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AIM AND SCOPE

World Journal of Virology (*World J Virol*, *WJV*, online ISSN 2220-3249, DOI: 10.5501) is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJV covers topics concerning arboviral infections, bronchiolitis, central nervous system viral diseases, DNA virus infections, encephalitis, eye infections, fatigue syndrome, hepatitis, meningitis, opportunistic infections, pneumonia, RNA virus infections, sexually transmitted diseases, skin diseases, slow virus diseases, tumor virus infections, viremia, zoonoses, and virology-related traditional medicine, and integrated Chinese and Western medicine. Priority publication will be given to articles concerning diagnosis and treatment of viral diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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REVIEW

Neurological manifestations of Zika virus infection

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Abstract

Zika virus (ZIKV) is a flavivirus (*Flaviviridae* family) transmitted mainly by *Aedes* mosquitoes. The virus was restricted to the African continent until its spread to south-east Asia in the 1980's, the Micronesia in 2007, the French Polynesia in 2013 and, more recently in the Americas in 2015, where, up to date, the World Health Organization (WHO) has estimated about 3-4 million total cases of ZIKV infection. During outbreaks in the French Polynesia and Brazil in 2013 and 2015, respectively, national health authorities reported potential neurological complications of ZIKV disease, chiefly an upsurge in Guillain-Barré syndrome, which coincided with ZIKV outbreaks. On the other hand, the emergence of ZIKV in Brazil has been associated with a striking increase in the number of reported cases of microcephaly in fetus and newborns, twenty times higher than in that reported in previous years. While investigations are currently assessing whether there is an actual association between neurological complications and ZIKV infections, the evidence was enough worrisome for WHO to declare a public health emergency of international concern. Here we present an updated review addressing what is currently known about the possible association between ZIKV infection and the development of severe neurological disorders.

Key words: Zika virus; Flavivirus; Microcephaly; Guillain-Barré syndrome; Transmission routes

Core tip: Zika virus (ZIKV), a mosquito-borne flavivirus, was restricted to Africa until its spread to south-east Asia, the Pacific, and, finally, to the Americas, where an estimated 4 million cases of ZIKV infection have been recorded, and where a worrisome possible association of ZIKV with the development of severe neurological disorders, such as Guillain-Barré Syndrome and microcephaly, have been reported. In this contribution we present an updated review addressing what is currently known about the possible association between ZIKV infection and the development of severe neurological disorders, remarking the urgent need for further investigations to clearly resolve this point.

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THE VIRUS

Zika virus (ZIKV) is a mosquito-borne *Flavivirus* classified into the *Flaviviridae* family. It is closely related to other important pathogens that affect human and animal health such as Japanese encephalitis virus, dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV) or St. Louis encephalitis virus[1]. ZIKV was first isolated in 1947 from the serum of a febrile sentinel rhesus monkey in the Zika Forest (Uganda) during the investigations performed to study the enzootic cycle of YFV. The virus was isolated for the second time from *Aedes africanus* mosquitoes collected at the same site one year later. In both cases, the virus was isolated by intracranial inoculation into infant mice[2].

ZIKV genome is constituted by a positive polarity RNA molecule of about 11 kb in length, comprising two untranslated regions flanking an open reading frame coding for a polyprotein of about 3420 amino acids. Similar to other flaviviruses, the ZIKV single polyprotein is expected to be post-translationally cleaved by host and viral proteases into three structural proteins [capsid (C), pre-membrane (prM), and envelope (E)] and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A NS4B, and NS5)[3] (Figure 1). The structure of mature ZIKV particle has been recently described[4] (Figure 2), and the virus particle has been observed to be structurally stable even at 40 ℃[5].

Phylogenetic analyses of the virus confirm its inclusion within the mosquito-borne flavivirus cluster with the presence of two major lineages: One includes the African strains, which is divided into two groups, the East and the West African clusters, and the other gathers the Asian and American strains[1]. ZIKV life cycle, as any other arbovirus, has several barriers to accumulate mutations as a consequence of the intrinsic constraints associated with dual replication in mammalian and invertebrate hosts, thus driving to a relatively slow fixation of mutations[1]. For instance, ZIKV strains collected over a few years interval in Central African Republic show minimal changes on their sequences[6].

Even though ZIKV strains from different continents and outbreaks showed up to 99% identity[1], nonsynonymous nucleotide differences have been described among them that, in other flaviviruses, have been implicated in viral infectivity. For instance, a full-length ZIKV genome amplified from fetal tissues obtained during the Brazilian outbreak presented five nonsynonymous mutations when compared with the French Polynesian isolate[7]. Three of these amino acid changes were found in NS1, implicated in immune evasion in the case of DENV[8], one in NS4B, related to the inhibition of type I interferon signaling in other flaviviruses[9,10], and one in a NS5 domain which has been shown to mask the viral RNAs from host recognition in the case of WNV[11,12]. In this line, it has been hypothesized the possible adaptation of the ZIKV virus to the human host by changes in non-structural proteins[13]. Thereby, Asian strains of ZIKV differ significantly from the African ones in codon usage in the NS1 region of the genome[14]. Codon usage by the pandemic strain is optimized for adaptation to human housekeeping cells, which could facilitate viral replication in human cells. In fact, codon optimization could result in higher viral titers and increased infectivity for mosquito vectors, as seen in other viruses[15].

Analysis of the polyprotein sequence predicted the presence of potential N-glycosylation sites in the ZIKV proteins prM, E and NS1[4,16-18]. Noteworthy, a 4 amino acid deletion corresponding to the envelope protein 154 glycosylation motif was found in several ZIKV strains, in a similar way to many other flaviviruses, such as West Nile virus strains[6]. Glycosylation has been associated in some instances with virulence[19,20], even though the functional importance of the N-glycosylations is not clear in related flaviviruses, since flaviviruses presenting or not this N-glycosylation can maintain the same antigenicity[21]. Additionally, glycosylation could play a role in replication and maturation[22]. In fact, it has been suggested that extensive mouse brain or cell culture passage could lead to the deletion of the potential glycosylation site, since there are differences on this site even between ZIKV isolates with different passage history, such as those of the prototypic strain ZIKV MR766[23,24]. Even more, it has been suggested that ZIKV may have experienced recombination in nature and that a loss of the N154 glycosylation site in the envelope protein was a possible adaptive response to the vector[25]. Therefore, a detailed analysis of whether and how these differences are directly related to virulence and pathogenicity has to be clearly elucidated for a better control of ZIKV infection.

TRANSMISSION

ZIKV is transmitted by mosquitoes of the genus *Aedes,* mainly of *Aedes aegypti* and *Aedes albopictus*, although the virus has been isolated from other genus such as *Anopheles, Culex,* and *Mansonia spp*[1]. Both *Ae. aegypti* and *Ae. albopictus* have a history of global expansion associated with trade and travel and are widely distributed[26].

Non-human primates are considered to serve as reservoir hosts for ZIKV, although the primary species have not been identified. ZIKV natural transmission cycle has been described to involve *Cercopithecus aethiops* and *Erythrocebus patas* monkeys in Africa[27], while ZIKV antibodies have been found among semi-captive and wild orangutans in Asia[28] (Figure 3). There is no current evidence of other animals than humans and non-human primates acting as amplifying hosts for ZIKV[29]. However, antibodies against ZIKV have been

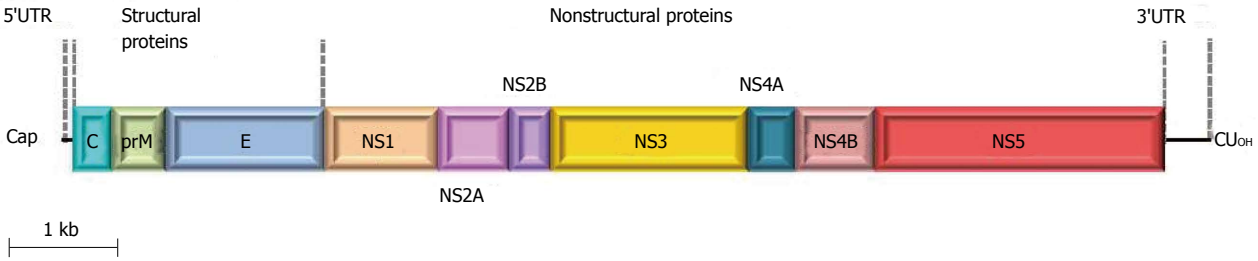

Figure 1 Schematic representation of Zika virus genome organization. The single open reading fram (boxes) that encodes both structural and non-structural proteins is flanked by two untranslated regions.

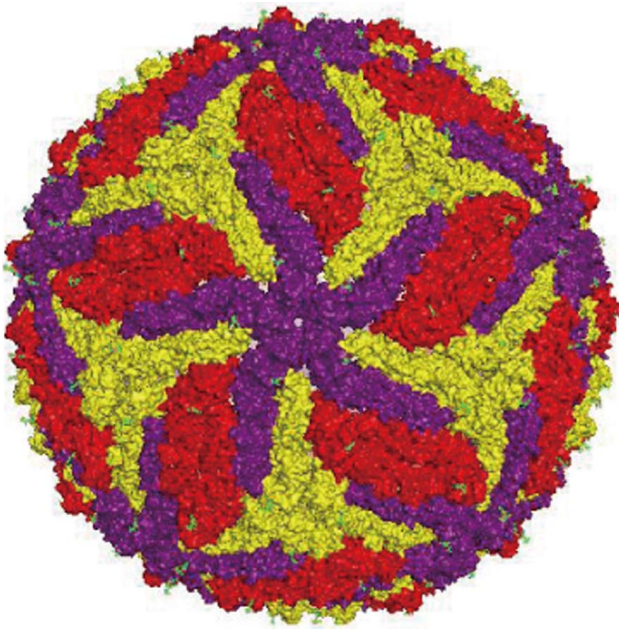

Figure 2 Schematic representation of Zika virus particle based on cryo-electron microscopy data[4].

found in many other vertebrate species, such as sheep, goats, cattle birds, rodents and even reptiles[1].

Even though mosquito transmission is the main cause of ZIKV outbreaks, other additional routes of transmission have been proposed: Breastfeeding, perinatal, sexual or by blood transfusion (Figure 3).

Horizontal transmission

The potential for viral transmission through blood transfusion was first suggested during the French Polynesia outbreak. Almost 3% of blood donors, who were asymptomatic at the time of donation, were found positive for acute ZIKV infection by specific reverse transcriptase polymerase chain reaction (RT-PCR)[30]. Moreover, in a very recent prospective study carried out in 72 pregnant women in Brazil, 26 tested positive for ZIKV RNA in blood samples[31]. These data point to the need for implementation of measures to prevent this way of infection in endemic areas, and, in other zones free of ZIKV, to advice people coming back from affected areas to delay blood donations[1].

Besides blood transfusion, sexual activity could be another risk factor for horizontal transmission. In this regard, ZIKV RNA and replicative virus have been found in semen[32-34]. In 2008, a case of sexual transmission was suspected to occur from an American scientist, who contracted ZIKV infection in Senegal, to his wife. Even though she had not left the United States during the previous year, she also developed clinical symptoms related to ZIKV infection. Even though, ZIKV was not investigated in the semen of the patient, virus infection was serologically confirmed in both[35]. A recent retrospective study in Italy detected ZIKV specific neutralizing antibodies in the sera of a couple with a suspected DENV infection, of which the female had not travelled to tropical areas during the previous year[36]. Later on, in early February 2016, the case of a ZIKV infected person after sexual contact in the United States has been reported[37]. In this line of investigations, the CDC received reports of 14 cases of suspected sexual transmission of ZIKV during February 2016, of which only two were laboratory-confirmed and four classified as probable cases of Zika disease. All reported cases belonged to women which only known risk factor was to have had sexual intercourse with symptomatic partners recently returned from an area with ongoing ZIKV circulation[38]. Up to date, and according to WHO, five countries have reported locally acquired infection in the absence of any known mosquito vectors, probably through sexual transmission (Argentina, France, Italy, New Zealand and the United States). Additionally, ZIKV RNA and infectious ZIKV in urine[39] and saliva[40] have been reported. All these data suggest that sexual transmission could play a role on ZIKV infection and transmission, even though this route seems unlikely to play a major role in ZIKV spread. In any case, the CDC have considered that ZIKV sexual transmission is of particular concern and, consequently, have published an interim guideline for prevention of sexual transmission of ZIKV[41].

