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REVIEW ARTICLE

Research Progress on the Functions of Non-Structural Protein 2 (NS2) of Classical Swine Fever Virus: A Review

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Abstract

Classical Swine Fever Virus (CSFV) is a significant pathogen that causes swine fever, and its non-structural protein, NS2, plays a crucial role in the viral life cycle. The NS2 protein not only participates in the processing of the viral polyprotein precursor but also significantly affects viral replication, assembly, and infection. In recent years, research on the functions of the NS2 protein has deepened, revealing its self-cleaving enzyme activity, interaction with the NS3 protein, and potential role in viral genome packaging. At the same time, the genetic variation of the NS2 protein and its adaptive changes under selective pressure provide an important theoretical basis for understanding the virus's adaptation mechanisms and vaccine development. This article aims to systematically review the latest research progress on the structural characteristics, processing mechanisms, and functional roles of the NS2 protein of CSFV, in order to provide reference and guidance for the development of future antiviral strategies.

Keywords: Classical Swine Fever Virus (CSFV), Genome packaging, Non-structural protein 2 (NS2), Proteolytic cleavage. Research progress, Viral replication

Introduction

Classical Swine Fever Virus (CSFV), as an important member of the *Pestivirus* genus in the *Flaviviridae* family, poses a serious threat to the global pig industry. According to the ICTV 2017 classification [1,2] (ICTV proposal 2017.010S), the species name of Classical Swine Fever Virus is *Pestivirus C*, whereas according to the ICTV 2022 classification [3,4] (ICTV proposal 2022.007S), it is *Pestivirus suis*. The classical swine fever (CSF) caused by this virus (CSFV) is an important infectious disease in the pig farming industry, characterized by high mortality and high infectivity. It is classified as a Category A notifiable animal disease by the World Organization for Animal Health (The current abbreviation is "WOAH", while the former abbreviation was "OIE") [5,6]. Since its discovery in Ohio, USA, in 1833, this disease has spread widely around the world, causing significant economic losses to the global pig industry [7-9].

In recent years, with the continuous deepening of research on CSFV, scientists have gradually recognized the importance of the NS2 protein in the viral life cycle. The NS2 protein is a hydrophobic membrane-spanning protein with intrinsic self-protease activity. It significantly contributes to the liberation of the NS3 protein [10].

The functions of the NS2 protein are diverse and complex, involving multiple aspects, such as viral replication, assembly, genome packaging, and viral particle formation [11,12]. Studies have shown that the NS2 protein is not only a core component of viral polyprotein processing but also

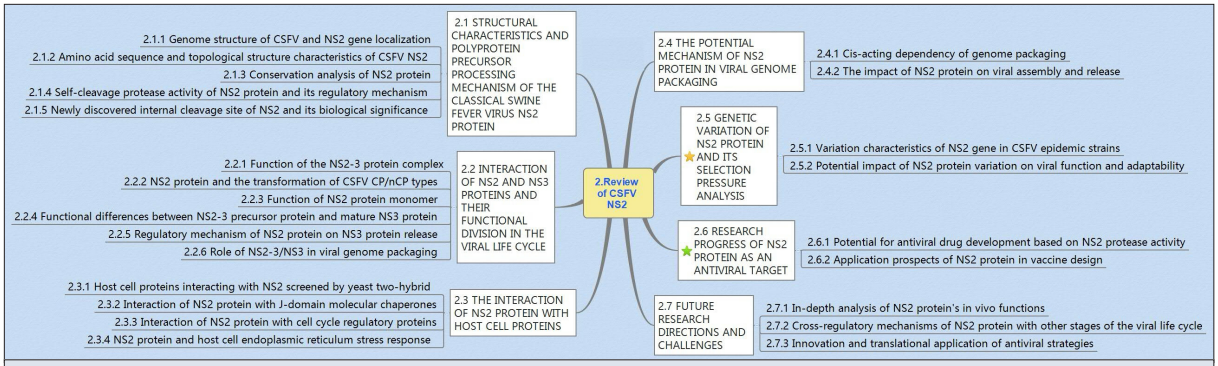

Fig 1. Study scheme on the research review of the NS2 protein of CSFV

influences the maturation and release of NS3 by regulating the cleavage process of NS2-3, thereby promoting effective viral replication [13]. In the early stages of CSFV infection, the NS2 protein fosters the release of NS3 through its self-protease activity, while the mature NS3 is a multifunctional enzyme essential for viral replication, possessing helicase, nucleoside triphosphatase (NTPase), and protease activities. This dynamic protein processing plays a key role at different stages of the viral life cycle, ensuring effective transmission and proliferation of the virus [10].

In recent years, significant progress has been made in the structural and functional studies of the NS2 protein. Through in-depth analysis of the NS2 protein and related molecules, scientists have revealed its multiple roles in the life cycle of CSFV [14,15]. For example, researchers have found that the interaction of the NS2 protein with the cellular protein DNAJC14 is a key regulatory factor for its self-protease activity, and the absence of DNAJC14 may lead to changes in the viral replication mechanism [13]. This finding provides a new perspective for understanding the functions of the NS2 protein, suggesting that there may be different cellular factors involved in regulating the activity of the NS2 protein.

