Mini-Review

Biological Relevance of C-terminal Elongation of the NS1 Protein of Influenza A Viruses

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Abstract | The non-structural 1 (NS1) protein plays a crucial role in moderating the virulence of influenza virus by multiple mechanisms. Due to C-terminal 'tail' (CTT) truncation of NS1, there are length variation types of NS1 in different subtype influenza viruses. CTT functions in several ways to defeat the cellular innate immune responses. Here, we discuss those different effects of CTT truncation or elongation of NS1 protein in different genetic backgrounds of influenza experimentations. Conclusively, it can be stated that CTT confers a positive role during the infection of influenza A viruses. However, this regulation is contributed by multiple factors and has variable impact on the pathobiology of influenza A viruses.

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Influenza A viruses (IAVs) are important zoonotic pathogens that pose a severe threat to animal and human health. IAVs are members of family Orthomyxoviridae and genus Influenza Virus A. They are classified into 18 hemagglutinin (HA) and 11 neuraminidase (NA) groups based on the antigenicity of these surface glycoproteins (Tong et al., 2013). IAVs consist of eight segmented, single-stranded RNA genomes of negative polarity that encode at least 11 proteins. The non-structural protein 1 (NS1) of influenza A viruses plays an important role with multiple accessory functions during virus infection (Munir et al., 2011, Hale et al., 2010). Broadly, the functions of NS1 protein can be divided into 4 categories, including (i) inhibition of IFN and the antiviral responses (ii) interference in the functions of host cellular proteins (iii) induction of apoptosis, and (iv) facilitate the synthesis of viral proteins. Cumulatively, these functions make NS1 protein as one of the most important virulent factor of influenza viruses. The NS1 usually encodes 230~237 amino acids (aa), which can be separated into four distinct domains. The first ~73 aa of NS1 constitute a unique N-terminal dsRNA-binding domain (RBD). A short inter-domain linker region (LR) then connects the RBD to the effector domain (ED), which encompasses residues 88–202. The remaining residues of NS1 form the C-terminal 'tail' (CTT) (Hale, 2014). The CTT contains four protein-binding regions (Crk/CrkL, PABI, PADF1 and PDZ). Recently, many research groups have studied the effect of CTT in the pathobiology of the influenza viruses and its interaction with the hosts. Based on the analysis of many NS1 protein sequences, seven different length variation types have been identified. The C-terminal elongation or truncation resulted in different length variations of the NS1 protein. Except for the 2009 pandemic virus, the majority of avian influenza viruses (H9N2 and H6N2), swine influenza viruses (H1N1, H1N2, and H3N2), as well as a few equine influenza viruses (H3N8), and

other influenza viruses contain this domain (Dundon, 2012; Dundon and Capua, 2009).