Vertical transmission

ZIKV RNA in breast milk was first detected during the outbreak in the French Polynesia[42] and, more recently, the presence of infective ZIKV particles, with substantial viral loads, in breast milk has also been described[43]. Nevertheless, since there is no evidence supporting viral transmission to babies by lactation, the CDC

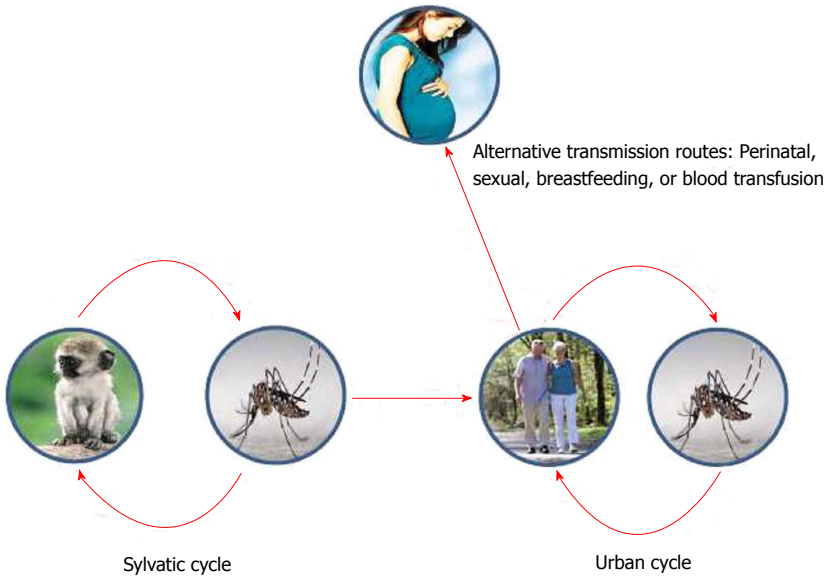

Figure 3 Schematic representation of Zika virus transmission cycle, with a sylvatic natural cycle between mosquitoes and monkeys, and an urban cycle between mosquitoes and human population.

encourage mothers to breastfeed their children, arguing that the benefits of it outweigh the risk of transmission (http://www.cdc.gov/zika/transmission/), as so do the Pan American Health Organization (PAHO/WHO) (http://www.paho.org), and several national health authorities. However, it should be noted that breast milk transmission has been previously documented in humans and experimentation animal models in other flaviviruses, such as DENV or WNV[44,45].

In any case, the most worrying aspect of recent ZIKV outbreaks is the increasing evidence pointing to mother-to-child viral transmission, which can lead to infants neurological disorders. As mentioned early, perinatal transmission was documented for the first time during the French Polynesia outbreak[42]. Sera from two mothers and their newborns were RT-PCR tested positive for ZIKV, although contamination during delivery could not be discarded. Later on, during the outbreak in Brazil, RT-PCR detection and histopathologic findings in tissue samples from two newborns with microcephaly who died within 20 h of birth and two miscarriages showed the presence of ZIKV. All four mothers had clinical signs of ZIKV infection during the first trimester of pregnancy, but not at the time of delivery or miscarriage[46]. Further reports in Brazil have described the presence of ZIKV RNA in fetuses and amniotic fluids[31,47,48]. Even though sporadic vertical transmission in humans has been previously reported in other members of the *Flaviviridae* family, such DENV[49] or YFV[50], the surprisingly high number of infants born with microcephaly in Brazil during the current outbreak, which could probably be the result of a possible vertical transmission, has urged the WHO to publish some advice for women who are pregnant, or planning to become pregnant, to take extra care to protect themselves from the bites of the mosquitoes that transmits ZIKV (http://www.who.int/features/qa/zika-pregnancy/en/).

Clinical features of the disease

ZIKV infection has been described to be symptomatic only in around 18% of the cases[51], causing a mild, self-limiting illness with an incubation period of up to 10 d[52]. Signs and symptoms generally include an onset of fever, maculopapular rash, arthralgia, myalgia, and conjunctivitis, and can be often mistaken with other arboviral infections, like dengue or chikungunya (Table 1). However, severe disease with hospitalization has not been commonly needed until now[1]. However, and even though a causal link has not been yet established, there seem to be growing evidences linking ZIKV infection to Guillain-Barré syndrome (GBS) and microcephaly in newborns. So that, due to this unexpectedly upsurge of severe neuronal complications, a case definition for ZIKV disease has been established by the WHO (http://www.who.int/csr/disease/zika/case-definition/en/) for the purpose of providing global standardization for classification and reporting of ZIKV cases. These interim guidelines distinguish between suspected cases, probable cases, and confirmed cases of ZIKV disease, showing the essential requirements for each of them[12] (Table 2).

GBS is a clinical syndrome of multiple autoimmune etiologies, which involve idiopathic peripheral neuropathy manifested as a progressive paralysis over 1-3 wk, with a 5% death rate and up to 20% of patients left with a significant disability[53-55]. Severe manifestation of GBS with respiratory failure affects 20%-30% of cases[56]. GBS is the most common and severe acute paralytic neuropathy, with en estimate incidence ranging 0.8-1.9 cases per 100000 people per year, with a 70% of these cases associated with previous infectious diseases. The syndrome was also first associated with ZIKV infection

Table 1 Clinical features of Zika virus disease

Mild symptoms	Other complications of the disease
Fever	Guillain-Barré syndrome
Rash	Microcephaly in fetuses and newborns
Joint pain	
Conjunctivitis	
Muscle pain	
Headache	

Table 2 Zika virus disease interim case definitions according to World Health Organization

Suspected case	Probable case	Confirmed case
A person presenting with rash and/or fever and at least one of the following signs or symptoms: Arthralgia, or Arthritis, or Conjunctivitis (non-purulent/ hyperaemic)	A suspected case with presence of: IgM antibody against Zika virus (with no evidence of infection with other flaviviruses) and An epidemiological link (contact with a confirmed case, or a history of residing in or travelling to an area with local transmission of Zika virus within 2 wk prior to onset of symptoms)	A person with laboratory confirmation of recent Zika virus infection: Presence of Zika virus RNA or antigen in serum or other samples, or IgM antibody against Zika virus positive and PRNT90 for Zika virus with titre ⩾ 20 and Zika virus PRNT90 titre ratio ⩾ 4 compared to other tested flaviviruses, and Exclusion of other flaviviruses

Available from: URL: http://www.who.int/csr/disease/zika/case-definition/en.

during the French Polynesian outbreak in 2013[57], where the incidence rate of GBS cases was about 20-fold higher than expected[58]. Likewise, in Colombia, during the ongoing outbreak, a three times higher number of GBS cases than the averaged expected cases during the 6 previous years has been reported. An association between the increase of GBS cases and ZIKV infection has also been reported in Venezuela (http://www.who.int/csr/don/12-february-2016-gbs-colombia-venezuela/en/). Very recently, two cases of GBS with confirmed ZIKV infection have been notified from the United States to the PAHO/WHO (http://www.who.int/csr/don/21-march-2016-gbs-usa/en/). According to the WHO, and in the context of ZIKV circulation, twelve countries or territories have reported an increased incidence of GBS and/or laboratory confirmation of a ZIKV infection among GBS cases (http://www.who.int/emergencies/zika-virus/situation-report/17-march-2016/en/). These data point to an alarming increase in the potential clinical severity of ZIKV infection[59].

In a case-control study performed during the French Polynesia outbreak, 42 patients were diagnosed with GBS at the Centre Hospitalier de Polynésie Française (Papeete, Tahiti, French Polynesia). Study control cohorts were age-matched, sex-matched, and residence-matched patients who presented at the hospital with a non-febrile illness (control group 1; n = 98) and age-matched patients with acute ZIKV disease and no neurological symptoms (control group 2; n = 70). Up to 98% of the patients with GBS had anti-ZIKV IgM or IgG, compared with 56% in control group 1[60]. Even though in this study a history of past dengue virus infection seemed not to differ significantly between patients with GBS and those in the two control groups, other reports have suggested that the simultaneous increase in dengue and chikungunya infections in the region may have contribute to the registered increase in GBS incidence[61]. The 42 GBS cases reported in the French Polynesia between November 2013 and February 2014 contrasted with the less than ten cases per year recorded during the previous four years (http://ecdc.europa.eu/en/publications/Publications/Zika-virus-French-Polynesia-rapid-risk-assessment.pdf), and suggests a possible association between ZIKV and GBS[60]. GBS was also the first important ZIKV-associated condition documented in Brazil, with 121 cases during the first half of 2015 (http://portalsaude.saude.gov.br/index.php/o-ministerio/principal/secretarias/svs/noticias-svs/19139-evento-desaude-publica-relacionado-aos-casos-de-febre-do-zika).

Even though GBS has also been associated to other arboviral infections, such as DENV[62,63], WNV[64], or CHIKV[65], it is believed to be a rare event. The onset of GBS presumably involves an autoimmune process[66], and although the possible factors determining the association of GBS and ZIKV have not yet been established, it has been suggested that sequential arbovirus infections may exacerbate the immune response and trigger an immunopathogenic process attacking peripheral nerves, and thus leading to the onset of GBS[58].

No matter what, the most concerning manifestation of ZIKV infection is the dramatic increase of reported cases of microcephaly in Brazil. Microcephaly is a head size smaller than expected for age, and is associated to different genetic factors, maternal malnutrition, intrauterine infection (including toxoplasmosis, cytomegalovirus, or rubella), and exposure to toxins during gestation (http://www.cdc.gov/ncbddd/birthdefects/microcephaly.html). Microcephaly is defined as an occipitofrontal head circumference below the third centile, or more than 2 standard deviations (SD) below the mean for sex, age, and ethnicity[67]. Anyway, the possible link of microcephaly with ZIKV is not still clear among researchers. The Latin American Collaborative Study of Congenital Malformations (ECLAMC) suggested that this increase in reported cases of microcephaly might largely be due to the intense search for cases of the birth defect, and to misdiagnoses, that arose from heightened awareness in the wake of the possible link with ZIKV; and the WHO had also stated that the causal relation of these disorders with ZIKV infection had not yet been scientifically proven[68].

According to the WHO data, between October 2015 and January 2016, Brazil reported 4783 cases of microcephaly and/or central nervous system malformation, while during the fifteen previous years the average number of cases reported in the country was 163 per year[69]. Although most of the Brazilian cases have not yet been confirmed, as only a few studies have investigated in detail the possible link between ZIKV infection and fetus cerebral damage, an increase in microcephaly and other fetal malformations has been widely reported in Brazil[31,70,71] and the French Polynesia[72]. In a retrospective analysis of data performed from the ZIKV outbreak in French Polynesia, eight cases of microcephaly were identified between September 2013 and July 2015. Seven of them occurred in a 4-mo period around the end of the ZIKV outbreak. With the development of a mathematical model, the study estimated a prevalence of risk of microcephaly associated with ZIKV infection in the first trimester of pregnancy of 95 out of 10000 infected women (around 1%) *vs* a baseline prevalence of microcephaly of 2 out of 10000[73]. Two additional cases, linked to a stay in Brazil, were detected in the United States[74] and Slovenia[7]. Even though no such a high increase has been observed in ZIKV Brazil endemic neighboring countries, a very recent report has diagnosed, for the first time in Colombia, one newborn with microcephaly and two with congenital brain abnormalities, which tested positive for ZIKV[75], and Panama has recently reported to the WHO a newborn with microcephaly and occipital encephalocoele who died a few hours after birth and also tested positive for ZIKV by RT-PCR.

