Analytical approach for flavivirus research

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Abstract- Flaviviridae is the family of viruses and has been studied for many decades. New analytical approaches i.e., Isolation, sequencing, structure elucidation also have been performed. Areas that need to be focused is drugs designing, many of the work have been done for it, but targets remains the non structural proteins, viral binding site still needs to be focused on. Development of new transgenic cell lines, so that it becomes resistant to infection from virus and inturn reduce flaviviral infection can also be potential site to work. Analytical methods to target the virus need to be developed, which could be use to find potential target site to inhibit viral replication.

Keywords- Flaviviridae, X-RD, TLC, MALDI, NMR, MS, RNAi, MHC

Introduction

Flaviviridae family comprises of three genera, Flavivirus, Pestiviruses, and Hepacivirus, which forms a large group of viral pathogens that causes infections to humans and animals. It addition to these genera, two groups of unassigned viruses, GBV-A and GBV-C, await formal classification within the family. Flavivirus consist of a large genus that includes dengue virus, *Japanese encephalitis virus (*JEV), *tick-borne encephalitis- virus* (TBEV), *West Nile virus* (WNV), and *yellow fever virus* (YFV). Pestiviruses comprises four species *Bovine viral diarrhoea viruses*, *Classical swine fever virus* (CSFV), formerly named *hog cholera virus*, Border disease virus (BDV).Pestiviruses can also be notable on the basis of their effect on tissue culture cells as cytopathic or non cytopathic (Tautz et al., 1998; Deregt and Loewen., 1995). Hepacivirus comprises of *Hepatitis C virus* species, which can be classified into six genetic groups (Büchen-Osmond., 2006). Flaviviridae virions are positive single stranded genome, encoded by structural and non structural proteins. Virions are spherical consisting of a nucleocapsid with an envelope and isometric core (Büchen-Osmond., 2006; Büchen-Osmond., 2006 Büchen-Osmond., 2006). Virions are measured 40-50 nm in diameter. Classification of the virus along with the disease it causes is mentioned in table 1.

Structure of Flaviviridae

Binding of the virus to the cell receptor play a very important role towards viral replication, as it is first step towards viral infection of the host cells, in order to penetrate the host cell and establish the infection (Altmeyer., 2004). After entering host cell virus establishes infection gaining controls on some of the host cell machinery. Initial replication requires some necessary viral components which a virus always carries in its genome so that it can initiate the process of duplication. Knowledge about the basic viral genome and its protein structure could be used for varied application such as drug design, conformational epitope mapping, vaccine designing, homology modeling etc. Also there are development in isolation technique and structural development. This review summarizes few of the important recent work done in improving the knowledge information related to the genus of Flaviviridae family.

Flavivirus

As mentioned in the classification table (table 1) the genome comprises of four structural and seven non-structural proteins. The dengue virus envelope protein has been isolated using Histidine tags by an inexpensive way which yields the protein that could be useful for diagnostics as wells as for vaccine development (Jaiswal et al., 2004). Structurally conserved functional domains as model for the development of 3D-model for the envelope glycoprotein of West Nile virus, from which epitopes for the virus can be used to identified as binding site for HLA (Human Leukocyte Antigen) proteins (Vijayasri and Agrawal., 2005). Far-UV circular dichroism spectroscopy analysis of capsid protein of Flavivirus indicates it to be comprising of four alpha helical (Jones et al.,