A deeper understanding of the multifaceted functions of the NS2 protein not only aids in uncovering the intricate pathogenic mechanisms underlying CSFV but may also offer significant insights and valuable clues that could be instrumental in the development of innovative antiviral drugs and effective vaccine strategies aimed at combating this viral infection *(Fig. 1)* [16]. With the continuous exploration of the NS2 protein and its role in the viral life cycle, specific inhibitors targeting its functions may be discovered in the future, providing new therapeutic ideas for controlling the spread and infection of CSFV. In summary, the NS2 protein has significant scientific value in the biological research of CSFV, and its in-depth study will help promote the continuous improvement of control strategies for CSFV.

Structural Characteristics and Polyprotein Precursor Processing Mechanism of The CSFV NS2 Protein

Genome Structure of CSFV and NS2 Gene Localization

CSFV belongs to the *Flaviviridae* family and the *Pestivirus* genus, and it exhibits serological cross-reactivity with Bovine Viral Diarrhea Virus (BVDV) and Border Disease Virus (BDV) of the same genus [17-19]. The genome of CSFV is a single-stranded positive-sense RNA, approximately 12.3 kb in length, containing a large open reading frame (ORF) that encodes a polyprotein of approximately 3,898 amino acids. This polyprotein is cleaved into four structural proteins (C, E^{rns}, E1, and E2) and eight non-structural proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) under the action of host cell and virus-encoded proteases [17].

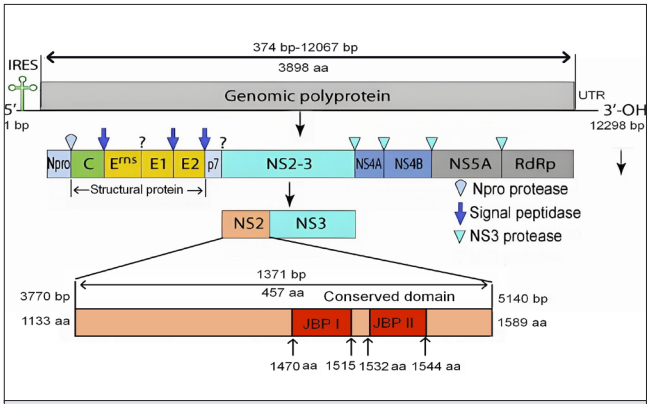

Fig 2. The genome map and NS2 gene location of CSFV. The overall structure of the CSFV genome can be categorized into three distinct components: the 5'-untranslated region (5'-UTR), which encompasses the internal ribosome entry site (IRES); the ORF responsible for polyprotein synthesis; and the 3'-untranslated region (3'-UTR). The NS2 gene is located in the middle of the polyprotein ORF, adjacent to the p7 gene on the left and the NS3 gene on the right. The gene contains two conserved domains, JBP I and JBP II, that bind to Jiv90 (host cellular molecular chaperone)

Among them, the non-structural protein NS2 is a hydrophobic transmembrane protein. Bioinformatics analysis indicates that the full length of the CSFV NS2 gene is 1371 bp, encoding a total of 457 amino acids, with a molecular weight of approximately 52 kDa [20]. To study the function of the CSFV NS2 protein, we mapped the genome structure of the CSFV Shimen virulent strain and the localization of the NS2 gene *(Fig. 2)* [12,15,21]. The NS2 gene resides centrally within the polyprotein ORF, flanked to the left by the p7 gene and to the right by the NS3 gene. This gene encompasses two conserved domains, JBP I and JBP II, which interact with Jiv90, a molecular chaperone of the host cell.

Amino Acid Sequence and Topological Structure Characteristics of CSFV NS2

The amino acid sequence of the CSFV NS2 protein shows high conservation among different strains. Comparative analysis of the amino acid sequences of NS2 proteins from multiple CSFV strains reveals that the conserved amino acid residues are mainly concentrated at the N-terminus and C-terminus, while the amino acid sequence in the middle region is relatively variable [21]. The N-terminus of the NS2 protein contains multiple conserved cysteine residues, which may participate in the formation of intramolecular or intermolecular disulfide bonds, playing an important role in maintaining the structure and function of the protein [22].

Bioinformatics analysis predicts that the NS2 protein has a complex topological structure, containing multiple transmembrane regions. Studies show that the N-terminus of the NS2 protein contains four transmembrane helices (TM1-TM4), and the C-terminus contains one transmembrane helix (TM5). Among them, TM1 - TM4 form a tight hydrophobic domain that anchors the NS2 protein to the endoplasmic reticulum membrane, while TM5 may participate in the interaction of the NS2 protein with other viral proteins or host cell proteins [21].