It is also noteworthy to mention that first experimental studies with ZIKV infection in two mouse model revealed that virus replication is mainly performed in brain cells, such as neurons and astroglial cells[76,77], which would be in line with a possible physiological mechanism linking ZIKV infection with microcephaly. Otherwise, a very recent study have showed that ZIKV infection of human cortical neural progenitors cells derived from induced pluripotent stem cell produced an attenuation of their growth, pointing to a possible mechanistic link between ZIKV and microcephaly[78]. On the other hand, it has been hypothesized that infection could damage the fetus either by evading the natural immunoprotective response of the placenta by direct transmission of the virus to the early embryo or fetus, or by the placenta itself provoking a response to the exposure, and thus contributing to, or causing, the brain defects[79]. In any case, the mechanism by which ZIKV may cause fetal microcephaly is still unknown and, thus, this point need to be clearly established.

Public health measures and future considerations

As in most flaviviral infections, there is no current specific antiviral treatment, vaccine or prophylaxis available for ZIKV. Treatment is generally symptomatic and based on analgesics, antipyretics, and antihistamines. This lack of specific measures against the virus emphasizes the importance of vector control strategies (Table 3). ZIKV is principally spread by mosquitoes, and not by person-to-person contact, although a limited number of cases of sexual transmission has been reported. Accordingly, vector control measures are analogous to those suggested in other mosquito-transmitted diseases[3], such as removing sources of standing water, insecticide application, avoidance of mosquito exposure, and implementation of accurate mosquito control programs. Besides these vector control approaches, development of effective ZIKV vaccines, and search for specific antiviral drugs are current challenges for Zika disease.

Table 3 Preventive measures

Vector control measures	Personal preventive measures
Removal of sources of standing water	Avoidance of mosquito exposure
Implementation of accurate mosquito control programs	Insecticide application
	Prevention of sexual transmission by use of preventive measures
	Travelling avoidance to risk countries during pregnancy

Since the WHO declared a public health emergency of international concern on the 1st of February of 2016, a list of preventive guidelines has been assessed, particularly during pregnancy. Recommendations for pregnant women considering travel to an area with ZIKV circulation and recommendations for screening, testing, and management of pregnant returning travelers are included in the CDC interim guidelines[80]. However, it should be taken into account that, even though ZIKV has been identified in a few cases in fetuses with microcephaly, this association does not demonstrate causality, and it will be necessary careful assessment to find the causal link between ZIKV infection and microcephaly[1,81]. Furthermore, in the case of newborns with microcephaly, the lack of data on short or long-term outcomes of neonatal or infant infection makes it difficult to take into consideration more subtle effects of ZIKV infection in the brain until later stages of childhood. Therefore, systematic and longer-term follow-up is mandatory to assess this point and to determine whether there are more fetal effects.

On the other hand, Zika's association with other viral infections in humans, such as dengue and chikungunya, has raised questions about the potential roles of these other viruses as cofactors for the more serious complications of ZIKV infection[82]. As the current ZIKV expansion is occurring in regions where dengue is endemic, pre-existing dengue immunity can cause increased ZIKV replication in patients, resulting in increased viremia and increased infectivity. In this sense, the possibility of immune enhancement by pre-existing heterologous anti-flavivirus antibodies, like DENV, has been hypothesized to increase viral replication[13]. Immune enhancement has been reported to play a major role in the pathogenesis of severe dengue infections[83]. In fact, ZIKV replication in cell culture were shown to be

enhanced by heterologous flavivirus antibodies[4]. In any case, the potential role of this immune enhancement by previous infection with other flaviviruses as cofactors for the more serious complications associated with ZIKV should be addressed in future research.

Beyond the considerable efforts exerted by the scientific community and the national and international health authorities focused on improving the knowledge on ZIKV infection, sufficient resources should be allocated to provide the necessary tools for assessing the potential mechanisms of ZIKV association to severe neurological diseases, such as GBS or microcephaly, as well as the development of more systematic diagnostic tools, vaccines, and design of antiviral therapies.

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MINIREVIEWS

Regulation of Wnt/β-catenin signaling by herpesviruses

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Abstract

The Wnt/β-catenin signaling pathway is instrumental in successful differentiation and proliferation of mammalian cells. It is therefore not surprising that the herpesvirus family has developed mechanisms to interact with and manipulate this pathway. Successful coexistence with the host requires that herpesviruses establish a lifelong infection that includes periods of latency and reactivation or persistence. Many herpesviruses establish latency in progenitor cells and viral reactivation is linked to host-cell proliferation and differentiation status. Importantly, Wnt/β-catenin is tightly connected to stem/progenitor cell maintenance and differentiation. Numerous studies have linked Wnt/β-catenin signaling to a variety of cancers, emphasizing the importance of Wnt/β-catenin pathways in development, tissue homeostasis and disease. This review details how the alpha-, beta-, and gammaherpesviruses interact and manipulate the Wnt/β-catenin pathway to promote a virus-centric agenda.

Key words: Herpesvirus; Herpes simplex virus-1; Varicella zoster virus; Cytomegalovirus; Epstein-Barr virus; Kaposi's sarcoma-associated herpesvirus; Wnt/β-catenin; Glycogen synthase kinase-3; Axin

Core tip: The Wnt/β-catenin signaling pathway is essential for many host cell functions. Herpesviruses have evolved to manipulate and control this vital pathway to promote viral propagation, evade host immune recognition and maintain latency.

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INTRODUCTION

Herpesviruses have been coevolving with vertebrates

for millions of years and have developed multiple mechanisms to avoid immune recognition and manipulate host signaling pathways to promote efficient viral replication. This is evident in the ability of herpesviruses to persist for the lifetime of the host while causing limited adverse effects[1]. In general, severe symptoms are only seen in those individuals who are immunocompromised[2]. Accumulating evidence suggests that herpesviruses interact with the Wnt/β-catenin pathway to regulate viral gene expression and alter host cell gene expression by manipulating downstream signaling components during both active infection and latency.

The Wnt/β-catenin pathway is responsible for a signaling cascade that is required during embryonic development and continues throughout the life of an organism. Nearly every tissue and organ depends on this signaling cascade for normal function. Correct Wnt/β-catenin signaling is crucial in the development of many organs including the brain, heart, lung, bone, liver, kidney and gut among others[3,4]. Many of these essential roles continue in adulthood in relation to tissue homeostasis, regeneration, maintenance and repair functions. Additionally, Wnt/β-catenin has been shown to be important in cell migration, genetic stability and apoptosis[5-8]. With such widespread influence on many diverse signaling cascades, dysfunctional Wnt/β-catenin signaling can have deleterious effects. Unregulated Wnt/β-catenin signaling was first linked to human disease in the 1990s when adenomatous polyposis coli (APC) protein was found to interact with β-catenin[9,10]. Since then, Wnt/β-catenin signaling has been implicated in many cancers[11-15], fibrosis[16,17], and metabolic disease[18].

Although conclusive data on the importance of Wnt/β-catenin signaling during the complete replication cycle of all herpesvirus members are lacking, accumulating data are beginning to reveal the importance of this pathway to viral replication, latency and pathogenesis. The potential to target the Wnt/β-catenin pathway for therapeutic intervention is enormous but is compounded by the complexity of the signaling cascade, the number of potential players involved during signaling activation and its importance to cellular homeostasis. Understanding how herpesviruses manipulate this pathway has increased our knowledge of this important pathway and may ultimately lead to novel antiviral therapies.

THE WNT/β-CATENIN SIGNALING CASCADE

Wnts are lipid-modified glycoprotein ligands that act in an autocrine or paracrine manner. Wnt signaling can be divided into three main signaling cascades: Canonical Wnt and two β-catenin-independent pathways, the non-canonical planar cell pathway[19] and the non-canonical Wnt/calcium pathway[20,21]. This review will focus on the canonical Wnt pathway but crosstalk of the three signaling cascades has been reported and is therefore unavoidable. Briefly, in the absence of Wnt stimulation, cytoplasmic β-catenin is phosphorylated and degraded by the ubiquitin-proteasome system (Figure 1). Upon binding of Wnt, phosphorylation of β-catenin is blocked allowing it to translocate to the nucleus where it complexes with transcription factors to upregulate Wnt target gene transcription (Figure 1). Canonical Wnt signaling is initiated when Wnts bind to a heterodimeric transmembrane receptor complex consisting of Frizzled (Fz) receptor and the co-receptors low-density lipoprotein receptor-related protein 5 (LRP5) and LRP6. The ligand interaction induces conformational changes and subsequent phosphorylation of target proteins. This results in recruitment and signaling through the scaffold protein Dishevelled promoting the inhibition of the destruction complex, which contains Axin, APC, β-catenin, casein kinase Iα/β (CKI Iα/β), and glycogen synthase kinase-3α/β (GSK-3α/β). APC directly interacts with β-catenin and Axin. Axin binds to the cytoplasmic tail of LRP6 and this complex is regulated through phosphorylation by GSK-3 and CK1. When the destruction complex is intact, Axin associated β-catenin is phosphorylated by CKI and GSK-3β at N-terminal Ser/Thr residues. Phosphorylated β-catenin is then recognized by the E3 ubiquitin ligase complex β-TrCP (Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase) and targeted for degradation by the proteasome. In the presence of Wnt ligand, signaling results in the dissociation of the destruction complex and loss of GSK-3 mediated phosphorylation of β-catenin. Axin is recruited to the phosphorylated tail of LRP preventing β-catenin phosphorylation and ubiquitination. As a result, β-catenin is free to accumulate and translocate to the nucleus where it interacts with members of the T cell factor/lymphoid enhancer-binding factor (TCF/LEF) family of transcription factors and transcriptional coactivators such as CREB-binding protein (CBP), E1A-associated protein p300, and Pygopus to initiate Wnt target gene expression[22]. β-catenin can also interact with many other transcription factors not linked to the TCF/LEF family but that do play important roles in cell maintenance and differentiation[23-25]. For more in depth reviews on Wnt/β-catenin signaling, the reader is referred to many of the excellent reviews available[23,26-29].

HERPESVIRUSES

The taxonomic order *Herpesvirales* includes over 130 herpesviruses divided into three virus families: *Herpesviridae* that can infect mammals, birds and reptiles; *Alloherpesviridae* that infect amphibians and bony fish; and *Malacoherpesviridae* that infects some invertebrates, including molluscs[30-32]. These classifications are based on genome size/structure and biological function. *Herpesviridae* is a family of

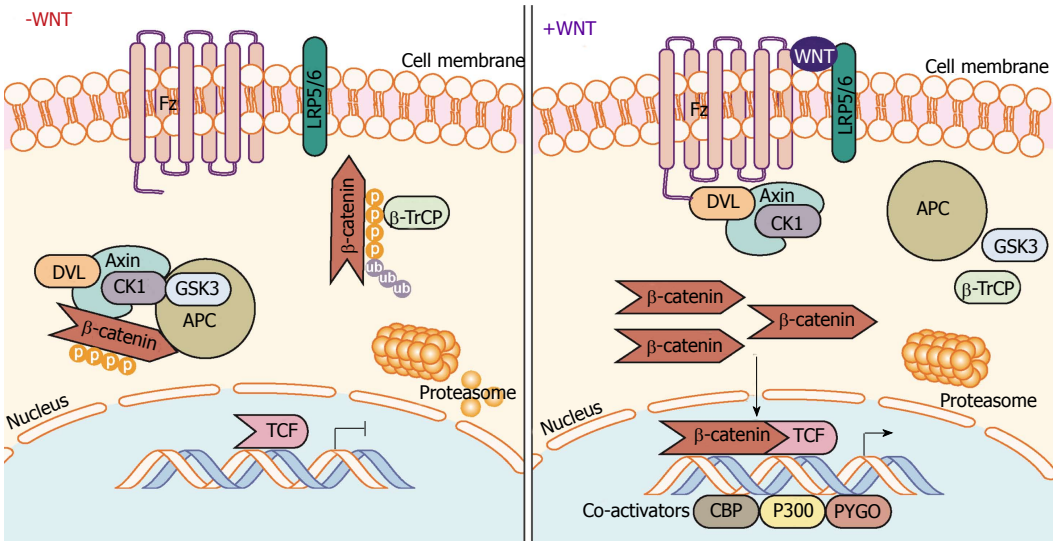

Figure 1 Canonical Wnt/β-catenin signaling pathway. In the absence of Wnt ligand stimulation, the β-catenin destruction complex - consisting of the proteins Axin, CK1, GSK-3α/β, APC, and DVL - phosphorylate β-catenin allowing β-TrCP to ubiquitinate β-catenin marking it for proteasomal degradation. When stimulated by Wnt ligands, engagement of the Fz receptor and co-receptors LRP5/6, induces signaling through DVL inhibiting the action of the destruction complex. This frees β-catenin from degradation pathways allowing β-catenin to translocate and accumulate in the nucleus. β-catenin mediated interaction with TCF family transcription factors and co-activators (CBP, *etc.*) and initiates transcription of target genes. APC: Adenomatous polyposis coli; β-TrCP: Beta-transducin repeat containing E3 ubiquitin protein ligase; CBP: CREB-binding protein; CK1: Casein kinase 1; DVL: Dishevelled; Fz: Frizzled receptor; GSK-3: Glycogen synthase kinase 3; LRP: Low-density lipoprotein receptor-related protein; TCF/LEF-1: T-cell factor/lymphoid enhancer-binding factor 1.

enveloped, DNA viruses that is further divided into 3 subfamilies (*Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*). A criterion for inclusion in the *Herpesviridae* family morphologically is centered on the virion structure[33]. The virion is spherical in shape and includes a core, capsid, tegument and envelope. The core contains the viral genome, which is a linear, double-stranded DNA molecule. The core is surrounded by an icosahedral capsid that is enclosed within a proteinaceous layer called the tegument. Finally, a lipid bilayer envelope surrounds the exterior of the tegument and completes the structure of the virion.

