD_{450nm} = 1.139 ± 0.056; $p < 0.01$).

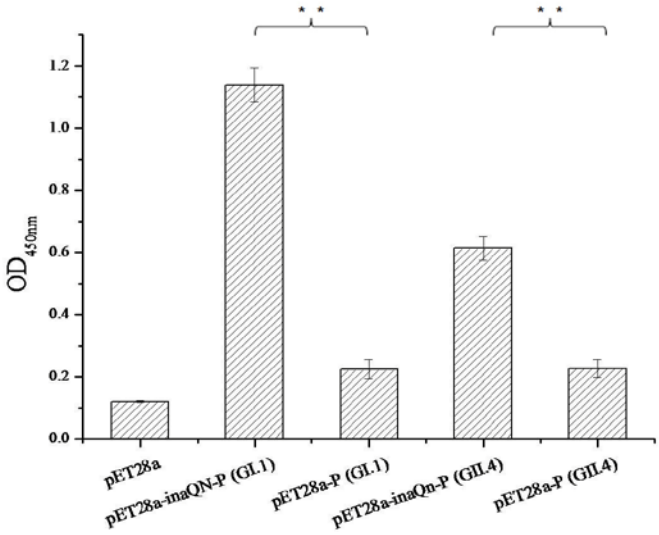

Figure 3. Membrane protein-PGM binding ELISA of the InaQN-P (GI.1) fusion protein, the InaQN-P (GII.4) fusion protein, and the P domains of GI.1 and GII.4. pET28a-inaQN-P (GI.1), pET28a-P (GI.1), pET28a-inaQN-P (GII.4), and pET28a-P (GII.4) represent the membrane proteins extracted from the corresponding recombinant *E. coli* BL21 (DE3). The recombinant *E. coli* BL21 (DE3) with the plasmid pET28a was used as a negative control. The anti-VP1 (GI.1) or anti-VP1 (GII.4) polyclonal antisera were used as the primary antibodies and the goat anti-mouse HRP-conjugated antibody was used as the secondary antibody.

3.3 Saliva-based HBGAs binding with surface-displayed P domain

To evaluate the binding ability to the HBGAs receptor, strains with plasmids pET28a-inaQN-P (GI.1), pET28a-P (GI.1), pET28a-inaQN-P (GII.4) and pET28a-P (GII.4) were interacted with saliva of blood types A, B, and O, respectively. The binding capacity between recombinant strains and HBGAs receptors was determined by comparing the whole-cell fluorescence intensities (Fig. 4). When exposed to saliva with blood types A and O, the recombinant strains harboring the InaQN-P (GI.1) or InaQN-P (GII.4) fusion protein had significantly higher fluorescence intensities than the groups without the InaQN anchor ($p < 0.01$). However, in the saliva with blood type B, the recombinant strain harboring the InaQN-P (GII.4) fusion protein showed high fluorescence intensity (2615.3 ± 86.9), whereas the recombinant strain harboring the InaQN-P (GI.1) fusion protein had low fluorescence intensity (522.0 ± 110.6). Recombinant strains without the InaQN anchor exhibited significant low fluorescence intensities. In addition, fluorescence intensities of the displayed P domain of GII.4 were higher than that of GI.1.

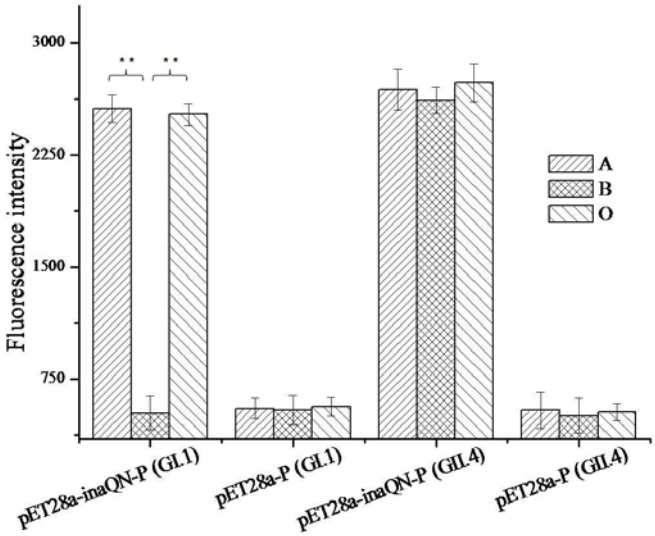

Figure 4. Saliva-based HBGA-binding profiles of the recombinant *E. coli* BL21 (DE3) with the pET28a-inaQN-P (GI.1), pET28a-P (GI.1), pET28a-inaQN-P (GII.4), or pET28a-P (GII.4). A, B, and O represent the saliva from individuals with A, B, and O blood types, respectively. Cells were treated with anti-VP1 (GI.1) or anti-VP1 (GII.4) polyclonal antisera, followed by the goat anti-mouse FITC-conjugated antibody.