2003). Disulphide bond analysis of the NS1 protein revealed presence of 12 half cystines being conserved among flavivirus suggesting their importance in structure and function of NS1 (Wallis et al., 2004), which could be analyzed by the NS1 antigen-capture Elisa for the early detection of the viral infection (Kumarasamy et al., 2007). Catalytically active form of protease CF40.Gly.NS3pro recombinant extracted using size exclusion chromatography acts as useful substrate for structure based drug design (Arakaki et al., 2002). Structural analysis of viral NS3 protease of *West Nile virus* and related serine protease to inhibit viral replication suggests induced fit to be common catalytic mechanism for ligand stabilization due to presence of two conformational state of active histidine (Robin et al., 2009). 3D structure of NS3 helicase domain for the Australian variant of West Nile virus suggest elongated molecular assembly of the protein as compared to hepatitis C virus (Mastrangelo et al., 2007). Active form of the NS3 protease is linked to 40 residues of NS2B cofactor showing highly flexible and disordered region(s). Proteolytic analysis determines that the region from residues D50 to E80 of NS2B interacts directly and strongly with the NS3 protease domain (Melino et al., 2006). The viral RNA-dependent RNA polymerase NS5 plays an important role in virus replication of dengue virus and represents an interesting target for the development of specific antiviral compounds. The three-dimensional structure of it can be used for structure-based drug-design (Yap et al., 2007). A model of interaction related to dengue c protein with RNA and viral membrane is proposed based on the asymmetric charge distribution of the protein (Ma et al., 2004). Interaction studies between the capsid protein of West Nile virus and host cell encoded phosphatase inhibitor revels that capsid binding site overlaps the region of I(2)(PP2A) required for the inhibition of PP2A activity, which results in increased PP2A activity, proving important for viral pathogenesis(Hunt et al., 2007). High mannose content is found at glycosylation position of the dengue virus, as well as loss of potential glycosylation site in the dengue 2 virus mutant in their fusion-from-within type(Johnson et al., 1994).

Hepacivirus

Much of the work for isolation of the structural and non structural protein for hepatitis virus, as well as for the detection of the viral components have been done *viz*., RT-PCR (Reverse Transcription-Polymerase Chain Reaction), QIAGEN EZ1 DSP Virus Kit(Schneider et al ., 2007) [27]. Recently, 3'-X-tail element, which is highly conserved in the Hepatitis C virus, is used for detection and viral assays (Drexler et al., 2009). Structure identifications of structural and non structural proteins of HCV are available in the PDB database, which could be used for designing inhibitors for the Hepatitis C virus (HCV). Targeting NS3 protease variants for structure based drug designing have been described by da Silveira NJ et al (da Silveira NJ et al., 2005).

Analytical Techniques

X-Ray crystallography

Small angle X-ray scattering analysis of full length NS3 to study the interaction of the helicase and protease disclose it to be an elongated assembly (Mastrangelo et al., 2007). Viral methyltransferase which are responsible for methylation of mRNA belong to either (guanine-N7)-methyltransferase or nucleoside-2'- methyltransferase. X ray crystallographic analysis of two flavivirus (Meaban and Yokose) classified them in the nucleoside-2'-methyltransferase group (Mastrangelo et al., 2006). Hanging-drop vapour-diffusion method at 291 K to isolate the putative receptor-binding domain (Domain III) for West Nile Virus was crystallized from inclusion bodies after several other crystallographic attempts (Yuan et al., 2005). Similar approach has been tried to isolate putative receptor-binding domain (Domain III) for Langat virus at 277 K (White et al., 2003).There are three class of viral fusion protein depending on the structure. Conditions to purification the E1 ectodomain of the Semliki virus before and after the membrane fusion interaction to generate good X-ray crystallographic results have been discussed by Gibbons DL et al (Gibbons et al., 2004). Viral fusion proteins of class II type has been characterized from tick-borne encephalitis envelope protein, which has been crystallized using nonionic detergent (Stiasny et al., 2004). X- ray crystallographic analysis of the soluble form of the E protein of tick borne encephalitis virus is currently under investigation of potential antiviral sites (Heinz et al., 1993;Heinz et al., 1991). Variations in the antigenic sites of these E proteins lead to the

attenuation of the protein, which could be of potential advantage for future drug discovery process (Heinz et al., 1993).

TLC method

Interaction of the envelope protein with cell surface receptor to initiate the initial step of the viral infection is studied with the help of (Thin Layer Chromatography) TLC/virus-binding assay, further characterization of the cell surface receptor lead to the purification of neolactotetraosylceramide, along with non reducing terminal disaccharide residue Galβ1-GlcNAcβ1 on the dengue susceptible cells, suggesting multivalent oligosaccharide to act as competitive inhibitor of the dengue 2 binding to cells (Aoki et al., 2006).