Humans can be infected by eight different herpesviruses. Herpesvirus infections are typically systemic, although some may be localized. Gene expression is tightly regulated and orchestrated in a temporal manner. Simplistically, immediate-early genes encoding regulatory proteins are expressed soon after infection, followed by expression of early genes that are important for replication of viral DNA. Finally, late genes encoding structural proteins are expressed. Due to various host immune evasion strategies, herpesviruses establish life-long latent infections in infected individuals. In an oversimplified model in regards to human infection, *Alphaherpesvirinae* establish latency in neurons, *Betaherpesvirinae* in monocytes and *Gammaherpesvirinae* in lymphocytes, monocytes, and macrophages[1,32,34].

HUMAN ALPHAHERPESVIRUSES

The subfamily *Alphaherpesvirinae* includes three members. The human herpesviruses 1 and 2 (HHV-1/2) also known as herpes simplex virus (HSV) (type 1/2) belong in the genus Simplexvirus while HHV-3 or Varicella-zoster virus (VZV) is classified in the genus Varicellovirus[32,33]. Infection can result in skin vesicles or mucosal ulcers and on rare occasions meningitis and encephalitis[2].

HHV-1 (HSV-1)

To date there have been no focused, thorough investigations of the role of Wnt/β-catenin on HSV-1/2. The studies that have been completed implicate individual members of the Wnt/β-catenin signaling cascade in viral pathogenesis. An example of this is the upregulation of the antiviral cytokine interferon-β (IFN-β) during HSV-1 infection. In adult immunocompetent mice, macrophages are essential for clearing HSV-1 from the blood; however, it was observed that macrophages from $Akt^{-/-}$ mice display poor clearance of HSV-1. The Akt1 family of serine/threonine kinases was shown to phosphorylate β-catenin at serine 552 allowing accumulation and β-catenin mediated induction of IFN-β[35]. Akt1 classically has been described as a β-catenin transcriptional promoter, exerting its effects by repressing GSK-3 mediated β-catenin proteasomal degradation[36]. Interestingly, the serine 552-phosphorylation site is distinct from the site typically targeted by GSK-3. The authors conclude that Akt1 is responsible for inhibiting GSK-3 phosphorylation of β-catenin on Ser9 and also for direct phosphorylation of β-catenin at serine 552 allowing for stabilization, enhanced nuclear translocation and transcriptional activity of β-catenin (Figure 2).

In a second study, Choi *et al*[37] observed that HSV-1 infection and replication was more efficient in a fibroblast-like murine cell line, L929. Knocking down Axin or treatment with Wnt3a conditioned media reduces

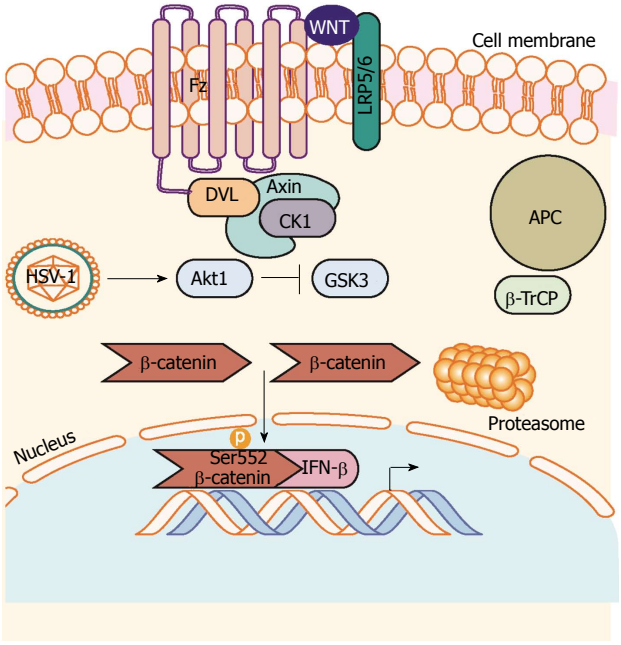

Figure 2 β-catenin mediated antiviral interferon response during herpes simplex virus 1 infection. HSV-1 infection induces activation of Akt1 activity. Akt1 phosphorylates β-catenin on Serine 552 inhibiting degradation signaling through GSK-3-mediated phosphorylation of β-catenin on Serine 9. β-catenin can then accumulate in the nucleus to induce transcription of β-catenin target genes such as the antiviral cytokine IFN-β. Akt1: Protein kinase B; HSV-1: Herpes simplex virus 1.

HSV-1 replication in L929 cells. They further showed that Axin expression minimalizes HSV-1 induced cell death, which in turn promotes increased HSV-1 replication. In a follow up study, this group observed that HSV-1 infection also induced autophagy but this is delayed in L929 cells ectopically expressing L-Axin[38]. The authors concluded that delay in induction of autophagy favors HSV-1 viral replication likely by suppression of HSV-1 mediated cell death. The implication is that HSV-1 replication is inversely related to Wnt signaling.

Lastly, Piacentini *et al*[39] demonstrated that HSV-1 infection disrupts synaptic function in cultured murine cortical neurons through GSK-3 activation and intracellular accumulation of amyloid-β protein. In a previous study this group showed that HSV-1 mediated increases in intracellular Ca^{2+} is the main mechanism for activation of GSK-3 in this model[39]. These studies suggest a possible link between HSV-1 pathogenesis and Alzheimer's disease.

To date, the involvement of Wnt/β-catenin signaling during VZV infection has been underinvestigated. Markus *et al*[40] observed an increase in canonical Wnt pathway transcription in infection of neurons derived from human embryonic stem cells. The Wnt pathway was unaffected during late VZV infection of fibroblasts. Intriguingly, like HSV-1 and -2, VZV will enter latency in neurons but will lytically replicate in fibroblasts suggesting a differing need for Wnt pathway modification by the virus in different stages of the viral life cycle[40]. Given the limited studies on Wnt/β-catenin signaling during alphaherpesvirus infections, how vital Wnt/β-catenin signaling is to viral replication and pathogenesis remains unknown. The studies mentioned above seem to portray a conflicting role of β-catenin in viral replication. More thorough studies using defined cell types and carefully delineated "branches" of the Wnt pathway will provide a clearer understanding.

HUMAN BETAHERPESVIRUS

The human *Betaherpesvirinae* subfamily consists of the three viruses: HHV-5 known as human cytomegalovirus (HCMV), HHV-6A/B, and HHV-7 (the latter two are commonly referred to as Roseolovirus)[32,33]. Infection is usually asymptomatic but infectious mononucleosis like symptoms are seen in HCMV infections and the development of a rash is associated with Roseolovirus. In immunocompromised individuals (organ transplant patients, HIV positive individuals, *etc*.) or during pregnancy, infection and/or reactivation of β-herpesvirus can have life-threatening consequences. Of these three viruses, HCMV is the most studied and is considered the prototypical betaherpesvirus. As little is known about Wnt/β-catenin regulation during infection by the polyphyletic Roseolovirus group, this portion of the review will focus exclusively on HCMV.

HHV-5 (HCMV)

The Wnt/β-catenin pathway is one of the many cellular pathways manipulated by HCMV to likely facilitate lytic viral replication. By dysregulating the physiological condition of the Wnt/β-catenin pathway, HCMV inhibits or severely hampers the processes of cellular replication, movement/migration, and differentiation among others[41,42].

HCMV infection of the placenta may cause impaired invasion of placental-derived cells toward maternal spiral arteries leading to shallow placentation and a deficit in oxygen/nutrient flow to the developing fetus[43]. The Wnt/β-catenin pathway is important in the differentiation of placental cytotrophoblasts into extravillous trophoblasts, the invasive lineage of cells that remodel maternal spiral arteries to establish blood flow to the placenta[44-46]. Using an *in vitro* model of first trimester cytotrophoblasts (SGHPL-4) infected with HCMV, Angelova *et al*[41] demonstrated that β-catenin protein levels decrease significantly during the late stages of infection roughly corresponding to expression of late proteins and packaging of nucleocapsids into an envelope to produce mature virions. This decrease in β-catenin protein is dependent on proteasomal degradation and occurs in all cellular pools including membrane, cytoplasm and nucleus. Remaining β-catenin, aggregates near the viral assembly compartment, a juxtanuclear region present during infection involved in virion assembly and egress; however, the reasons for this are currently unclear. Transcriptional targets of β-catenin, such as Dickkopf-related protein 1 (Dkk1) and Cyclin D, also exhibit transcriptional repression as a result. However,

β-catenin mRNA levels actually increase in the same timeframe[41]. Consistent with these results, Ueland *et al*[47] showed that plasma levels of DKK-1 were significantly lower in solid organ transplant patients with HCMV DNAemia. In contrast, Langemeijer *et al*[48] reported that HCMV infection increases transcriptional activation of β-catenin in a glioblastoma cell line that is dependent on expression of the virally encoded G-protein coupled receptor, US28. These different results may be explained by the use of different cell types and methods to detect β-catenin activity.

The mechanism by which HCMV depletes membrane stores of β-catenin is currently unknown although infection extensively remodels cellular membranes[49]. As for cytoplasmic and nuclear stores of β-catenin, HCMV exerts control at the level of the β-catenin destruction complex as disruption of this complex with lithium chloride (LiCl), a GSK-3β inhibitor can inhibit the degradation and depletion of β-catenin during infection. It should be noted that inhibition of β-catenin degradation does not rescue transcriptional function of β-catenin[41]. This may be due to further regulation of transcriptional activity of β-catenin, for example through regulation of β-catenin coactivators like TCF/LEF-1, by the virus or due to undetected post-translational modification of β-catenin. Viral regulation of the destruction complex appears to be mostly mediated at Axin1, the rate-limiting protein in the β-catenin destruction complex in the cytoplasm. PolyADP Ribose Polymerase 5a and 5b (PARP5a/b), also called Tankyrase (TNKS as a combination of isoforms 1 and 2), PARsylates Axin1 leading to degradation through the ubiquitin proteasome pathway. During HCMV infection, TNKS PARsylation activity is inhibited allowing for stabilization of Axin1 and stabilization of the β-catenin destruction complex leading to the degradation of β-catenin seen during infection (Figure 3)[50]. This suggests that HCMV requires a complete and competent β-catenin destruction complex for degradation of β-catenin.