Conservation Analysis of NS2 Protein

The NS2 protein exhibits a high degree of conservation in CSFV and other *Pestivirus* species, and its structural characteristics play an important role in the viral life cycle. Studies indicate that the NS2 protein contains a key protease active site, a feature commonly found in different *Pestivirus* species [23]. This conservation suggests a core function of the NS2 protein in the viral replication process, particularly in the processing of polyprotein precursors. Specifically, the self-protease activity of the NS2 protein enables it to mediate the cleavage of the NS2-3 precursor, thereby regulating the release of the NS3 protein, which plays an essential role in viral replication and assembly [10]. The conservation of the NS2 protein provides an important basis for understanding its function in the viral life cycle.

Self-Cleavage Protease Activity of NS2 Protein and Its Regulatory Mechanism

The self-cleavage protease activity of the NS2 protein is not only an essential feature of its function but also has a profound impact on the viral life cycle. Through self-protease activity, the NS2 protein can cleave the NS2-3 precursor, releasing the functionally diverse NS3 protein [24]. The NS3 protein is involved not only in viral replication but also plays a vital role in viral assembly and release. Notably, the cleavage activity of the NS2 protein is influenced by different stages of the viral life cycle and cytokines. For example, the action of certain cytokines may enhance or inhibit the cleavage activity of NS2, thereby regulating the efficiency of viral replication and assembly [10]. This regulatory mechanism provides new insights into understanding the biological characteristics of CSFV and offers potential targets for developing antiviral strategies against CSFV.

Newly Discovered Internal Cleavage Site of NS2 and Its Biological Significance

Recent studies have identified a new internal cleavage site of the NS2 protein, mediated by the NS3/4A protease, specifically located at L188-G189. This cleavage site shows a certain degree of conservation among multiple *Pestivirus* species, indicating its importance in viral function [25]. Although the impact of this cleavage site on viral replication and viral particle formation is limited in *in vitro* cell experiments, its function *in vivo* requires further investigation. This finding offers new insights into the multifunctionality of the NS2 protein and may reveal the complex regulatory mechanisms of the virus within host cells. Future research is expected to clarify the specific biological significance of this cleavage site and its role in the viral life cycle, providing a new theoretical basis for the prevention and control of CSFV.

Interaction of NS2 and NS3 Proteins and Their Functional Division in the Viral Life Cycle

Function of the NS2-3 Protein Complex

The NS2 protein exists mainly in two forms after CSFV infects host cells: the NS2-3 protein complex and the NS2 monomer. The NS2-3 complex is a key molecule in the replication and viral particle assembly processes of *Pestivirus* life cycle [26].

In BVDV, the NS2-3 protein complex contains 1140 amino acid residues and has a size of 120 kDa. The NS2-NS3 complex of BVDV can be detected in infected cells, while the production of NS2 and NS3 monomers of BVDV is

completed by the proteolytic activity of the BVDV NS2 protease [27]. Studies on BVDV, which belongs to the same genus as CSFV, indicate that the NS2-NS3 complex is essential for the formation of viral particles [26]. Still, only the NS3 monomer is necessary for viral replication [28,29]. NS3 has helicase [30], NTPase [31], and protease activities [24], making it an essential protein for CSFV genome replication.

The NS2-3 complex is necessary for viral particle formation. Inserting a ubiquitin gene or an internal ribosome entry site between NS2 and NS3 of BVDV can disrupt the NS2-3 complex. This type of mutation does not affect viral replication but leads to defects in the generation of progeny viral particles. However, supplementing the NS2-3 complex with exogenous expression plasmids can restore progeny virus production [26]. Research on CSFV shows that during viral replication, the NS3 protein is released from the NS2-3 complex and joins the replication complex to function as an RNA helicase, thereby participating in viral replication [32,33]. During viral assembly, the NS2-3 complex recruits the NS4A protein to participate in the formation of progeny viruses on the endoplasmic reticulum [34].

NS2-3 can be cleaved by NS2 into NS2/NS3 monomers. Meyers *et al*. discovered the cIns sequence in BVDV [35], and Rinck et al.[36] identified it as the Jiv90 protein. Lackner et al.[37] demonstrated that NS2 has cysteine protease activity, Jiv90 regulates viral replication [38], and the binding domains of NS2 and Jiv90 were identified as JBP I and JBP II, respectively [23]. Balint et al.[39] found that the insertion sequence enhances NS2-3 cleavage. Agapov et al.[26] confirmed that the NS2-3 complex is essential for viral assembly. The NS2-host interaction mechanism still needs further research.