MALDI

Matrix Assisted Laser Desorption /Ionization-Time Of Flight (MALDI-TOF), mass spectrometry, MALDI post-source decay, and mald-tms has been used to study the 12 half cystines of NS1 separated from size exclusion method (Wallis et al., 2004).To understand the pathogenesis of *West Nile Virus* associated meningoencephalitis, proteomic profiling of the rat neurons and subsequent identification of the expression altered protein is performed with MALDI-TOF MS using peptide mass fingerprinting and database searching (Dhingra et al., 2005). Proteomic analysis of the hepatic cells to analyze secreted proteins during the dengue virus infection has been studied using electrophoresis liquid chromatography coupled with LC-MS/MS succeeded by MALDI-TOF(Higa et al., 2008). SDS (sodium dodecyl sulfate) polymer-filled capillary gel electrophoresis (CE-SDS) for testing the purity of the GB virus-C fusin proteins proved efficient method for protein purity method as compared to normal SDS-PAGE coupled with scanning densitometery, but is less accurate as compared to MALDI-TOF MS(Kundu et al., 1997).

HPLC

Separation and characterization of the three structural proteins, E, C and M, from tick-borne encephalitis virus has been done by means of a two-step high-performance liquid chromatography (HPLC) technique, followed by peptide mapping (Winkler et al., 1985). Isolation and Identification of the macrophage binding site of the suppressor cytokine (SC) induced by dengue 2 virus with help of SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and HPLC revealed, suppressor cytokine receptor(SCR) to be the β chain of the αβ polypeptide chain of the SCR (Mukherjee et al., 1993).

NMR

Structural difference for domain III envelope protein between Omsk hemorrhagic fever virus and tick borne flaviviruses have be studied by NMR structure of the former structure(Volk et al., 2006).Structure of dengue capsid protein has been determined NMR (Nuclear Magnetic Resonance) method using secondary structure sorting approach (Ma et al., 2004). A model of interaction related to dengue c protein with RNA and viral membrane is proposed based on the asymmetric charge distribution of the protein (Ma et al., 2004). NMR studies of the NS3 protease NS4A cofactor complex of the hepatitis C virus proteins revealed that there are long structural changes of the loop and the strand region ofV51- D81 residues of NS3 protein as NS4A binds to NS3 (McCoy et al., 2001).

Additional method of Assay

An assay to specifically detect the presence of St. Louis encephalitis virus (SLEV) and *Eastern equine encephalitis virus* (EEEV) duplex TaqMan real-time reverse transcriptase polymerase chain reaction (PCR) assay has been developed by Hull et al(Hull et al., 2008) [47]. *Modified Shell Vial Culture* (MSVC) protocol has been applied for the detection of flaviviruses by Caceda ER et al(Caceda et al., 2007). Sulfated derivatives of two glycan *viz.,* an alpha-D-(1-->4)-glucan with an alpha-(1-->4) linked branch attached to O-6 branch points showed strong anti-dengue virus bioactivities and increase in the degree of substitution the more potent the derivatives is (Qiu et al., 2007). Melting temperature and color multiplexing based method to detect all the serotype of dengue has been demonstrated by Lo CL et al (Lo et al., 2007). Detection of West Nile Virus can be done by VectTest antigen-capture assay which is a reliable method for large scale detection of the virus (Lindsay et al., 2003). Hernandez R and group have devised a simple, inexpensive micro-preparative HPLC method to purify and analyze nM quantities of DNA based on size, charge as well as overall G+C content

of the PCR product(Hernandez et al., 2004). Capsid and surface viral proteins have be isolated and their N-terminal been sequenced from tick-borne encephalitis and Venezuelan equine encephalomyelitis viruses (Akimenko et al., 1999). 2D-DIGE has been used to analyze proteome of early stage viral infected cell to understand the modification of expressions in host cell during the infection, which would help research in the field of antiviral therapy (Pastorino et al., 2009; Dhingra et al., 2005).