The non-canonical pathways of Wnt signaling, although lacking direct β-catenin regulation, seem to play a role in regulation of the canonical Wnt/β-catenin pathway during HCMV infection. Wnt5a interacts with the tyrosine-like orphan kinase 2 ROR2 and physiologically activates the Wnt/Planar Cell Polarity pathway and Wnt/Ca^{2+} pathway[42]. During HCMV infection, infected cells become insensitive to normal Wnt5a ligand signaling but ROR2 expression is significantly increased. Uninfected trophoblasts invade toward a Wnt5a gradient *in vitro* but are incapable of doing so when infected despite the increased presence of ROR2. The increase in ROR2 expression inhibits canonical signaling by repressing β-catenin TCF/LEF-1 transcriptional activity. Knockdown of non-canonical ROR2 that is overexpressed during infection can rescue some function of the canonical Wnt/β-catenin pathway in trophoblasts suggesting that the canonical and non-canonical Wnt pathways are deeply intertwined, especially during HCMV infection[42].

Targeting of Wnt/β-catenin signaling with select pharmacological inhibitors can inhibit viral replication suggesting that some level of β-catenin or a member of the canonical Wnt pathway may be necessary for viral replication[51]. Why HCMV infection overrides normal Wnt/β-catenin signaling is unknown, but some research indicates involvement of repurposing the molecular members of the pathway to further HCMV replication. Activity of GSK-3 has been implicated in assembly of the viral nucleocapsid in simian CMV (infecting Chimpanzees and Orangutans). Phosphorylation of the viral assembly protein precursor (pAP) by GSK-3 may induce conformational changes in the protein and stabilize pAP interaction with the major capsid protein during capsid assembly[52]. Additionally GSK-3 (along with other members of the β-catenin destruction complex) has been identified as a target for phosphorylation by the viral kinase UL97[53]. However, inhibition of UL97 activity during infection does not seem to rescue β-catenin degradation suggesting that UL97 phosphorylation of GSK-3 is not the primary mechanism by which HCMV depletes β-catenin stores (our unpublished data). Further research must be conducted to determine the importance of molecular mechanisms of Wnt/β-catenin on viral replication itself.

HCMV infection has recently been associated with a diverse array of diseases and disorders such as diabetes[54], atherosclerosis[55], and some cancers (reviewed in[56,57]), along with the abovementioned issues with infection during pregnancy on the placenta and developing fetus. As data show that HCMV infection undermines normal functioning of canonical Wnt/β-catenin and non-canonical Wnt signaling in diverse ways, differing perhaps by infection of a multitude of diverse cell types, it becomes key to better characterize this viral regulation.

HUMAN GAMMAHERPESVIRUSES

The human *Gammaherpesvirinae* family includes two members: Human herpesvirus 4 (HHV-4) commonly known as Epstein-Barr virus (EBV) and HHV-8 or Kaposi's sarcoma-associated herpesvirus (KSHV)[32,33]. They are further classified under the genera Lymphocryptovirus and Rhadinovirus, respectively. EBV was one of the first viruses to be associated with human cancer when it was originally identified in Burkitt's lymphoma. Since then, it has become associated with B cell malignancies and epithelial cell associated cancers. KSHV was discovered in 1994 when samples from AIDS-associated Kaposi's sarcoma came back positive for viral DNA sequences[58]. Diseases associated with KSHV include B cell malignancy primary effusion lymphoma (PEL), Castleman's disease and the endothelial lesion, Kaposi's sarcoma.

HHV-4 (EBV)

The accumulation of β-catenin is seen in EBV-infected epithelial and B cells. In the earliest report, Shackelford *et al*[59] reported that β-catenin was not degraded in

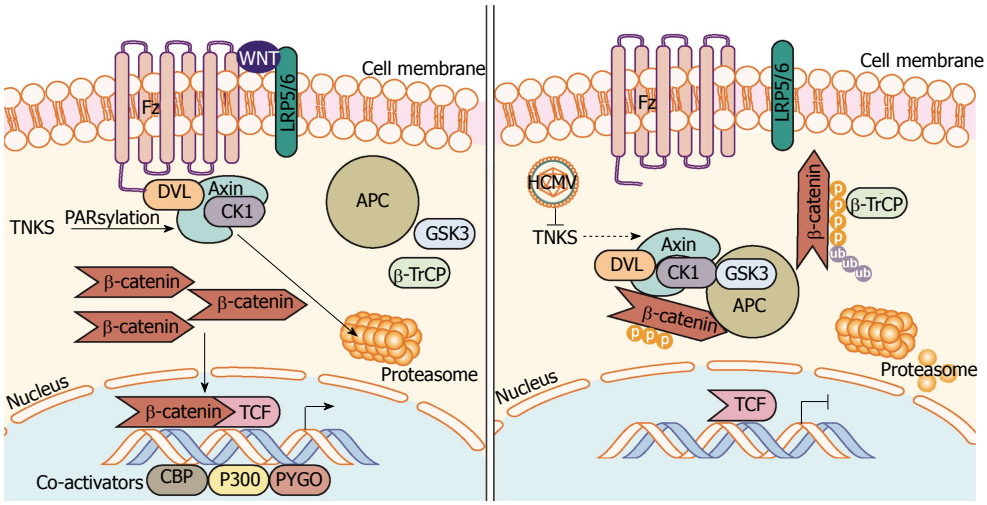

Figure 3 Human cytomegalovirus inhibits PARsylation activity of tankyrase 1 and 2 (PARP5a/b) to enhance infection. Regulation of Axin is the rate-limiting step in the assembly and function of the β-catenin destruction complex. PARsylation, a process driven by NAD^+, marks Axin for proteasomal degradation, inhibiting β-catenin destruction complex formation resulting in β-catenin accumulation and transcription in the nucleus. HCMV infection causes inhibition of TNKS (PARP5a/b) PARsylation activity, which inhibits PARsylation of Axin and increases its stability. Further inhibition of TNKS PARsylation activity or knockdown of TNKS significantly aids in HCMV replication. An increase in stable Axin permits β-catenin destruction complex formation, increased β-catenin degradation, and subsequent inhibition of β-catenin-mediated transcription. HCMV: Human cytomegalovirus; NAD^+: Nicotinamide adenine dinucleotide; PARsylation: Poly-ADP ribose modification; PARP5a/b: Poly-ADP ribose polymerase 5a/b; TNKS: Tankyrase 1 and 2 (PARP5a/b).

lymphoid cells during an EBV type Ⅲ latent infection. The authors postulate that these observations may be due to ubiquitinating enzymes or the dysregulation of other oncogenes. Interestingly, this effect was not observed during EBV type Ⅰ latency infection[59]. Shortly after, a second group showed that telomerase-immortalized human foreskin keratinocytes have increased β-catenin accumulation after infection with EBV[60]. The mechanism was shown to be dependent on latent membrane protein 2A (LMP2A) activation of Akt and Akt-mediated inactivation of GSK-3, independent of phosphorylation at Ser9. Treatment with LiCl led to β-catenin accumulation in the cytoplasm, translocation into the nucleus and activation of a TCF-responsive reporter. In a follow-up study, the immunoreceptor tyrosine-based activation and PY motifs of LMP2A were found to mediate the accumulation and nuclear translocation of β-catenin[61]. Using LMP2A ΔPY mutants, they showed that β-catenin levels and translocation to the nucleus decreased along with epithelial cell differentiation. The authors concluded that LMP2A mediated epithelial cell differentiation appears to be inversely correlated with β-catenin activation in this model.

EBV latent membrane protein 1 (LMP1) has also been associated with an increase in β-catenin levels in EBV-infected BL cells[62]. Jang *et al*[63] reported that an E3 ubiquitin ligase, a human homolog of *Drosophilia* seven in absentia (Siah-1), is repressed by LMP1. Siah-1 binds APC and in a GSK-3 independent manner, degrades β-catenin. However, another study using transient and stable expression of LMP1 sequences failed to find evidence that LMP1 induces Wnt/β-catenin signaling or promotes the accumulation of β-catenin[64]. To further verify their observations, they proceeded to show that there was little evidence for interactions between LMP1 and β-catenin. The authors proposed that differences in cell lines and LMP1 sequences used may account for the conflicting results in these two studies.

Lastly, EBV-mediated dysregulation of Wnt/β-catenin was associated with idiopathic pulmonary fibrosis (IPF)[65]. EBV detection in alveolar epithelial cells has been associated with poor prognosis. Pathogenesis is believed to occur in IPF due to repetitive epithelial cell injury that may be mediated by EBV. Using transcriptomic data, the authors identified altered Wnt/β-catenin pathway transcripts. Specifically, Wnt5b expression was altered. The authors conclude that EBV may be using a non-canonical Wnt/β-catenin pathway that includes CUX1 and the EBV early gene Rta.

HHV-8 (KSHV)

Fujimuro *et al*[66] first observed the association between Wnt/β-catenin and KSHV in 2003. They made the observation that in latently KSHV-infected B cell lines derived from PEL, β-catenin accumulated at high levels in the cytoplasm. KSHV infection of PEL cells results in a high KSHV latency rate suggesting that the increased levels of β-catenin may be linked to expression of KSHV latency associated proteins. The latency-associated nuclear antigen (LANA) protein proved to be the protein responsible, as siRNA transient knockdown specific to LANA, decreased levels of LANA and β-catenin[67]. LANA was originally shown to be involved in the tethering of KSHV episomal genomes to host chromosomes to aid in viral DNA replication[68,69]. Using a yeast-two hybrid system, paired with coimmunoprecipitation assays, LANA was also found to possess the ability to bind to GSK-3α and GSK-3β[67].

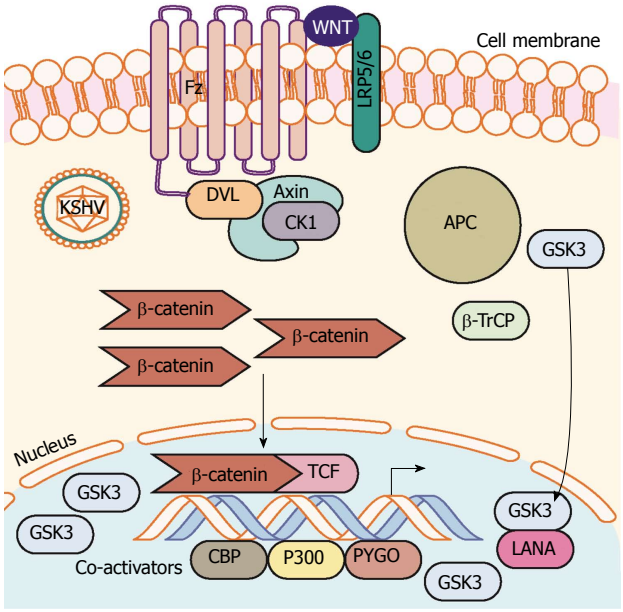

Figure 4 Kaposi's sarcoma-associated herpesvirus latency upregulates β-catenin. Establishment of KSHV latency involves expression of the latency associated protein LANA. LANA binds with and translocates GSK-3 into the nucleus after phosphorylation by GSK-3. This translocation of cytoplasmic pools of GSK-3 prevents β-catenin destruction complex formation and stability of cytoplasmic β-catenin. β-catenin translocation to the nucleus occurs resulting in increased β-catenin-mediated transcription. LANA: Latency-associated nuclear antigen; KSHV: Kaposi's sarcoma-associated herpesvirus.

In addition to mediating the phosphorylation of β-catenin as part of the destruction complex in the cytoplasm, GSK-3 also translocates to the nucleus during apoptotic stimuli in a cell cycle-dependent manner. Nuclear levels of GSK-3 protein increase in the nucleus of PEL cells specifically during the S phase of the cell cycle[66]. This results in lower GSK-3 within the destruction complex and more unphosphoryated β-catenin that translocates to the nucleus and activates target gene expression (Figure 4). The authors proposed that LANA promotes the accumulation of GSK-3 in the nucleus, reducing the total amount of GSK-3 in the cytoplasm.