NS2 Protein and the Transformation of CSFV CP/nCP Types

BVDV, a member of the *Pestivirus* genus, has two biotypes: cytopathogenic (CP) and non-cytopathogenic (nCP). The CSFV wild-type strain usually does not produce cytopathic effect (CPE). Genomic recombination can lead to the transformation from nCP to CP [40,41]. commonly seen in the insertion of a ubiquitin gene in the NS2-3 region [42]. In 1998, Meyers G et al.[35] discovered that the CP-type BVDV NADL strain had a 270bp cellular sequence cIns inserted between NS2-3, and its deletion resulted in the loss of CPE. Additionally, Kummerer et al.[43] found that CP type originated from mutations in nCP type, with changes in the NS2 gene being a key pathway. In 1999, Moser et al.[44] constructed a CSFV deletion clone and found that NS2 is non-essential but regulates viral replication. The deletion of the NS2 gene does not affect the replication of infectious RNA in cells; however, the NS2-deleted strain can cause CPE. In 2001 Aoki et al.[45] and in 2004 Aoki et al.[46] found that nCP-type CSFV can assist in the replication of defective particles, and CPE is associated with the accumulation of NS3. In 2007, Moulin et al.[34] confirmed that NS2-3 is essential for viral assembly, and CSFV lacking NS2 cannot complete the packaging of viral particles. In 2008, Gallei et al.[47] constructed a chimeric virus, demonstrating that the chimeric virus's CP-type virulence was weakened. Studies indicate that NS3 promotes the development of CPE, while NS2 may inhibit this effect. In 2011, Lamp et al.[20] found that nCP CSFV maintains low levels of mature NSP by delaying the processing of NS2-3 and downstream NSP, which may be related to its persistent infection characteristics. In contrast, CP CSFV accumulates NSP and causes CPE due to the high expression and rapid processing of NS3.

Function of NS2 Protein Monomer

The NS2 protein is highly conserved, a hydrophobic transmembrane protein located on the endoplasmic reticulum membrane, containing one self-protease responsible for the cis-cleavage at the NS2-3 junction, and its N-terminus can regulate viral replication [12]. Studies show that the different forms of NS2 and NS3 present *in vivo* are closely related to the CPE of the virus [29,39].

Current research on the NS2 protein of CSFV indicates that NS2, when expressed in cells, does not induce apoptosis but can regulate the host cell cycle to arrest in the S phase and upregulate the expression of IL-8 [14,15].

In 2011, Guo et al.[21]'s research showed that the CSFV NS2 protein contains two intrinsic signal peptide sequences, which are involved in the translocation of the NS2 protein to the endoplasmic reticulum. The NS2 protein may also have at least four transmembrane domains. The NS2 protein contains 457 amino acid residues. It exhibits self-cleaving protease activity, which is released from the NS2-3 complex through a cis-cleavage process, and anchors itself to the endoplasmic reticulum via its hydrophobic structure. The amino acid residues His1447 and Cys1512 are essential for maintaining NS2 protease activity [21,24]. NS2 regulates the number of NS3 monomers in the cell through its self-cleavage process, thus preventing excessive RNA replication that leads to the accumulation of viral RNA, causing CPE. Therefore, NS2 acts as a switch for *Pestivirus* replication and assembly [34]. *In vitro* experiments also demonstrate that although NS2 is not related to genome replication, it can extend the half-life of RNA replication complexes. Recent studies on NS2 monomers also show that it can participate in various life processes within the cell. First, NS2 can accelerate the degradation of Cyclin A by the proteasome while increasing the translation level of Cyclin A, leading to the host cell division process being stalled in the S phase, providing an optimized

intracellular environment for viral replication [14]. Second, NS2 can induce endoplasmic reticulum stress responses and activate NF-κB, leading to the upregulation of IL-8 expression, synergistically antagonizing type I interferon responses. Meanwhile, research by Tang et al.[15] proves that CSFV NS2 protein expression can also increase the expression of the anti-apoptotic protein Bcl-2, resisting the apoptosis response induced by MG132, thus playing a role in the inflammatory response and the persistent infection of CSFV.

Functional Differences between NS2-3 Precursor Protein and Mature NS3 Protein

In the life cycle of CSFV, the functions of the non-structural protein NS2-3 precursor and mature NS3 protein exhibit significant differences. The NS2-3 precursor plays a key role in the later stages of viral particle generation, while the mature NS3 protein is primarily responsible for the viral replication process [34]. Specifically, the NS2-3 precursor serves not only as the basis for viral assembly but also as an important bridge in the formation of viral particles, promoting the aggregation and assembly of other structural proteins. Research shows that during the viral life cycle of CSFV, only the uncleaved NS2-3 precursor can effectively support virus production, indicating that the self-protease activity of NS2 plays a crucial role in the viral life cycle [10].

On the other hand, the mature NS3 protein exhibits various enzymatic activities, including helicase [30], NTPase [31], and protease [24]. These functions are essential for viral RNA replication. Studies indicate that NS3 can promote the synthesis and replication of viral RNA through its helicase and NTPase activities. During the replication process, NS3 interacts with other non-structural proteins, such as NS4A, to form an efficient replication complex. This interaction is crucial for ensuring the effective replication of viral RNA and subsequent viral particle formation [48].

In summary, the NS2-3 precursor and mature NS3 protein play different roles in the viral life cycle, with the former focusing on viral assembly and the latter concentrating on RNA replication. This functional division provides an essential basis for understanding the replication and assembly mechanisms of CSFV.

