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Short communication

Characterization of the genome sequence of an oncolytic Newcastle disease virus strain Italien

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ABSTRACT

We determined the complete genome sequence of strain Italien, a virulent and oncolytic strain of Newcastle disease virus (NDV) by direct nucleotide sequencing of RT-PCR products, a size of 15,186 nucleotides (nt). Comparison of six coding genes and non-coding regions of Italien with those of the other 25 sequenced strains revealed NDV Herts/33 was the most similar strain with Italien. The gene encoding the RNA dependent RNA polymerase was the most highly conserved, while the gene encoding phosphoprotein was the most highly variable. The HN and F proteins of Italien have been modeled on the crystal structure in order to study the structural characteristics. Interaction between the HN protein and the heptad repeat B (HRB) region of F protein was analyzed in silico by molecular docking predicted five critical residues I133, V142, D143, R480, and K567 on HN protein. Identification of amino acid residues that could be crucial for this interaction provides working hypotheses for subsequent studies.

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1. Introduction

In recent years, tremendous advances have been made in the development of oncolytic virotherapy to destroy the malignant cells. Newcastle disease virus (NDV), one of the virotherapeutic agents has shown oncolytic therapeutic efficacy in preclinical studies (Csatary et al., 1993, 1999; Freeman et al., 2006; Phuangsab et al., 2001) and an oncolytic strain, PV701 is currently in clinical trials (Laurie et al., 2006; Lorence et al., 2007). Our previous experiments showed that another oncolytic NDV strain, Italien, which was modified by a bispecific fusion protein effectively reduced the side-effects in vivo, while sustaining the anti-tumor effects (Bian et al., 2006). To improve the selectivity and efficacy, oncolytic virus can be genetically integrate a cancer cell-selective targeting, or "armed" to express a therapeutic protein. Thus, a better understanding on the complete genome of oncolytic NDV Italien might be able to facilitate the development of more effective therapeutic virus.

NDV is a member of genus *Avulavirus* of the family *Paramyxoviridae*, order *Mononegavirales* (de Leeuw and Peeter, 1999). The virus is enveloped and has negative-sense, non-segmented RNA genome encoding six proteins including nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase (HN) and RNA dependent RNA polymerase (L). Two addition proteins, V and W protein are expressed by RNA-editing of the P gene (Steward et al., 1993).

NDV strains have been classified as high virulent (velogenic), intermediate (mesogenic), or nonvirulent (lentogenic) on the basis of conventional in vivo pathogenicity indices for the chicken (de Leeuw et al., 2003). The molecular basis for pathogenicity of NDV is mainly determined by the amino acid sequence of the protease cleavage site of the F protein and by the host cell proteases (Peeters et al., 1999). Cleavage of the precursor glycoprotein F0 to F1 and F2 is required for progeny virus to become infectious. If NDV strain replicates in given human tumor cells to full maturation, oncolytic effects can be predicted by production of infectious progeny virus particles, thus killing tumor cells fairly quickly. NDV are now considered to be safe platforms for the development of oncolytic therapies due to the lack of adverse consequences in clinic trials (Freeman et al., 2006; Kelly and Russell, 2007; Pecora et al., 2002; Sinkovics and Horvath, 2000).

On the basis of phylogenetic analysis with the partial nucleotide sequences of the F gene, NDV has been classified into 10 genotypes (I–X). Genotypes VI and VII can be further divided into seven (VIa–g) and five (VIIa–e) subgenotypes, respectively. Com-

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plete genomes of about forty NDV strains have been sequenced which are defined into three different genome lengths 15,186 nt, 15,192 nt (Y. Huang et al., 2004; Z. Huang et al., 2004), and 15,198 nt (Czeglédi et al., 2006). The lengths of all of these NDV genome obey the "rule of six" strictly (Calain and Roux, 1993; Peeters et al., 2000).

The two surface glycoproteins, F and HN are presented on the envelope. HN can recognize sialic acid-containing receptors on cell surfaces and promote the fusion activity of the F protein allowing the virus to penetrate the cell surface (Lamb and Kolakofsky, 2001). The F protein can mediate viral membrane fusion with target cell membranes, undergoing a series of structural transitions in this process. A structural study of F (Zaitsev et al., 2004) and a peptide inhibition experiment (Gravel and Morrison, 2003) have suggested that F and HN hold each other in the switched-off state and that the globular head region of HN from 124 to 151 binds to the membrane-proximal HRB region of F. When HN binds to sialic acid receptors, the association of the HN dimer is changed which promotes F into its fusogenic state, sending its fusion peptide into the cell membrane. Previous studies have defined several crystal structures of HN protein of NDV strain Kansas (Crennell et al., 2000), including HN alone (PDB Entry 1E8T) and in complex with either an inhibitor (PDB Entry 1E8V) or with the β-anomer of sialic acid (PDB Entry 1E8U). But there is only one 3D structure of F protein of NDV (PDB Entry 1G5G) (Chen et al., 2001) that is a partially proteolyzed structure of a soluble form of strain Queensland V4. In other Paramyxoviruses, two crystal structures of the fusion protein have been determined. One is the crystal structure of the secreted, uncleaved ectodomain of the human parainfluenza virus 3 (hPIV3) F protein (PDB Entry 1ZTM) (Yin et al., 2005), but this structure contains the postfusion six-helix bundles (6HB) structure. Another is the crystal structure of the parainfluenza virus 5 F protein in the metastable, prefusion conformation, stabilized by the addition of carboxy-terminal trimerization domain (PDB Entry 2B9B) (Yin et al., 2006).