Further studies revealed that the C-terminal region of LANA displayed limited homology to the domain of Axin that binds GSK-3β and is functionally similar to Axin[67]. LANA protein mutants were used to study the binding potential between LANA and GSK-3β[70]. These studies showed that changing Phe^{291} in the coding sequence of LANA to Leu (F291L mutant), leads to a reduction in binding to GSK-3 by 90%. The interaction of the various components of the destruction complex is mediated by phosphorylation, which also mediates the interaction of LANA and GSK-3. GSK-3 and LANA interactions require the LANA C-terminal GSK-3 interacting domain and GSK-3 phosphorylation of the LANA N-terminus. Within this region are four consensus GSK-3 phosphorylation sites [(Ser/Thr)xxx(Ser/Thr)p]. Mutation of the four consensus sites prevented GSK-3 binding to LANA, suggesting that this is a phosphorylation mediated event[70]. Additionally, as GSK-3 substrates typically must be primed prior to phosphorylation by GSK-3, a mutant (R96A) was used to determine if GSK-3 phosphorylation of LANA could proceed without priming. Results showed that, under *in vitro* conditions, GSK-3 phosphorylation of LANA requires priming kinases. The reader is referred to two comprehensive reviews detailing the manipulation of GSK-3 by KSHV[71,72].

Additional studies revealed that CKI and mitogen-activated protein kinase could each function as priming kinases for GSK-3 phosphorylation of LANA[73]. To summarize, KSHV latency protein LANA, promotes nuclear accumulation of GSK-3 to promote dysregulation of β-catenin. Functionally, there is increased expression of cyclin D1, and when β-catenin reporters have been tested, there is increased activity[74]. Surprisingly, it was also determined that most of the GSK-3 in the nucleus of LANA-expressing cells is in an inactive phosphorylated form suggesting that despite increased GSK-3 present in the nucleus, there is a decrease in nuclear GSK-3 activity. Inhibitor of the MyoD family a (I-mfa) and the human I-mfa domain-containing protein (HIC) has been shown to be negative inhibitors of the Wnt pathway. Kusano *et al*[75] showed that LANA interacts with HIC and I-mfa in the 995-1102 amino acid region of LANA. This site is located near the GSK-3 binding site and inhibits the LANA mediated transactivation of a β-catenin construct. Furthermore, this interaction decreases LANA-GSK-3 complex formation resulting in a decrease in Wnt/β-catenin signaling associated transcription. Thus manipulation of the Wnt/β-catenin pathway may play a key role in LANA-mediated oncogenesis in KSHV-infected cells.

Lastly, a recent paper reports that KSHV viral IFN regulatory factor 4 (vIRF4) targets the β-catenin/TCF transcription complex[76]. Using a TOPFlash system, the data suggests that LANA and vIRF4 are negative regulators of each other. Expression of LANA alone resulted in increased β-catenin protein and transcriptional levels, but introducing vIRF4 reduced the levels of LANA-mediated β-catenin/TCF activation. The authors also observed that that this effect was not dependent on β-catenin protein stability. In conclusion, the study suggests that KSHV employs vIRF4 to block the progression of the cell cycle at the G_1-S phase to aid in viral replication.

It has been proposed that dysregulation of the viral gene program leads to nonlytic expression[4]. Angelova *et al*[77] show a novel pathway that KSHV uses to upregulate the Wnt/β-catenin pathway. The KSHV virally-encoded G-protein coupled receptor (vGPCR) inserted into a retroviral vector was transduced into endothelial cells. The authors observed increased cyclin D1, Wnt7A and pygopus 1 (Pygo) expression in vGPCR expressing cells as compared to non-expressing control cells. Additionally, β-catenin was found to accumulate in the nucleus of vGPCR expressing cells and β-catenin/LEF1-dependent TOPFlash reporter constructs displayed increased activity. Initial data suggests that vGPCR-

Table 1 Wnt/β-catenin molecular manipulations by human *Herpesviridae*

Virus	Pathway component	Stabilization, activation or inhibition of pathway component	Outcome	Ref.
Alphaherpesvirinae				
HSV-1	β-catenin	Stabilized	β-catenin stabilized, increased transcriptional activity of β-catenin	[35]
	Axin	Stabilized	Reduced host cell apoptosis	[37,38]
	GSK-3	Stabilized	Phosphorylation of APP	[39]
Betaherpesvirinae				
HCMV	β-catenin	Inhibited	β-catenin degradation, decrease in β-catenin transcriptional targets	[41]
	Axin	Stabilized	TNKS PARsylation activity inhibited resulting in β-catenin degradation	[50]
	ROR2	Activated	Repression of β-catenin TCF/LEF-1 transcriptional activity	[42]
	GSK-3	Stabilized	Stabilization of pAP and promotion of HCMV replication	[52,53]
Gammaherpesvirinae				
EBV	β-catenin	Stabilized	Accumulation of β-catenin in type III latency	[59]
	GSK-3	Inhibited	LMP2A activation of Akt inactivates GSK-3 resulting in β-catenin accumulation	[60,61]
	APC	Activated/inhibited (conflicting results)	LMP1 represses Siah-1 promoting β-catenin accumulation. LMP1 does not promote β-catenin stabilization	[63,64]
KSHV	β-catenin	Stabilized/inhibited (dependent on viral stage?)	Increased transcriptional activity, induction of viral latency/inhibition of LANA mediated transactivation of β-catenin	[66,76,77]
	GSK-3	Inhibited	LANA promotes nuclear accumulation of GSK-3	[67,70]

GSK-3: Glycogen synthase kinase 3; HCMV: Human cytomegalovirus; TNKS: Tankyrase 1 and 2 (PARP5a/b); TCF/LEF-1: T-cell factor/lymphoid enhancer-binding factor 1; pAP: Protein precursor; LMP2A: Latent membrane protein 2A; APC: Adenomatous polyposis coli; LMP1: Latent membrane protein 1; LANA: Latency-associated nuclear antigen; KSHV: Kaposi's sarcoma-associated herpesvirus.

induced activation of the Wnt/β-catenin is through the PI3K/Akt pathway, similar to what is seen in HSV and EBV. This conclusion was contrary to prior work suggesting that this effect may be mediated through COX2 activity; it was found that PI3K/Akt inhibition potently inhibited Wnt/β-catenin activity in endothelial cells and prevented formation of capillary endothelial tubes *in vitro*[77].

SPECULATION AND QUESTIONS

Despite numerous studies addressing the role of Wnt/β-catenin signaling in herpesviruses, there are still many questions to address. It seems at odds that the gammaherpesviruses would institute a program promoting the accumulation of β-catenin whilst the other family members inhibit the accumulation of β-catenin. The range and complexity of the Wnt/β-catenin pathway makes a simple answer unlikely; but factors such as stage of viral infection and cell type are obvious candidates. As we understand more about herpesviruses, it is conceivable that the herpesvirus family can change the regulation and function of such an important pathway at different times during the viral life cycle. Control over apoptosis, cytoskeletal rearrangement, migration and differentiation are all vital components of viral control over the host cell that would be required at different times post infection.

As mentioned previously, dysregulation of the Wnt/β-catenin pathway is tightly associated with numerous human cancers. In fact, most of the human herpesviruses can be thought of as oncomodulators, whether in a direct manner such as in the expression of oncogenic viral proteins in gammaherpesvirinae infection or through indirect generation of oncostimulatory microenvironments by virally induced inflammation or cellular metabolic shifts caused by alpha- and betaherpesvirinae infection. Why would an evolutionarily successful viral family induce cancer in its host? Ultimately, herpesviruses are successful because they coexist with their host. The development of cancer due to the persistence of a herpesvirus is likely an infrequent event that is complicated by others factors such as altered host cell metabolism and possibly the presence of other pathogens. For example, HCMV is now known to alter host cell metabolism during infection[78-81]. The changes are very similar to the Warburg-effect first identified in cancer cells.

Interestingly, a recent publication may bridge the different actions of viruses on the Wnt/β-catenin pathway during different stages of infection. Data from Marcato *et al*[82] suggests that the TCF/β-catenin complex is instrumental in mounting an effective antiviral response. They linked two observations, namely, that IFN-β is needed during the innate antiviral response and that murine models lacking IFN-β are susceptible to viral infections. In this paper, the authors show that inhibiting GSK-3 using LiCl increases IFN-β expression if β-catenin interacts with the IFN-β promoter by recruitment of TCF/β-catenin complexes to the promoter region. Using Rift Valley fever virus, a RNA virus belonging to the Bunyaviridae family, they showed pathogenicity is correlated to viral targeting of the β-catenin pathway.

Viral manipulation of Wnt/β-catenin signaling may be impeded using small molecules inhibitors that target the Wnt/β-catenin pathway. In fact, Chan *et al*[83] have shown results displaying the potential of this treatment. The authors used ICG-001, a small molecular Wnt modulator (CBP/β-catenin antagonist) to inhibit the growth of tumor spheres in a model of nasopharyngeal

carcinoma. This epithelial malignancy is associated with EBV latent infection. It is hypothesized that ICG-001 targets the cancer stem cells within the tumor reducing growth due to alterations in signaling cascades. To date, no studies have looked at the direct effects of small molecule inhibitors as antivirals, but targeting the Wnt pathway is being explored in many other diseases and should be examined in the context of herpesvirus infection (reviewed in[28]).

CONCLUSION

Human herpesviruses exploit the Wnt/β-catenin signaling pathway to ensure successful replication and survival in host cells (Table 1). The manipulation of such an important signaling cascade by herpesviruses should not be surprising as this pathway dictates the expression of many essential transcriptional pathways. The current literature provides an incomplete picture of why herpesviruses alter the Wnt/β-catenin pathway when they do. A deeper understanding of why herpesviruses induce changes in the Wnt/β-catenin pathway when they do, would provide vital information about the viral purpose of manipulating this pathway and how to interfere with this host manipulation controlled by the virus. As we understand more about virally induced aberrant Wnt/β-catenin we can develop better antivirals and possibly apply this knowledge to other human diseases associated with the Wnt/β-catenin pathway.

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ORIGINAL ARTICLE

Basic Study

Antiretroviral naive and treated patients: Discrepancies of B cell subsets during the natural course of human immunodeficiency virus type 1 infection

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Author contributions: Tsachouridou O participated in recruiting patients, designing the study and in the writing of the manuscript; Skoura L, Zebekakis P and Daniilidis M contributed equally to this work and have designed, organized the study and edited the text and approved the final version of the manuscript; Margariti A, Bougiouklis D and Pilalas D contributed equally to this work and have processed the samples and executed the flow cytometry and extracted/interpreted the results and wrote part of the manuscript; Georgiou A and Galanos A contributed equally to this work and have performed the statistical analysis of the study, wrote part of the manuscipt and approved the final version of the article; Metallidis S participated in recruiting patients and drafting, writing and revised on the content of the manuscript; all authors have read and approved the final version of the article to be published.

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Abstract

AIM

To evaluate alterations of memory B cell subpopulations during a 48-wk period in human immunodeficiency virus type 1 (HIV-1) patients.

METHODS

Forty-one antiretroviral naïve and 41 treated HIV-1 patients matched for age and duration of HIV infection were recruited. All clinical, epidemiological and laboratory data were recorded or measured. The different B cell subsets were characterized according to their

surface markers: Total B cells ($CD19^+$), memory B cells ($CD19^+CD27^+$, BMCs), resting BMCs ($CD19^+CD27^+CD21^{high}$, RM), exhausted BMCs ($CD19^+CD21^{low}CD27^-$, EM), IgM memory B ($CD19^+CD27^+IgM^{high}$), isotype-switched BMCs ($CD19^+CD27^+IgM^-$, ITS) and activated BMCs ($CD19^+CD21^{low+}CD27^+$, AM) at baseline on week 4 and week 48.