Regulatory Mechanism of NS2 Protein on NS3 Protein Release

The NS2 protein plays a crucial role in regulating the release of the NS3 protein, with its self-protease activity and interaction with cytokines ensuring the timing and efficiency of NS3 release. Specifically, NS2 catalyzes the cleavage of the NS2-3 precursor through its self-protease activity, releasing the mature NS3 protein [10]. The timing of this process is crucial for viral replication and assembly. Studies indicate that the self-protease activity of NS2 is co-regulated by cytokines, and this regulatory mechanism plays a vital role in ensuring the temporal coordination of viral replication and assembly processes [49].

Additionally, the NS2 protein enhances its regulatory ability on NS3 release through interactions with the cell membrane and binding with other non-structural proteins. For example, the interaction between NS2 and NS3 is achieved by forming a membrane-bound complex, and the stability of this complex directly affects the efficiency and timing of NS3 release [50]. Research shows that only by releasing NS3 at the appropriate time can the virus effectively carry out RNA replication and particle assembly. This regulatory mechanism not only improves the efficiency of the viral life cycle but also provides potential targets for future antiviral therapies [50].

In-depth research on the regulatory mechanism of NS2 on NS3 release can provide new insights into understanding the viral life cycle and assist in developing therapeutic strategies against CSFV.

Role of NS2-3/NS3 in Viral Genome Packaging

In the process of genome packaging of CSFV, the NS2-3 precursor and mature NS3 protein play different but complementary roles. Studies show that the mature NS3 protein must be encoded by the same RNA molecule (i.e., cis-acting) to ensure efficient genome packaging. The expression of NS3 is directly related to the efficiency of viral genome packaging; effective genome packaging can only be achieved when NS3 coexists with its encoding RNA [10].

Moreover, the roles of NS2-3 precursor and mature NS3 in genome packaging also show significant differences. The NS2-3 precursor is not only a necessary component required for viral particle assembly, but also provides support for the release of NS3. The mature NS3 primarily handles RNA binding during the packaging process, ensuring the integrity and specificity of the viral genome. In this process, the presence and activity of NS2 assist the function of NS3, ensuring the efficient packaging of the viral genome and the successful assembly of viral particles [13,48].

It is worth noting that in 2019, Dubrau et al.[51] demonstrated that the genus *Pestivirus* can achieve NS2-3 independent virus particle formation through a few key mutations (such as NS2 V439D/T444V, NS3 M132A, NS4A A48T, and NS5B D280G). These mutations coordinate the transition between RNA replication and virus packaging by regulating the conformation of the NS3/4A complex and the function of NS5B.

Based on these findings, the NS2-3 precursor and mature NS3 protein, along with their conserved amino acid

residues, play an indispensable role in the packaging of the virus genome. Their collaboration ensures the assembly specificity and integrity of the virus particles, providing important scientific evidence for understanding the biological characteristics of the CSFV and developing corresponding antiviral strategies.

The Interaction of NS2 Protein with Host Cell Proteins

Host Cell Proteins Interacting with NS2 Screened by Yeast Two-Hybrid

Kang et al.[52] screened multiple host cell proteins interacting with NS2 using the yeast two-hybrid system: GOPC, HNRNPH1, DNAJA1, ATP6, CSDE1, CNDP2, FAN CL, TMED4, DNAJA4, MOAP1, and PNMA1. These proteins are primarily associated with apoptosis, stress response, redox balance, and metabolism.

Interaction of NS2 Protein with J-Domain Molecular Chaperones

In CSFV, the self-cleavage activity of the NS2 protein depends on the assistance of the host protein Jiv (also known as a J-Domain protein interacting with virus protein). The Jiv90 fragment serves as the active form of Jiv and plays a key role in viral replication. Studies have found that Jiv90 is directly associated with the function of CSFV NS2: overexpression of Jiv90 significantly promotes viral replication, while knockdown of its expression inhibits viral replication. This interaction occurs in the cytoplasm (not the endoplasmic reticulum) and affects the efficiency of viral RNA replication [11].

Interaction of NS2 Protein with Cell Cycle Regulatory Proteins

The normal operation of the cell cycle is crucial for cell growth, proliferation, and differentiation. Viral infection often interferes with the cell cycle process to meet its own replication and proliferation needs. After cells are infected with CSFV, the expression of the NS2 protein is upregulated, which inhibits the activity of CDK1 by interacting with cell cycle regulatory proteins, leading to the cell cycle being unable to progress normally and stagnating in the S phase [14]. The S phase of the cell cycle is the period of DNA synthesis, and the virus may utilize the abundant DNA synthesis materials and related enzymes in the host cell during this period to replicate its own genomic RNA. Meanwhile, cells stalled in the S phase show reduced sensitivity to interferons (Interferon, IFN), which may help the virus evade the host's immune clearance and promote viral infection and proliferation [53].