Through large scale sequence analysis of influenza viruses, four residues at the C-terminal of NS1 were considered to be a PDZ domain ligand (PL) whose the typical characteristics were the X-S/T-X-V type. The PDZ domains are protein–protein recognition modules that organize diverse cell-signaling assemblies. The PDZ domain-containing proteins play important roles in the transportation, localization and assembly of supramolecular signaling complexes, to organize cell polarity, receptors, and downstream effectors (Javier and Rice, 2011; Sheng and Sala, 2001). In WSN H1N1 (a mouse adapted strain of the influenza virus), the 1918-like PDZ-ligand binding motif (PBM) (KSEV) and H5N1 highly pathogenic avian influenza (HPAI) viruses-like PBM (ESEV) enhanced the pathogenicity in mice (Jackson et al., 2008). The PBM ESEV can specifically associate with the PDZ proteins scribble, Dlg1, MAGI-1, MAGI-2, and MAGI-3. However, other PBM moieties including RSKV, KSEV, and EPEV ineffectively bind with these PDZ proteins. ESEV PBM-mediated binding of NS1 to Scribble and Dlg1 disrupts the cellular tight junction (Golebiewski et al., 2011). Another approach is to use ESEV inhibited apoptosis during infection through disruption of Scribble's pro-apoptotic function (Liu et al., 2010). At the same time, ESEV can also regulate IFN-beta by binding with MAGI-1 (Kumar et al., 2012) and terminate the NF-kB activation by associating with the PDlim2 protein (Yu et al., 2011). This modulates the phosphorylation status of human Src kinase (Laura et al., 2011). These functions can contribute to the severe disease associated with highly pathogenic H5N1 influenza A viruses. Thus, C-terminal truncation of NS1 may attenuate the virulence or replication of influenza virus. In H5N1, the HPAI virus-like PBM (ESEV) and human influenza virus–like PBM (RSKV) and C-terminal truncation does not have a significant impact in mice and chickens. Thus, it contributes little to the virulence of H5N1 viruses. However, these variants have different replication in different host cells. These results suggest that loss of PDZ binding did not affect the virus and other segments play more important role through function compensation. These findings also indicated that this PDZ motif modulates viral replication in strain- and host-dependent manners (Zielecki et al., 2010). A similar result was found in H7N1 (Soubies et al., 2010). In Italy, most H7N1 HPAI viruses harbored a C-terminally truncated NS1 protein, resulting from a point mutation that introduced a premature stop codon at position 225 (Dundon et al., 2006). In contrast to the full-length NS1 protein, the 6 aa truncation had no impact on virus replication in duck or chicken cells in vitro. Interestingly, the viruses with full-length NS1 induced an interstitial pneumonia in chickens (Soubies et al., 2013). Lohrmann et al (2013) have explored the effect of NS1 protein with a C-terminal extension of seven amino acids on the viral characteristics. The results revealed that the NS1 extension did not have any impact in most experimental systems and only conferred minor growth advantages compare with wild type virus. In our study, 13 aa truncation had no impact on virus replication and the production of IFN-beta mRNA. Of note, the C-terminal extension of seven amino acids increased replication, increased the levels of inflammatory cytokines, and facilitated transmission in chickens (Kong et al., 2014). This mechanism remains unclear. While in line with our data, C-terminal PBM of NS1 was determined as a transmission determinant of influenza virus in guinea pig (Kim et al., 2014).) Like H9N2 avian influenza virus, the 2009 pandemic H1N1 had an 11 aa truncation in the C-terminal of NS1. Extension of NS1 to 230 aa results in an NS1 protein carrying GTEI at positions 227 to 230, which deviates from the X-S/T-X-V-type PDZ ligand motifs. This extension had only minor effects on replication, virulence or transmissibility of the 2009 pandemic H1N1virus (Hale et al., 2010). Other PBM moieties (RSEV/RSKV/ESEV) were engineered into the 2009 pandemic H1N1. These changes increased the replication and pathogenicity of pandemic (H1N1) 2009 influenza virus (Ozawa et al., 2011). A similar 11 aa truncation also persisted in classical swine H1N1 influenza virus. The EPEV or GSEI was introduced and was involved in viral virulence in mice (Wang et al., 2012). These findings suggested that the C-terminal tail of the NS1 protein modulates the viral virulence of classical H1N1 swine influenza virus to some extent.

Except the PDZ domain, other protein-binding regions also contributed to viral replication. Another important discovery was that the H3N2 influenza virus contains an amino-acid sequence (ARSK) (226-229aa) very similar to the histone's ARTK sequence. The experiment showed that this sequence similarity is functional — the NS1 tail can serve as a substrate for the histone-modifying enzyme Set1, which is a lysine methyl transferase. The NS1 tail contributed to

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evading the host's immune system by binding directly to the transcription-elongation complex PAF1C (Marazzi et al., 2012). When the C-terminal tail of NS1 of H3N2 influenza virus was truncated, the influenza virulence was attenuated. The Src homology 3 (SH3) domain-binding motif of NS1 binds with Crk/CrkL to modulate host cell signaling. This can enhance influenza A virus replication by inducing PI3K signaling (Heikkinen et al., 2008). In addition, the CTT contained PABII-binding domain that can inhibit the cellular mRNA nuclear export machinery (Satterly et al., 2007). Although the 2009 pandemic H1N1 restored PABIII binding ability, the virulence was attenuated.

In summary, the NS1 tails moderate the influenza A virus pathogenicity and transmissibility through a versatile function. This function is a strain- and host-dependent manner. Regardless of C-terminal elongation or truncation of NS1, natural variations exist and modulate the function of the influenza virus.

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