RESULTS

Mean counts of BMCs were higher in treated patients. There was a marginal upward trend of IgM memory B cell proportions which differed significantly in the treated group (overall trend, P = 0.004). ITS BMC increased over time significantly in all patients. Naive patients had of lower levels of EM B cells compared to treated, with a downward trend, irrespectively of highly active antiretroviral therapy (HAART) intake. Severe impairment of EM B cells was recorded to both treated (P = 0.024) and naive (P = 0.023) and patients. Higher proportions of RM cells were noted in HAART group, which differed significantly on week 4^{th} (P = 0.017) and 48^{th} (P = 0.03). Higher levels of AM were preserved in HAART naive group during the whole study period (week 4: P = 0.018 and 48: P = 0.035). HIV-RNA viremia strongly correlated with AM B cells (r = 0.54, P = 0.01) and moderately with RM cells (r = -0.45, P = 0.026) at baseline.

CONCLUSION

HIV disrupts memory B cell subpopulations leading to impaired immunologic memory over time. BMC, RM, EM and ITS BMC were higher in patients under HAART. Activated BMCs (AM) were higher in patients without HAART. Viremia correlated with AM and RM. Significant depletion was recorded in EM B cells irrespectively of HAART intake. Perturbations in BMC-populations are not fully restored by antiretrovirals.

Key words: B cell subpopulations; Time-trend; Memory cells; Human immunodeficiency virus infection; Highly active antiretroviral therapy

Core tip: During the progress of human immunodeficiency virus (HIV) infection and viral replication functional irritations of memory B-cell (BMC) compartment occur. Depletion of BMCs is one hallmark of deregulation in HIV-1 infection. Diminished levels of IgM^+ BMCs are also noted. Additionally, resting BMCs are severely impaired and defective B-cell subsets, like exhausted and activated BMCs circulate in peripheral blood. Significant fluctuations of these B cells' frequencies are recorded over time and antiretroviral therapy may play a role on this observation. Assessing these populations could potentially lead to improvement in assessing vaccine responses and tracing vulnerable patients to certain infections.

Tsachouridou O, Skoura L, Zebekakis P, Margariti A, Georgiou A, Bougiouklis D, Pilalas D, Galanos A, Daniilidis M, Metallidis S. Antiretroviral naive and treated patients: Discrepancies of B cell subsets during the natural course of human immunodeficiency virus type 1 infection. *World J Virol* 2016; 5(4): 155-160 Available from: URL: http://www.wjgnet.com/2220-3249/full/v5/i4/155.htm DOI: http://dx.doi.org/10.5501/wjv.v5.i4.155

INTRODUCTION

During the chronic human immunodeficiency virus (HIV) infection and viral proliferation functional irritations of B-cells take place like impairment of isotype switching, polyclonal activation, divergences in the frequencies of circulating B cell-populations and diminished immune reactions to immunization[1-3].

The deprivation of memory B cells ($CD19^+CD21^+$-$CD27^+$, BMC) reflects some dysfunction in HIV patients[3,4]. Reduced IgM^+ BMCs ($CD19^+CD27^+IgM^{high}$) is also observed[2,3]. During the course of the infection, resting BMCs ($CD19^+CD21^{high}CD27^+$, RM) are severely impaired[5]. Furthermore, dysfunctional B-cell subsets, accounting for activated BMCs ($CD19^+CD21^{low}CD27^+$, AM) and exhausted BMCs ($CD19^+CD21^{low}CD27^-$, EM), rise in these patients, while they appear at very low frequencies in healthy subjects[4].

The phenotype of B cells that serves the immune response to several antigens has been ambivalent[6-9]. The conflict primarily is targeted on the surface markers of the B cells that respond to the antigens[3,6,7,10-12]. It is currently challenged that IgM BMCs are merely in charge of antibody production; since other memory subsets such as isotype switched B cells ($CD19^+CD27^+IgM^-$) also produce anti-polysaccharide antibodies *in vitro*[2].

Highly active antiretroviral therapy (HAART) reduces polyclonal B-cell activation but has only a constricted effect on the remediation of B-cells and remains to be elucidated which certain perturbations can be repaired[3,5]. The loss of memory is confirmed by in the reduction of antigen specific BMCs post vaccine administration, which is not reconstituted by HAART[13,14]. RM cells are preserved if HAART is initiated immediately in HIV confirmation of infection[14].

Aim of this study was to record and evaluate alterations of BMC subpopulations during a 48-wk period in HIV-1 patients. Moreover, we prospectively studied the impact of HAART on these cell populations seeking for significant changes.

MATERIALS AND METHODS

This is a longitudinal study including 82 HIV patients matched for age and duration of HIV infection, 41 of whom were antiretroviral naive and 41 were under HAART, with successful viral suppression (HIV-1 viral load < 34 copies/mL). All rest data, including epidemiological (age, gender, HIV-1 transmission route, co morbidities) and laboratory results, HIV-1 viral load, current CD4 T-cell count, nadir CD4 cell count were

Table 1 Patients' characteristics at enrollment

	HAART naïve patients (n = 41)	Treated patients (n = 41)	P value
Age (yr)	31.76 ± 7.16	34.15 ± 6.17	0.168
Gender (male/female), n (%)	41 (100.0)/0 (0.0)	36 (87.8)/ 4 (12.2)	0.065
Years on HIV infection	3.27 ± 2.78	3.6 = 98 ± 4.41	0.415
Nadir CD4 cell count	573.6 ± 223.4	326 ± 187.3	0.0005
ART duration in months	NA	34.4 ± 14.18	NA
HCV infection, n (%)	2 (4.8)	3 (7.3)	0.345
HBV infection, n (%)	6 (14.6)	4 (9.7)	0.167

All quantitative data are presented as mean ± SD, median (IQR). HAART: Highly active antiretroviral therapy; VL: HIV RNA viral load; HIV: Human immunodeficiency virus; ART: Antiretroviral therapy; NA: Not applicable; HCV: Hepatitis C virus; HBV: Hepatitis B virus.

recorded.

The Aristotle's University Ethical Committee approved the protocol and a written informed consent was obtained from all participants. All study individuals were asked to give blood sample on day 0 and on week 4 and 48.

Mouse anti-human fluorochrome-conjugated monoclonal antibodies of Immunostep Company® were used: CD19-PerCP, CD27-PE, IgM-FITC, IgD-FITC and CD21-FITC to count total B cells and BMC subsets combined properly. One hundred microliter of blood samples after adding 10 μL of the above combined monoclonal antibodies were incubated in the dark for ten minutes. In turn, red blood cells were thawed upon ingestion of 2 mL of Lysis Buffer (BD Biosciences, San Jose, CA) and incubated for another twenty minutes in room temperature. B cells were assessed pre vaccination. The different B cell subsets were characterized as follows: Total B cells ($CD19^+$), BMCs ($CD19^+CD27^+$), EM ($CD19^+CD21^{low}CD27^-$), IgM memory B ($CD19^+CD27^+IgM^{high}$), RM ($CD19^+CD27^+CD21high$), IgM memory B ($CD19^+CD27^+IgM^{high}$), AM ($CD19^+CD21^{low+}CD27^+$) and ITS ($CD19^+CD27^+IgM^-$) at baseline and on weeks four and forty eight. Results were expressed as B cell absolute counts or as a percentage of the total B cell population.

Sample processing and result extraction was performed in the XL Epics cytometer (Beckman Coulter™ Company, Florida, United States). The input capture, cell staining and flow cytometry were performed immediately in a blinded manner.

Statistical analysis

The comparison of variables at each time point was performed using the independent samples t test or the Mann-Whitney test in case of violation of normality. To indicate the trend in the one year period, the median percentage changes after 4 wk and 48 wk respectively were calculated. All tests are two-sided, a P-value of < 0.05 was used to denote statistical significance. All analyses were carried out using the statistical package SPSS vr 16.00 (Statistical Package for the Social Sciences, SPSS Inc., Chicago, Ill., United States).

RESULTS

The demographics, clinical and rest data of eighty two HIV individuals are illustrated in Table 1.

In order to confirm whether the percentages of B-cell subsets were altered among treated and antiretroviral naive HIV-1 individuals, the percentages of memory B, activated memory, resting memory, exhausted memory as well as isotype-switched and total B-cells were assessed. Significant differences were observed between the groups in relation to B cell subsets.

Mean counts of BMCs ($CD19^+CD27^+$) were higher in treated patients throughout the 48 wk (P = 0.987, NS), with a gradual declining trend by the end of the 48th week. Mean fraction of IgM memory B ($CD19^+CD27^+IgM^{high}$) cell-population found higher in the treated group at baseline. There was a marginal upward trend of the proportions which differed significantly in the treated group (overall trend, P = 0.004) (Figure 1). Isotype-switched BMC ($CD19^+CD27^+IgM^-$) were slightly elevated in patients without HAART compared to the other group. The time trend variation was equivalent in both groups, irrelevantly of HAART intake (P = 0.808). ITS B cell compartment raised significantly in all patients, concerning baseline levels (overall significance, P = 0.0005) (Figure 1).

HAART patients preserved higher proportions of EM B cells ($CD19^+CD21^{low}CD27^-$) compared those without treatment, with a downward trend along with the progression of the disease, irrespectively of HAART intake. These changes were not significant among groups (overall significance, P = 0.876). Significant depletion of EM B cells was recorded to both ART-naive (P = 0.023) and rest individuals (P = 0.024) (Figure 1). The fraction of RM cells ($CD19^+CD21^{high+}CD27^+$) in patients under HAART were higher and significantly different on week 4th (P = 0.017) and 48th (P = 0.03). The fluctuation over time of RM was nearly the same though, in both groups (P = 0.201) with treated patients having a significant overall increase (P = 0.003). Patients HAART-naive maintained higher levels of AM ($CD19^+CD21^{low+}CD27^+$) during the whole study period, with the downward trend being significant in the treated group (P = 0.004) (Figure 1).

HIV-RNA viremia strongly correlated with AM B cells (r = 0.54, P = 0.01) and moderately with RM B cells (r = -0.45, P = 0.026) at baseline, supporting the impact of viral replication on these subsets (data not shown).

DISCUSSION

HIV infection impels to a broad amplitude of B cell defects, like cell switching, depleted numbers of B cells, production of uncommon B cell populations and disfunctional immune responses even in patients under HAART[3,6,7]. Furthermore, it is generally accepted to augments the risk of several infections. Very scarce and