NS2 Protein and Host Cell Endoplasmic Reticulum Stress Response

In 2010, Tang et al.[54] constructed subclones of different segments of NS2 and transfected host cells. Through confocal microscopy, they found that the NS2 protein is localized in the endoplasmic reticulum of host cells and contains at least two internal signal peptide sequences and four transmembrane regions. The transfected NS2 protein is rapidly degraded in host cells; however, the proteasome inhibitor MG132 can prevent the rapid degradation of the NS2 protein. Their research also found that the CSFV NS2 protein can induce an endoplasmic reticulum stress response in host cells and activate the nuclear transcription factor NF-κB, suggesting that the rapid degradation of CSFV NS2 protein by the proteasome may be related to the endoplasmic reticulum stress response.

The Potential Mechanism of NS2 Protein in Viral Genome Packaging

Cis-Acting Dependency of Genome Packaging

Genome packaging is a crucial step in the viral life cycle, involving the effective encapsulation of the viral genome. For the CSFV, the NS2 protein plays a key role in this process. Studies have shown that genome packaging depends on the cis-encoded mature NS3 protein, which can prevent the encapsulation of defective genomes, thereby improving the quality of viral particles. This was validated through trans-packaging experiments of viral subgenomes, which showed that genome packaging could only proceed effectively when the NS3 protein is encoded by the RNA molecule itself [10]. Although the NS2-3 precursor can provide support in the trans mode to facilitate the assembly of viral structural proteins, the encapsulation of the genome requires the cis expression of NS3. This finding highlights the pivotal role of NS3 in viral genome packaging and suggests a more intricate NS2-3/NS3 functional model, underscoring the complexity of the viral packaging mechanism. In this process, NS3 is not only a multifunctional enzyme with helicase and protease activities but also participates in genome encapsulation through its mature form. The self-cleavage process of NS2 is also considered a crucial mechanism regulating the release of the NS3, thereby ensuring the efficient release of mature NS3 during the early stages of the viral life cycle. This regulatory mechanism may serve as a downstream quality control to prevent the packaging of defective genomes and coordinate the encapsulation of RNA molecules before membrane acquisition [48,49]. Therefore, the role of NS2 in genome packaging is not limited to providing precursors but also indirectly determines the quality and efficacy of viral particles by influencing the maturation and release of NS3.

The Impact of NS2 Protein on Viral Assembly and Release

The NS2 protein also plays an important regulatory role in the processes of viral assembly and release. Research has shown that the NS2 protein indirectly affects the assembly and release of viral particles by regulating the release of the NS3 protein. Specifically, the proteolytic activity of NS2 and its interactions with other proteins may be involved in the formation of the viral membrane and the maturation of viral particles. In studies of HCV, NS2 is not only responsible for the self-cleavage of the NS2-NS3 precursor but also promotes the recruitment of envelope protein E2, thereby affecting viral assembly [55]. Furthermore, interactions between NS2 and other non-structural proteins have also been shown to be crucial in viral assembly. For example, the direct interaction between NS2 and NS3 proteins, as well as the binding with envelope proteins such as E1 and E2, plays an important role in connecting and stabilizing structures during the formation of viral particles. Through these interactions, NS2 helps establish a complex protein network, providing the necessary support for effective viral assembly [56,57].

Overall, the role of the NS2 protein in viral assembly and release is multifaceted. It not only affects packaging efficiency by regulating the release of NS3 but may also promote the maturation and release of viral particles through interactions with other key proteins. Understanding the comprehensive function of NS2 in the viral life cycle will provide an important theoretical basis for developing new antiviral strategies.

Genetic Variation of NS2 Protein and Its Selection Pressure Analysis

Variation Characteristics of the NS2 Gene in CSFV Epidemic Strains

Genomic analysis of Japanese CSFV epidemic strains from 2018 to 2020 revealed significant non-synonymous mutations in the NS2 protein, indicating that this gene is subject to positive selection. This phenomenon reflects the genetic variation that occurs in CSFV during the epidemic process, allowing it to adapt to the host environment and strengthen its transmission capability. Compared to highly conserved non-structural proteins such as NS3 and NS4A, the variation characteristics of the NS2 protein are more pronounced, which may be closely related to its role in viral adaptive adjustment. Specifically, as a multifunctional non-structural protein, NS2 is involved in viral replication, assembly, and immune escape, and its variation may provide the virus with new adaptive advantages. In the variations of the epidemic strains, studies found that most mutation sites related to NS2 are located within its functional domain. These mutations not only affect the structure and function of NS2 but may also influence its interactions with other non-structural proteins, thereby affecting the overall biological characteristics of the virus [22].

Moreover, the variation characteristics of the NS2 gene in epidemic strains also indicate that the virus may adapt to new ecological niches through selective mutations in different host environments. The accumulation of these mutations may support the transmission and pathogenicity enhancement of CSFV [58,59]. The emergence of positive selection, especially during periods of high epidemiological incidence, further highlights the importance of NS2 in the CSFV life cycle and its key role in adaptive evolution.

Potential Impact of NS2 Protein Variation on Viral Function and Adaptability

The variation of the NS2 protein not only affects its own function but may also have profound effects on the overall adaptability of the virus. Firstly, these variations may alter the protease activity and self-cleavage efficiency of NS2, changing its interactions with other non-structural proteins such as NS3. For example, the NS2 protein is believed to play an important role in viral assembly, and its self-cleavage function directly affects the release of NS3, which is an indispensable component in the viral replication process. Therefore, variations in NS2 may indirectly affect the virus's replication capability and assembly efficiency by influencing its self-cleavage process [55].

