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Original Article

Caspase Cleavage Motifs of Influenza Subtypes Proteins: Alternations May Switch Viral Pathogenicity

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Abstract

Background and Aims: The caspases are unique proteases that mediate the host cell apoptosis during viral infection. In this study, we identified the caspase cleavage motifs of H5N1 and H9N2 influenza viruses isolated during 1998-2012.

Materials and Methods: Amino acid sequences of the eleven proteins encoded by the viruses as the caspase substrates downloaded from NCBI. The caspase cleavage motifs predicted at the three scanning P_4P_1, $P_4P_{2'}$, and $P_14P_{10'}$–trained support vector machine classifier.

Results: Data showed that H5N1 and H9N2 viruses were represented the same cleavage motif pattern for some of the viral proteins substrates. The HA, NP, and NS1 of H9N2 viruses were found to possess additional cleavage motifs from 2005, when an outbreak wave of H5N1 viruses was expanded through Asia.

Conclusion: Moreover, the cleavage motif of PB1-F2 protein was differing in both subtypes. The results indicated that the caspase activity of PB1-F2 protein may be involve in the viral pathogenicity.

Keywords: Influenza virus; subtypes; Caspase Cleavage Motif; pathogenicity

Introduction

Influenza virus A (IAV) belongs to the *Orthomyxoviridae* family and the viral genome consists of eight segments of negative-sense, single-stranded RNA that encode at least ten proteins, including hemagglutinin (HA) and neuraminidase (NA), nucleoprotein (NP), three polymerase proteins (PB2, PB1, and PA), two matrix proteins (M1 and M2), with two nonstructural proteins (NS1 and NS2) (1). Most of these viruses have become established in bird spices resulting in mild to severe disease and pose a threat of zoonotic infection (2). Cleavage of HA determines viral pathogenicity, because it is necessary for the spread of infection. For low pathogenic IAV strains, the cleavage site usually consists of a single arginine, whereas highly pathogenic IAV HAs generally have multiple basic residues in this position (3-5). Although highly pathogenic IAVs have been reported in human influenza outbreak with a high case mortality rate, but the Spanish influenza pandemy causative agent has only a single arginine at the cleavage site. It has been speculated that another mechanism might have played a role in the devastating influenza outbreak. So, understanding the pathogenesis of the viruses is critical to effectively prevent and limit the degree of infectivity in human populations. Little is known about human response patterns to low pathogenic avian viruses and it will be interesting to find what mechanism may influence virus transmission and establishment of infection in a new host.

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Direct transmissions of the avian influenza viruses from avian to human and development of multiple reassortant genotypes introduce the viruses as potential pandemic candidate by the World Health Organization. The generation of new variants may related to co-circulation of at least these two subtypes and also some positive and negative selection pressures that may occurring on environment (6, 7). Recently isolation of a high lethality H9N2 virus for mice has been reported from chickens in northern China without producing observable clinical signs of disease or death in infected chickens (8). Several studies on interaction between influenza viruses and hosts revealed that the infection causes apoptosis or programmed cell death in natural hosts. A variety of host and viral factors are involved in induction of apoptosis that may depend on the cell type (8-12). The regulation of some host apoptotic factors like cysteinyl-aspartate-kinases (caspases) activation interacts with influenza virus replication and pathogenicity (13, 14).

Generally, during the highly regulated form of cell death, a cascade of caspases is activated and lead to cleavage of many structural and regulatory proteins and involve many physiological and pathological processes (15, 16). Changes in cellular morphology and metabolism include pronounced chromatin condensation, genomicl DNA fragmentation, and alterations in nuclear shape are hallmarks of the apoptotic process. Since 1998 that a list of caspase substrates cleaved by proteases has been published (17), at least more than 280 caspase targets including proteins involved in scaffolding of the cytoplasm and nucleus, signal transduction and transcription-regulatory proteins, cell cycle controlling components and proteins involved in DNA replication and repair are identified. To date, several studies have been demonstrated that influenza virus infection triggers apoptotic cell death involving caspase-dependent death receptor and mitochondorial/cytochrome c pathways, tumor suppressor protein p53, PKR cascade, and transcription factors (18-22). The role of viral NA, PB1-F2, and NS1 genes in host cell apoptosis modulation has been identified, invariably by facilitating the cleavage of latent transforming growth factor (TGF)-β into the active form, permeabilizing the mitochondrial membrane leading to cytochrome c release, and suppression of type I IFN production (23-28).