4 Discussion

The limitation knowledge about the HuNoVs receptors was one of the constraints for the study of viral infection [17]. Thus far, researches related to the viral receptors are usually relied on the use of VLPs, P particles or animal caliciviruses [4]. The VLPs or P particles maintains the receptor binding ability of HuNoVs (Tan et al. 2008). However, the procedures for preparing them *in vitro* are complicated. For other animal caliciviruses, the biological characteristics are different from HuNoVs. In addition, it is hard to separate the complex of virus and its receptor by simple methods [21, 22]. Therefore, so far, mining viral receptors from various environmental samples or host cells is difficult. To address this issue, in our previous study, bacterial cell surface display systems were constructed using the P domains from HuNoVs GI.1 and GII.4 as passenger motifs, respectively. The results indicated an excellent antigenicity [18]. For further evaluating the function of the surface display systems, the surface display efficiency and functional receptors-binding ability were detected in this study. In Fig. 2, the InaQN-P (GI.1) and InaQN-P (GII.4) fusion proteins were observed in the membrane extracts of recombinant *E. coli* cells, respectively. No obvious signs of extensive proteolysis were detected in Western blot analysis. These results demonstrated that the expression and surface display of InaQN-P fusion proteins was very stable. In Fig. 3, the membrane extracts of cells expressing InaQN-P (GI.1) or InaQN-P (GII.4) fusion proteins retained high antigenicity. In Fig. 4, displayed P domain had high binding capacity to the saliva-based HBGAs receptors. In summary, these results provided strong evidence that the heterologous P domain of VP1 from HuNoVs was not only anchored successfully onto the cell membrane, but also retained the receptors-binding function. The characters of this displayed viral capsid protein on *E. coli* was similar with the natural HuNoVs. It is a good candidate surrogate for mining viral receptors from host cells or environmental samples.

Previous studies demonstrated that the prototype Norwalk virus (GI.1) binds to the saliva of A and O secretors, whereas the GII.4 strain binds to that of A, B and O secretors [6, 13, 23]. The amino acid residues of the P domain which interact with the HBGAs are highly conserved among strains within the same group but not between the GI and GII groups of HuNoVs [6]. This is due to the HuNoVs in

each of the two groups that have unique HBGA binding interfaces formed by a different set of amino acids. The evolution analysis of HuNoVs suggests that these two groups are two species which may have developed their HBGA binding ability independently [13]. This is supposed to be explained their difference in the receptor-binding properties. Moreover, the different modes for the interaction between HBGAs and the two groups of HuNoVs result in a better binding affinity in GII HuNoVs, compared with GI HuNoVs [24-27]. In the present work, the InaQN-P (GI.1) fusion protein showed a high binding ability with the saliva of A and O secretors, but had negative results with the saliva of B secretors. The InaQN-P (GII.4) fusion protein had a high binding ability with the saliva of A, B and O secretors (Fig. 4). In addition, the displayed P domain of GII.4 had a higher binding ability than that of GI.1. Therefore, the displayed P domain could recognize the viral receptors in different blood types of human saliva. These results were consistent with previous studies.

The cell surface display system can be a good approach for mining viral receptors, especially the viruses that remain uncultivated *in vitro*. The advantages of surface-displayed viral capsid protein to mine unknown viral receptors are as follows: (i) The viral capsid protein displayed on the bacterial surface can directly interact with the viral receptor. (ii) The *E. coli* surface display system is much more efficient. (iii) The *E. coli* culture is easier to maintain. (iv) The cloning and the expression methods are straight forward, simple, and less time-consuming.

5 Conclusions

In conclusion, we demonstrated that the surface-displayed P domain from the capsid protein of HuNoVs conferred the bioengineered *E. coli* BL21 (DE3) with a high antigenicity and HuNoVs receptor binding ability. The bacterial display system reported in this study proved to be extremely valuable as bioengineer alternative organisms for mining viral receptors.

Acknowledgement

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