Secondly, variations in NS2 may also facilitate the virus's escape from immune surveillance, thereby enhancing its adaptability in diverse host environments. This adaptive change may lead to the virus exhibiting different pathogenicity and transmission capabilities in different hosts. For instance, specific mutations may enable the virus to replicate more easily in specific hosts or enhance its resistance to the host immune system, thereby increasing the virus's transmission potential and pathogenicity [60]. In summary, the genetic variation of the NS2 protein is not only a result of CSFV adaptive evolution but also a key factor for its successful transmission in different hosts. Future research should further investigate the specific effects of these variations on viral function, providing new insights for the development of vaccines and therapeutic strategies against CSFV.

Research Progress of NS2 Protein as An Antiviral Target

Potential for Antiviral Drug Development Based on NS2 Protease Activity

The NS2 protein of CSFV plays a vital role in the virus's life cycle, particularly in the cleavage and maturation of viral proteins. Studies have shown that the NS2 protein possesses self-protease activity, which facilitates the cleavage between NS2 and NS3, a process crucial for viral replication [10]. Therefore, the self-protease activity of NS2 provides a highly promising target for designing specific inhibitors. By inhibiting NS2-3 cleavage, it is possible to effectively block viral replication, thus offering new ideas for antiviral drug development.

In current research, the screening and structural optimization of small-molecule inhibitors and protease inhibitors have become hot topics. Many researchers are exploring compounds that can target the NS2 protein, which not only need to have high affinity for NS2 but also effectively inhibit its enzymatic activity, thereby reducing the virus's infectivity. For example, by utilizing high-throughput screening technology, researchers have identified several small-molecule inhibitors that can significantly reduce the replication levels of CSFV and Hepatitis C Virus [61]. Additionally, with the advancement of structural biology, researchers have begun to utilize computer simulation and molecular docking techniques to design more specific and efficient NS2 inhibitors, which are expected to play a crucial role in future clinical applications.

In summary, the development of antiviral drugs based on NS2 protease activity has significant theoretical significance and practical application potential. With a deeper understanding of NS2's functions, it is expected that more effective antiviral drugs against CSFV will be developed in the future, providing new solutions for controlling CSFV outbreaks [62].

Application Prospects of NS2 Protein in Vaccine Design

In 2002, Armengol [63] discovered that NS2 contains T cell epitopes. The conservative nature and key functions of the NS2 protein make it an important candidate target for vaccine design. During the vaccine development process, the NS2 protein, as a non-structural protein, plays a crucial role in the virus's life cycle, especially during the replication and assembly stages. Research has found that the NS2 protein is highly conserved in CSFV, indicating that it has a relatively stable structure and function across different viral strains, providing a solid foundation for its application in vaccine design [21].

Research on NS2 mutation sites helps improve the broad-spectrum and immunoprotective effects of vaccines. By analyzing the variations of NS2 in different viral strains, researchers can identify key immunogenic epitopes that can induce a broad immune response in the host. For instance, using modern bioinformatics tools, researchers can design recombinant vaccines containing multiple conserved epitopes, which can not only enhance protection against specific viral strains but also improve cross-protection against other related viruses [64].

Moreover, studies have shown that attenuated CSFV strains containing specific NS2 modifications (leading to high expression of NS3) can induce high levels of specific neutralizing antibodies (humoral immunity) in natural host pigs, demonstrating their potential for vaccine development [47]. Future research can focus on optimizing the development of CSFV attenuated strains of NS2 protein to enhance the immunogenicity and safety of vaccines, thereby providing a more solid foundation for CSFV vaccine development [65].

In conclusion, the application prospects of the NS2 protein in vaccine design are broad. With advancements in science and technology, vaccines targeting the NS2 protein are expected to provide new strategies and tools for controlling CSFV outbreaks in the future.

Future Research Directions and Challenges

In-Depth Analysis of NS2 Protein's *In Vivo* Functions

The NS2 protein plays an important role in the life cycle of CSFV, and an in-depth analysis of its *in vivo* functions is a key focus of future research [66]. To reveal the biological significance of NS2 intramolecular cleavage and its regulatory mechanisms, researchers can utilize animal models and *in vivo* infection experiments to explore NS2's functions. Specifically, researchers can use transgenic mice or transgenic pig models to effectively observe the impact of NS2 on host immune responses and NS2's role in viral replication. Additionally, in-depth studies on the interactions between NS2 and host cell factors can reveal how these NS2-host cell factor interactions affect the virus's pathogenicity. Therefore, exploring these interactions and their mechanisms is important. This will help us understand the specific role of NS2 in viral pathogenesis.