NDV Italien was isolated in 1944 and it seems to be an oncolytic strain based on its efficient replication in human tumor cells, killing the tumor cells within 24 h (Bian et al., 2006). The aim of the present study is to sequence the complete genome of NDV Italien and analyze it phylogenetically. The six coding genes and non-coding genes were compared with that of 25 NDV strains for which complete genome sequences have been deposited in GenBank. Finally, homology models of F and HN proteins were constructed and interaction between the globular head of HN and the HRB region of F was predicted by molecular docking. Bioinformatic data can used to understand the conformational changes of residues involved to form the catalytic site and the binding-site of HN, as well as the association of F and HN.

2. Materials and methods

2.1. Virus incubation and collection

NDV strain Italien, obtained from Prof. Volker Schirrmacher (Division of Cellular Immunology, German Cancer Research Center), was inoculated in 10-day-old specific pathogen-free chicken embryos (Merial Vital Laboratory Animal Technology Company, Beijing) via allantoic cavity after diluted to 10^3–10^6 folds. After 72 h of incubation, allantoic fluid was harvested. Allantoic fluid was clarified by centrifugation at $4000 \times g$ for 30 min at 4 °C then the supernatant was overlaid on 20% sucrose solution and centrifugation at 25,000 rpm for 4 h at 4 °C (SW-28 rotor, Beckman). The virus pellets were reconstituted with TNE buffer (100 mM Tris, pH 7.2, 100 mM NaCl, 1 mM EDTA) and stored at −80 °C for use.

2.2. RNA isolation and RT-PCR

Virus genome RNA was isolated with TRIzol LS (Invitrogen) according to the manufacturer's instructions. Reverse transcription reactions were carried out with ReverTra Ace reverse transcription kit (TOYOBO) according to the manual by pipetting 2 μl purified RNA and 1 μl 10 μM primers. RT products were amplified by high fidelity DNA polymerase KOD-plus (TOYOBO) with six pair primers in 50 μl volume to generate overlap DNA fragments. The number of cycle is 20–25 cycles. After the reaction finished, DNA fragments were identified by the agarose gel electrophoresis and reclaimed by gel extraction kit (QIAGEN). All the fragments were inserted into pMD-19T vector (TaKaRa).

2.3. Sequence determination

All gene fragments were sequenced by ABI 3730 automatic sequencer (ABI) in Biological Engineering Technology & Services Co., Ltd., Shanghai. Overlapping fragments were sequenced in both directions. The results were aligned and analyzed by MEGA 4.0.

2.4. Molecular modeling and docking of HN and F proteins

With the homology comparison method, 2B9B was chosen as template structure for F protein and 1E8T for HN protein. With the BLASTP program on NCBI website, the amino acid sequences of stain Italien HN and F were blasted with the monomer sequence of 1E8T and 2B9B, respectively. The alignment between the sequence of F and that of its template structure was subsequently optimized according to the structure homology between 1G5G and 2B9B. Based on these alignment, the homology models building of Italien HN dimer and F trimer were carried out using the automodel function of MODELLER 9v1 (Sali and Blundell, 1993) and the quality of models was assessed using the DOPE (Discrete Optimized Protein Energy) method of MODELLER 9v1. The best structures were optimized by 2000 steps conjugated gradient minimization using DISCOVER module of INSIGHTII2000 (Molecular Simulations Inc., San Diego, CA, USA). Subsequently, the monomer model of Italien HN was chosen as macromolecular and truncated C-terminal end of F protein was chosen as ligand contained HRB from T458 to V491 according to previous studies (Gravel and Morrison, 2003; Young et al., 1999). One hundred computational docking experiments were performed by using AutoDock3. The best docking model was optimized by using DISCOVER with molecular mechanism minimization and molecular dynamics annealing, which was subjected to STING web server (Higa et al., 2004) to analyze the interaction residues.

3. Results and discussion

3.1. Complete sequence analysis

The 25 NDV strains with complete genome sequences that were used for comparison analysis in this study are listed in Table 1. We amplified and determined the genome sequence of NDV Italien by direct nucleotide sequencing of RT-PCR products. Primers for RT-PCR are shown in Table 2. Our result showed that the length of the genome of strain Italien is 15,186 nt. The complete sequence was deposited into the GenBank (accession number EU293914). The phylogenetic analysis was performed based on the nucleotide sequence of the complete genome and its six genes, NP, P, M, F, HN and L. The phylogenetic trees were somewhat variable depending on the specific gene fragment analyzed (data not shown), however, NDV Italien was always in the same cluster with NDV Herts/33

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