Current trends in Drugs development

Two important proteins have been identified in cellular response to the viral infection for flavivirus *viz*., Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and viperin. TRAIL is expressed in dengue virus infection, and it is proved to be one of the contenders against dengue viruses (Warke et al., 2008). Viperin is expressed by *Japanese encephalitis virus* (JEV) and *Sindbis virus* (SIN), in JEV it is negatively controlled to counteract its antiviral nature whereas, viperin exhibits potent antiviral activity against SIN (Chan et al., 2008). Thiazolidine studies related to design protease inhibitor done by Sudo K et al showed RD4-6250 of it to be most potent inhibitor of protease (Sudo et al., 1997). A genetic vaccine for *West Nile virus* (WN) synthesized with the WN premembrane-envelope gene sequences, the transmembrane and carboxyl terminal domains of lysosome associated membrane protein encoded as a chimera provided a long term antibody induction and neutralization as compared to unspecifically targeted vaccine (Anwar et al., 2005). mRNA display selection technology with cyclization used to study HCV translation has potentially be used to develop anti-HCV drug (Litovchick et al., 2008).

RNA Interference Studies

RNA interference is mechanism where 21-22 nucleotides small RNA is used to control the expression of gene. This expression can be targeted towards human in the ways *viz*., useful way or harmful way. RNA silencing when triggered by viral infection can be used to establish the viral illness (Akira and Tetsuro., 2008; Gomase and Tagore; 2008), or Viral suppressors of RNA silencing (VRS) can be used to silencing of the gene (Li et al., 2006). It can be used as a inhibit replication of the viral genomes, including flavivirus (Pacca et al., 2009). Much of the work has been done to show that replication of arthropod borne viruses, dengue type 2 viruses, can be inhibited in the vector of the viruses(Adelman et al.,2001; Sanchez-Vargas et al., 2004). Silencing can be done when the expression of the viral specific small interfering RNA is expressed at the critical site of the viral replication (Travanty et al., 2004). A number of transgenic mosquito lines that transcribe dsRNA constitutive promoters have be designed which reduces the viral competence and subsequently the chances of viral transmission to humans (Caplen et al., 2002). Genetically modified mosquitoes (*Aedes aegypti*) showed high level of viral competence which can be used as a replacement strategy to control the viral infection (Franz et al., 2006). Interference method is also looked up for the inhibition of the viral genome in the host cells and can be used for drug development. Mosquito vector species has been genetically modified by Hermes element from house fly so as to reduce the competence of the virus in the vector species (Jasinskiene et al., 1998). RNA interference studies with *West Nile virus* infection explains the role of ubiquitin ligase in the internalization of the virus and monocarboxylic acid transporter as replication resistant factor which can be used for flaviviruses as potential antiviral targets (Krishnan et al., 2008). Studies on the *West Nile virus* have also explored retrovirus-mediated siRNA delivery systems to analyze specific viral functions and can have impending therapeutic actions (Yang et al., 2008; Ong et al., 2006). Role of microRNA (miR-122) as one of the potential site in viral replication is one of the recent developed method (Pan et al., 2007).

Current trends in oncogenesis

Studies on *Hepatitis C Virus*, NS4B protein in relation to Ha-ras gene shows that the protein plays an important role in the malignant transformation of cell (Park et al., 2000). Role of NS5A in oncogenesis suggest the down regulation of protein kinase R (PKR) thereby surpassing the effect of interferon's (Giménez-Barcons et al.,2005; Gale et al., 1998). HBx protein is also thought to play role in tumor induction, by inhibiting Bcl2 pathway and suppressing the tumor suppressor protein p53, along with these many other signaling pathway are also triggered e.g., MAP, JAK and STAT pathway (Schuster et al.,2000; Wei et al., 2006; Arbuthnot et al., 2000).