Cross-Regulatory Mechanisms of NS2 Protein with Other Stages of the Viral Life Cycle

The NS2 protein plays a role in viral replication. It may also participate in cross-regulatory mechanisms during other

stages of the viral life cycle, including assembly and release. Researchers should focus on the regulatory network of NS2 during these stages to gain a deeper understanding of its functions. For example, high-throughput omics technologies can identify key host proteins that interact with NS2 and analyze how these interactions affect different stages of the viral life cycle. Furthermore, structural biology techniques can also elucidate the multifunctional mechanisms of NS2, revealing its specific roles in viral assembly and release. Existing studies have shown that specific amino acid residues in the Influenza A Virus NS2 protein significantly impact the virus's survival and replication. This indicates that NS2's role in the viral life cycle extends beyond a single stage [67]. Therefore, a thorough understanding of NS2's regulatory roles at various stages of the viral life cycle will provide crucial insights for the development of new antiviral strategies.

Innovation and Translational Application of Antiviral Strategies

Developing new antiviral drugs and vaccines based on the functional characteristics of the NS2 protein is crucial for effective treatment [62]. This approach represents an important direction for future research. Researchers can utilize the unique structure and functions of the NS2 protein to design specific inhibitors targeting the NS2 protein, effectively blocking viral replication and transmission. Additionally, applying NS2-related research findings in clinical and pig farming settings is crucial for controlling the spread of CSFV. For instance, interfering with the interaction between NS2 and host cell factors may effectively reduce the virus's pathogenicity. This approach offers new insights for developing innovative antiviral strategies. At the same time, integrating modern biotechnologies, such as gene editing and vaccine vector technologies, can enhance the immunogenicity and safety of vaccines. This improvement will promote their application in the farming industry. Through these innovative studies, the threat of CSFV to the swine industry is expected to be significantly reduced in the future. This reduction will contribute to animal health and food safety.

Conclusion

CSFV, as an important viral animal disease agent, affects not only the pig farming industry but also involves food safety and public health. In recent years, research on the non-structural protein NS2 of CSFV has gained increasing attention, and the revelation of its multiple key functions in the viral life cycle marks a significant milestone in our understanding of this virus. The core role of NS2 in polyprotein processing, NS3 protein release, and viral genome packaging highlights its importance in viral biology.

Recent studies have shown that the discovery of new cleavage sites within the NS2 protein further enriches our understanding of its multifunctionality. These cleavage sites not only provide new perspectives for studying the viral life cycle but also offer a biological basis for the virus's adaptive evolution. As observed in various studies, the genetic variations of NS2 reflect the selective pressures the virus encounters in the host environment, providing important evidence for vaccine design and the development of antiviral strategies. By deeply analyzing the mechanisms of NS2's variations, researchers can better predict the virus's mutation trends, thereby enhancing the effectiveness and durability of vaccines.

As research on NS2 as an antiviral target continues to deepen, future studies are expected to promote the development of new antiviral drugs and vaccines. The multifunctionality of NS2 makes it an ideal research subject, capable of providing key clues to reveal the virus's pathogenic mechanisms and transmission pathways. To achieve this goal, further *in vivo* functional validation and mechanism analysis will be the focus of future research. Researchers need to establish more comprehensive model systems to fully reveal the specific roles of NS2 in the pathogenicity and transmission of CSFV, providing theoretical support and practical guidance for controlling CSFV.

In the current research context, balancing different research viewpoints and findings has become an important challenge we face. Although existing studies provide multifaceted evidence for NS2's functions, caution must be exercised in experimental design and data interpretation. Different research groups may employ different experimental methods and models, resulting in discrepancies in understanding the functions of NS2. Therefore, in future research, establishing unified experimental standards and data analysis frameworks will help integrate different research findings and form a more consistent scientific consensus.

In summary, the NS2 protein plays a crucial role as the core of CSFV research. By exploring its multiple functions and roles in the viral life cycle, we can not only broaden our scientific understanding of CSFV but also lay the foundation for developing effective prevention and control strategies. Future research should focus on addressing current scientific issues and continuously advancing prevention and treatment strategies for CSFV.

Highlight Keypoints

1. Subcellular localization and ER Stress induction: The NS2 protein is mainly localized in the host cell endoplasmic reticulum, activating the NF-κB signaling pathway by inducing endoplasmic reticulum stress, leading to the degradation of Cyclin A protein.

2. Cell cycle regulation: The NS2 protein promotes the degradation of Cyclin A through the proteasome pathway, causing cell cycle arrest at the S phase and significantly inhibiting the proliferation activity of host cells.

3. Protease activity and viral replication: NS2 has its own protease activity, capable of cleaving the NS2-3 precursor protein into NS2 and NS3 monomers, a process that is crucial for viral RNA replication.

4. CPE: NS2 regulates viral replication and inhibits the development of CPE; the absence of the NS2 gene does not affect the replication of infectious RNA in cells, but NS2-deficient strains can cause CPE.

5. Virulence-related functions: The cleavage efficiency of the NS2-3 protein is directly related to the pathogenicity of the virus and is one of the key molecules determining the virulence of CSFV.

Declarations

Availability of Data and Materials: Data availability is not applicable to this article as no new data were created in this study.

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