In this study to understand whether the cleavage sequence corresponds to the caspase activation may involve in influenza virus pathogencity, the highly pathogenic H5N1 and the low pathogenic H9N2 viruses caspase cleavage motifs were identified. We also discussed their changes during two past decades.

Methods

The full-length sequences for PB2, PB1, PB1-F2, PA, HA, NA, NP, M1 and M2, as well as NS1 and NS2 proteins of H9N2 and H5N1 viruses isolated in Asia during 1997-2012 periods were downloaded from NCBI database. The caspase substrate cleavage sites were predicted by applying CASVM, a web server for support vector machine algorithm (29). The presence of aspartic acid at P_1 position (XXXD) was selected as default. Considering the influence of adjacent amino acid residues on substrate cleavage all of the three scanning P_4P_1, $P_4P_{2'}$, and $P_14P_{10'}$ –trained SVM classifier were used for prediction.

Results

The caspase cleavage motifs of both H9N2 and H5N1virus proteins predicted using CASVM are summarized in table 1. The responsible caspase for each substrate was determined according to corresponding amino acid sequences and divided as initiator, effector, and inflammatory caspases. The viruses were represented similar cleavage motif patterns for the polymerase proteins substrates. The HA, NP, NS1, and PB1-F2 of H9N2 viruses were found to possess additional cleavage motifs based on the time and region of isolation. According to the bioinformatics comparison the majority of the Far East H9N2 viruses showed the conserved cleavage site at the

Table 1. Caspase cleavage motifs predicted in H9N2 and H5N1 influenza viruses isolated in Asia during 1997-2012 with CASVM.

Protein	Predicted caspase cleavage motifs		Responsible caspase
	H9N2	H5N1	
PB1	$AQTD_{16}$, $DVTD_{102}$, $DSMD_{105}$, $ESAD_{452}$, $LVSD_{523}$, $MDED_{549}$, $MEYD_{588}$	AQTD, DVTD, DSMD, ESAD, LVSD, MDED, MEYD	caspase-2, caspase-8, caspase-10
PB1-F2	$SQAD_{51}$[a]	$QGQD_{7}$, $RLMD_{41}$	caspase-8
PB2	$DDVD_{186}$, $LIPD_{396}$, $MGVD_{416}$, $EPED_{610}$, $ILTD_{676}$	DDVD, LIPD, MGVD, EPED, ILTD	caspase-2, caspase-3, caspase-6, caspase-8
PA	$DLYD_{41}$, $DFED_{313}$, $DCKD_{316}$, $DVSD_{319}$, $IELD_{356}$, $SLTD_{459}$	DLYD, DFED, DCKD, DVSD, IELD, SLTD	caspase-2, caspase-3, caspase-6, caspase-7, caspase-8, caspase-9
HA	$SCSD_{83}$[a], $LKTD_{210}$, $DFHD_{380}$	DFHD, $ILRD_{71}$, $CDLD_{62}$, $GMVD_{366}$, $KAID_{393}$, $KMED_{433}$	caspase-2, caspase-8
NP	$ILYD_{42}$, $ENVD_{302}$[a], $DAMD_{305}$[a], $EEYD_{427}$	ILYD, EEYD, $SAFD_{73}$	caspase-2, caspase-8, caspase-10
NA	$MLVD_{140}$, $VMTD_{170}$, $SWPD_{387}$	VMTD, SWPD, $SGPD_{181}$, $IITD_{196}$, $CYPD_{226}$, $TETD_{336}$[b]	caspase-6, caspase-8
NS1	$DESE_{4}$, $YLTD_{22}$, $SNED_{139}$[c]	$ELGD_{30}$, E(D)ESD, YLTD, SNED	caspase-2, caspase-8
M2	$SAVD_{15}$, $VDVD_{17}$, $DVDD_{18}$	SAVD, VDVD, DVDD	caspase-2, caspase-3, caspase-6, caspase-7, caspase-8

[a] the motif was added in Middle East isolates from 2005-2012.
[b] the motif was removed from 2005-2012.
[c] the motif was added from 2005-2012.

examined periods whereas, all the Middle East and Indian sub-continent countries viruses contained additional motifs especially in 2005-2006 isolates indicating that they have similar source of origin. It may reflect a geographical parameter for restriction of the viruses in the areas. The different HA and PB1-F2 cleavage motifs were predicted in all of H5N1 viruses isolated from 1997-2012. Similar patterns were revealed for NP, NS1, and NA proteins for H5N1 and H9N2 viruses one or two motifs may be add or removed. The M1 and NS2 proteins appeared to lack any caspase cleavage motifs for H9N2 and H5N1viruses.