MHC Class-I binding peptides

Currently, new research paradigm in vaccine design is emerging, following essential discoveries in immunology and development of new Major Histocompatibility Complex (MHC) Class-I binding peptides prediction tools. MHC molecules are cell surface glycoproteins, which take active part in host immune reactions. The involvement of MHC class-I in response to almost all antigens and the variable length of interacting peptides make the study of MHC Class I molecules very interesting. MHC molecules have been well characterized in terms of their role in immune reactions (Singh et al., 2002; Cui et al., 2006; Bhasin et al., 2003). They bind to some of the peptide fragments generated after proteolytic cleavage of antigen (Kumar et al., 2007). This binding acts like red flags for antigen specific and to generate immune response against the parent antigen. So a small fragment of antigen can induce immune response against whole antigen. Human papillomavirus viral peptides are most suitable for subunit vaccine development because with single epitope, the immune response can be generated in large population. TAP (Transporter associated with antigen processing) is a transporter associated with MHC class I restricted antigen processing. The TAP is heterodimeric transporter belong to the family of ABC transporter, that uses the energy provided by ATP to translocate the peptides across the membrane (Gomase et al., 2008). The subset of this transported peptide will bind MHC class I molecules and stabilize them. These MHCpeptide complexes will be translocated on the surface of antigen presenting cells (APCs). This theme is implemented in designing subunit and synthetic peptide vaccines (Gomase et al., 2008).

Applications of Vaccinomics

Vaccinomics is one of the leading roles of omics providing research in vaccine development, in order to provide best protection for children. Vaccines are developed against many disorders like polio, mumps, measles, hepatitis A, hepatitis B, rubella, diphtheria, pertussis, tetanus, HiB, influenza, meningococcal disease, chicken pox, pneumonia and rotavirus. It is noticed that vaccines do not guarantee complete protection from a disease. However, this is because the host's immune system simply doesn't respond adequately or at all. This may be due to a lowered immunity in general (diabetes, steroid use, HIV infection) or because the host's immune system does not have a B-cell capable of generating antibodies to that antigen. Adjuvants used for boosting immune response. Most often aluminium adjuvants are used, but adjuvants like squalene are also used in some vaccines and more vaccines with squalene and phosphate adjuvants are tested. Vaccinomics is one of the pioneering fields for providing new ideas for vaccine development. This provided new research paradigm in this area, with development of new vaccines against various diseases such as meningitis. This is particularly useful in developing countries where large number of people suffer from theses disease (Gomase and Tagore., 2008).

Conclusion

Flaviviridae family has been studied for many decades. Isolation, sequencing, structure elucidation also have been performed. Areas that need to be focused is drugs designing, many of the work have been done for it, but targets remains the non structural proteins like NS2, NS3. RNA dependent RNA polymerse (RdRP), viral binding site still needs to be focused on. Development of new transgenic mosquitoe lines, so that it becomes resistant to infection from virus and inturn reduce infection can also be potential site to work. New computational methods to target the virus need to be developed, one of such upcoming example is proteochemometris, which could be use to find potential target site to inhibit viral replication.

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Table 1- Classification of Flaviviridae

Genus		**Disease**	**Structural(S) & Non-structural proteins(NS) classification**
Flavivirus	*Tick borne Viruses*	Yellow fever, Dengue, West Nile, St Louis encephalitis, Japanese encephalitis, Tick-borne encephalitis (Gould and Solomon., 2008).	C,E prM,M are S And NS1, NS2a, NS2b, NS3, NS4a, NS4b & NS5 are NS.
	Mosquito borne viruses	Hemorrhagic disease, encephalitis, and jaundice in human (Harakuni et al., Chaturvedi and Nagar., 2008; Tomori., 2004).	
Pestivirus	*Bovine viral diarrhoea viruses*	Bovine Viral Diarrhea, Mucosal Disease in cattle(Neyts., 1999).	C, E^{rns}, E1, E2,are S And p7, N^{pro} ,NS2, NS3, NS4A, NS4B, NS5A, NS5B are NS
	Classical swine fever virus	Classical Swine Fever(Neyts., 1999; Moennig and Plagemann., 1992).	
	Border disease virus	Border Disease in Sheep(Neyts., 1999; Moennig and Plagemann., 1992).	
Hepacivirus	*Hepatitis C virus*	Hepatitis, cryoglobulinemia, mesangiocapillary glomerulonephritis (Armas-Merino, R.,1999)	C, E1, E2, p7 are S And NS2, NS3, NS4A, NS4B, NS5A, NS5B are NS