Discussion

The pathogenesis of influenza infection is based on the ability of the causative viruses to kill host cells by apoptosis or cytolysis. Studies reported that H5N1 and H9N2 infections are capable of inducing apoptosis in host cells (9-11, 18, 21). Following an apoptotic stimulus, caspases cascade is activated lead to regulate

virus replication in the infected cells. Here we characterized the cleavage sites of H9N2 and H5N1 viruses isolated during the first emergence to now in Asia and found that the variation in caspase cleavage sites of H5N1 viruses was less than H9N2 viruses. As shown in table 1, the caspase recognition motifs VDVD in M2, DDVD in PB2, and EEYD in NP proteins are conserved amongst H9N2, H5N1 viruses. These motifs were also reported for H7N1 subtype (13). The lack of caspase cleavage motif for M1 and NS2 proteins of these viruses is in agreement with that reported for avian H7N1 subtype. O'Neill *et al* (30) have been shown a regulatory role of NS2 protein in the transcription and replication of the viral RNA genome as well as directly involving of both M1 and NS2 in the nuclear export of vRNPs during the viral life cycle. The correlation between the lack of M1 and NS2 caspase cleavage site and avian influenza virus replication is not clear. The prediction also revealed that caspase cleavage motifs of H9N2 HA, NP, and NS1 proteins alter based on the isolation time in which at least an additional motif detect in these substrates for Middle eastern viruses isolated in 2005-2006. The pattern is similar to phylogenic analysis of H9N2 HA and NS genes isolated in Asia (6, 7). All the three proteins are determinants for viral pathogenicity through virus-host interaction by specific binding to sialic acid receptors on the cell surface, regulation of viral RNA synthesis, and suppression of host defense mechanism. It has been demonstrated that during the last two decades, several distinct sub lineages of H9N2 viruses have become established and classified in genotypes series A-G (31). Among them G1-like viruses detected in humans have been dominated in Middle East whereas, BJ/1/94 sub lineage as well as the genotypic complexity of H9N2 viruses shown in China. From 1997, H5N1 epizootic of domestic poultry has circulated in South-East Asia and during the late of 2005 a new transmission and outbreak wave was rapidly expended. Studies revealed that the H9N2 viruses have undergone gradual and complex evolution or reassortments with highly pathogenic H5 and H7 viruses to generate multiple novel genotypes (4, 31). The cleavage motifs DEHD in HA, DAMD and ENVD in NP, SNED in NS1, and SQAD in PB1-F2 are presented only in Middle East H9N2 viruses during 2005-2012. The co-circulation of these influenza virus subtypes raises concern about the risk of the emergence of new pathogenic viral strains. Previous studies on evolution of H9N2 Asian isolates have been revealed that an amino acid at position 210 in HA is under positive selection at the 90% confidence level (6). Substitution of N to D at this position leads to identification of additional caspase cleavage motif LKTD. In case of NS1 amino acid sequences some simultaneously changes became fixed in the Middle East recent isolates (7). The same substitution at position 139 in effector domain of NS protein leads to identification of additional cleavage motif SNED. In this study different caspase cleavage motif in PB1-F2 protein predicted for H5N1 and H9N2 subtypes. The protein expressed in some influenza A virus strains plays an important role in viral pathogenesis, induction of apoptosis, and down-regulation of the host immune response to influenza infection by enhancing the anti-IFN effects of the polymerase proteins (25, 26). The previous studies suggested that influenza virus infection causes apoptosis in natural hosts and plays direct and or indirect roles in the pathogenesis of influenza virus. The highly virulent H5N1 virus induced apoptosis in mammalian alveolar epithelial cells and chicken cells resulted in the deregulation of adaptive immune responses (20, 21). The low pathogenic H9N2 virus can induce apoptosis in chicken macrophages by the Fas/(FasL)-mediated extrinsic pathway (18, 19) and human alveolar epithelial cells in a time and dose-dependent manner (11). The widespread prevalence of H9N2 subtype in the Middle East region (6, 31) and isolation of high lethally H9N2 for mice (8) suggested the need for more understanding of the virus pathogenicity. The different subtypes of influenza viruses have taken advantage of apoptosis induction by caspase targeting of the protein. Based on the role of PB1-F2 protein we suggest that the existence of subtype-specific differences in caspase cleavage sites

may modulate pathogenicity during the influenza infection process in target cells. It seems that the viruses utilize caspase activity to directly facilitate own replication in host cells that adds another complexity in the host-apoptosis-virus triangle.

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