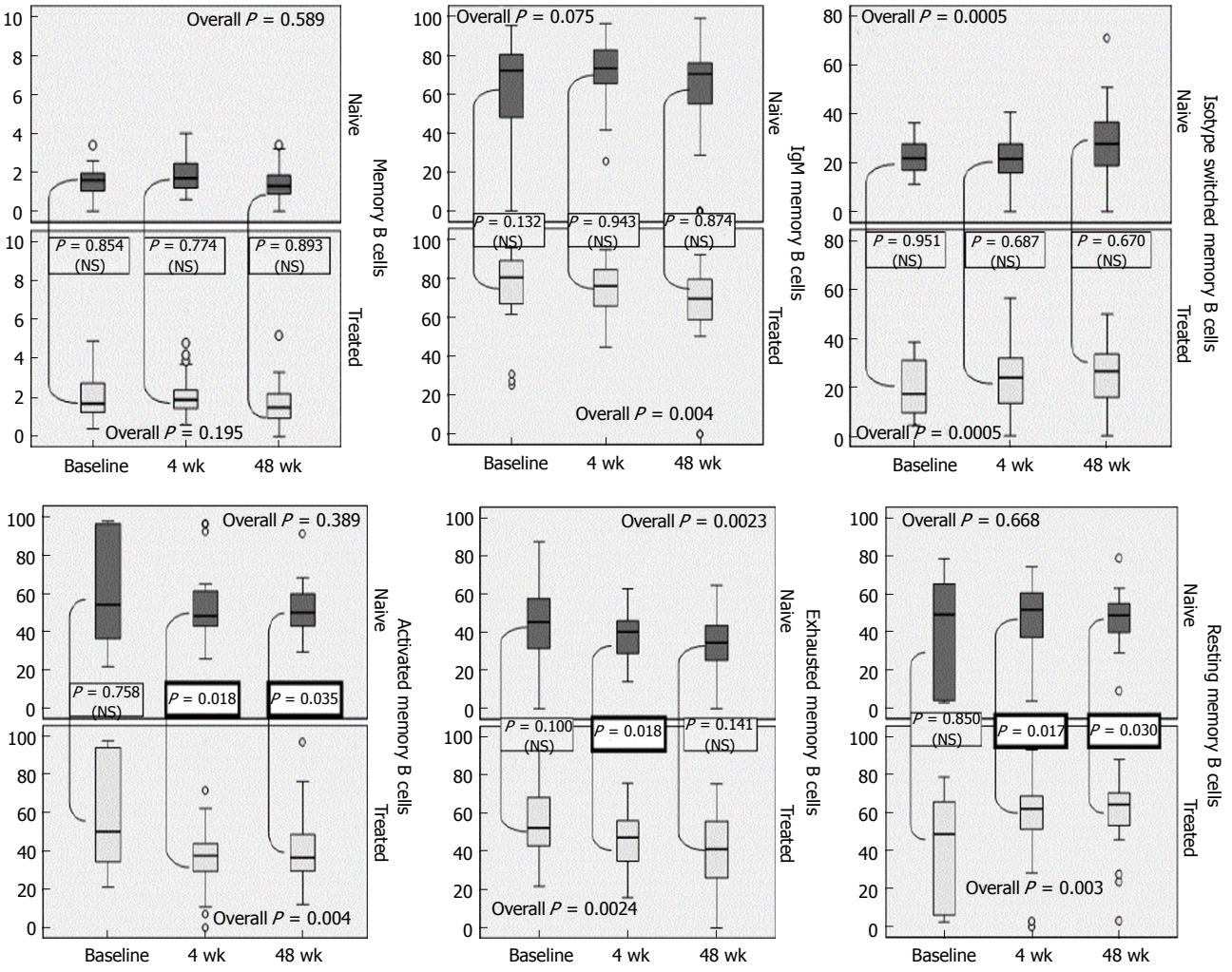

Figure 1 Time trend alterations of memory B cells.

conflicting data are currently available illustrating the significance of B cell subsets that mediate satisfactory and protective immune responses[6,7].

Our study promotes the scouting and assessment of specific BMC subpopulations interfered in humoral responses, confirming few other authors claiming that, not solely ITS and IgM BMC, but also AM and RM, might contribute to impaired protection against certain bacteria[7-12,14]. Recent studies focus on B cell memory and immunological response post immunizations, though most constitute solely cross-sectional studies[8-12,14].

BMCs were increased in patients on HAART compared to naive on an annual basis, as confirmed in other studies as well[2,15]. We showed rise of BMCs in both groups, which depleted throughout time in a similar pattern, that is in line with previous studies[7].

Interestingly, slight rise in IgM BMC was confirmed in all patients. Patients on treatment preserved high frequencies of them, confirming authors suggesting that HAART preserves the levels of this specific subset[15]. Both treated and naive patients maintained their IgM BMC over time, which is controversial in literature[15].

EM B cells are believed to be increased in naive patients[2,6]. Although in our study we did not confirmed the former observation, gradual decrease has been recorded irrespectively of HAART. Patients under HAART had decreased AM cells compared to those without treatment, explaining the effect of HAART which restricts their expansion during the progress of HIV infection[2]. We additionally confirmed that AM B cells are preserved in continuous viral replication[13]. Even though effective HAART is regarded to have no impact on RM, in our study maintenance of high levels especially in the treated group, implies that some restoration may be feasible upon HAART initiation, regardless being not during primary infection[5,14].

Studies have shown that isotype switched B cells are not affected in healthy individuals[7], but these are dramatically impaired in HIV infection irrespectively of HAART[15]. Despite few studies that confirmed high frequencies of ITS B cells in patients under HAART[2,15], our study confirmed more recent authors[15] showing no effect of HAART, which underlies the need for further investigation on this specific memory subset.

HAART introduction lead to further investigation on these populations, concluding that most divergences are reversible, implying that viremia has a causal relationship. Viremia has been associated with the

elevated frequency of AM cells[16]. However, the impact of HIV viremia has not been fully explained, apart from in limited studies[14]. Our study in lineage with other authors has shown that viremia was linked to certain B cell populations[14].

In conclusion, the data of our study points out that significant divergences occur in specific BMC populations in HIV patients. Natural course of HIV infection has an immunological impact on distinct B cells, sparking modifications on their absolute numbers and functions in the peripheral blood of HIV adults. Furthermore, HAART administration affects subsets like RM and AM which are significant in secondary immune responses, while has controversial implication in other BMC-compartments. We propose that evaluation of BMC might implicate in immunization and have clinical utility in forecasting all susceptible HIV adults to bacterial and other viral infections.

The significance of the paper lies to the fact that HAART prompt initiation may alter few of the disturbances that HIV infection itself promotes. Similar findings for the significance of immediate initiation of antivirals have been published recently, which insist that HAART is necessary to be started once the diagnosis of HIV infections has been confirmed[17].

Additionally, concerning the HIV vaccine development design, new scientific trends lean towards the role of B cells in HIV pathogenesis and their possible use to design and develop a proper vaccine for preventing HIV infections. Multiple studies try to assess and isolate the responsible B cell subsets that interfere to the pathogenesis of HIV infection and will lead to the vaccine development[18,19].

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COMMENTS

Background

Human immunodeficiency virus (HIV) causes several phenotypic and functional perturbations on B lymphocytes, like hyperactivation leading to hypergammaglobulinemia. Simultaneously, B cells also display hyporesponsiveness to vaccines. Memory B cell (BMC) react and secrete antibodies specific to the antigen faced, with improved pertinence when meeting the same antigen twice, offering protection against several infections. Highly active antiretroviral therapy (HAART) partially restores B cell perturbation, especially when initiation is prompt. Moreover, antivirals are cannot retrieve humoral response in HIV adults, and re-immunization might be mandatory. Optimizing vaccine strategy may improve BMC responses to immunizations. The role of some recently described BMC subsets is not fully understood and remains to be elucidated. Health care providers should consider prompt HAART initiation in order to restrict HIV-associated BMC impairment.

Research frontiers

Previous studies have assessed the role of early antiretroviral therapy (ART) administration showing that some populations may be benefited and protected from functional and phenotypic perturbations, with conflicting results.

Innovations and breakthroughs

This study aimed to assess over time the fluctuation of significant BMC subsets for humoral responses in HIV patients and not in single time slot. The study revealed certain time-trend differences among antiretroviral naive and treated patients.

Applications

Literature is still ambiguous concerning the precise role of certain BMC populations and the significance of HAART in restoring or preventing some disturbances on them. ART intake affects subsets like resting BMC (RM) and activated BMC (AM) which are significant in secondary immune responses, while has controversial implication in other BMC. Evaluation of BMCs might intervene in immunizations and have clinical utility in pointing out the susceptible HIV adults to bacterial and other infections.

Terminology

$CD27^+$ BMC comprise of $CD21^+$ cells (RM) and $CD21^-$ cells (AM). RM are depleted while AM rise during the natural course of HIV infection. The classical $CD27^+$ BMC are classified as ITS and un-switched subpopulations, while the isotype-switched BMC subset represent BMC that have switched their immunoglobulin from IgM and IgD to other classes. TLM ($CD19^+CD10^-$ $CD27^-$ $CD21^{low}$), rise in HIV infected patients.

Peer-review

The article is well prepared and makes a pleasant and useful reading for those in the field.

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ORIGINAL ARTICLE

Basic Study

Role of RNA secondary structure in emergence of compartment specific hepatitis B virus immune escape variants

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Author contributions: Datta S designed the study to test the hypothesis, performed predictions, analyzed the models and wrote the manuscript; Chakravarty R reviewed the results and edited the manuscript.

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Abstract

AIM

To investigate the role of subgenotype specific RNA secondary structure in the compartment specific selection of hepatitis B virus (HBV) immune escape mutations.

METHODS

This study was based on the analysis of the specific observation of HBV subgenotype A1 in the serum/plasma, while subgenotype A2 with G145R mutation in the peripheral blood leukocytes (PBLs). Genetic variability found among the two subgenotypes was used for prediction and comparison of the full length pregenomic RNA (pgRNA) secondary structure and base pairings. RNA secondary structures were predicted for 37 ℃ using the Vienna RNA fold server, using default parameters. Visualization and detailed analysis was done using RNA shapes program.

RESULTS

In this analysis, using similar algorithm and conditions, entirely different pgRNA secondary structures for subgenotype A1 and subgenotype A2 were predicted, suggesting different base pairing patterns within the

two subgenotypes of genotype A, specifically, in the HBV genetic region encoding the major hydrophilic loop. We observed that for subgenotype A1 specific pgRNA, nucleotide 358^{U} base paired with 1738^{A} and nucleotide 587^{G} base paired with 607^{C}. However in sharp contrast, in subgenotype A2 specific pgRNA, nucleotide 358^{U} was opposite to nucleotide 588^{G}, while 587^{G} was opposite to 359^{U}, hence precluding correct base pairing and thereby lesser stability of the stem structure. When the nucleotides at 358^{U} and 587^{G} were replaced with 358^{C} and 587^{A} respectively (as observed specifically in the PBL associated A2 sequences), these nucleotides base paired correctly with 588^{G} and 359^{U}, respectively.

CONCLUSION

The results of this study show that compartment specific mutations are associated with HBV subgenotype specific alterations in base pairing of the pgRNA, leading to compartment specific selection and preponderance of specific HBV subgenotype with unique mutational pattern.

Key words: Hepatitis B; Compartmentalization; Peripheral blood leukocytes; pgRNA; RNA secondary structure; G145R

Core tip: We have previously shown that, in our study population, distribution of hepatitis B virus (HBV) subgenotypes A1 and A2 is highly biased in the serum/plasma and peripheral blood leukocyte (PBL) compartments respectively. Analysing the predicted base pairing patterns of pregenomic RNAs (pgRNAs), specific for HBV subgenotype A1 and A2, we demonstrate that the potent immune escape mutation G145R evolves specifically in the context of HBV subgenotype A2. The PBL compartment is exposed to strong anti-HBs immunity, and thus G145R is highly advantageous for the virus to persist. This explains the exclusive preponderance of subgenotype A2 in the PBL compartment, sharply contrasting the prevalence of subgenotype A1 in the serum/plasma.

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INTRODUCTION

Viral compartmentalization signify infection, persistence and replication of viruses in off-target cells/tissues or anatomical compartments of the host, and this phenomenon is now believed to be a crucial event in many important viral infections, including hepatitis B virus (HBV), human immunodeficiency virus, hepatitis C virus, *etc.*[1-5]. Recent molecular evolutionary studies have demonstrated that viruses evolve independently under the influence of unique immunological milieu in a given compartment, leading to the selection and emergence of specific viral variants, which endow the virus with an advantage to survive and persist in that particular compartment[2,6,7]. Such compartment specific viral evolution have extremely important implications in emergence and re-emergence of immune-escape mutants, antiviral resistant mutants, their long term persistence and transmission through different non-conventional routes[1,7].

HBV is the prototype member of the *Hepadnaviridae* family of enveloped viruses with a very unique partially double-stranded DNA genome[8]. Despite having a DNA genome, HBV exclusively uses an RNA intermediate (the *pregenomic* RNA or the *pgRNA*) and a virus encoded reverse transcriptase to replicate its genome through a complex mechanism of primer shifting[8]. Even though HBV is classically considered to be a hepatotropic virus, HBV related nucleic acids and proteins have long been detected in different tissues, suggesting that it replicates and propagates in various non-hepatic tissues[1]. Interestingly, some of these extrahepatic sites have been shown to act as reservoirs and also the source of reinfection after surgical and therapeutic interventions[9,10]. Recently, ours and other research groups have provided convincing evidences that the HBV strains and their mutational signature pattern present in different extra-hepatic compartments, are often characteristically distinct from the HBV strains circulating in the serum/plasma/hepatic compartments and that immune escape/drug resistance mutations are significantly more frequent in different extrahepatic compartments in HBV carriers[2,4,11].

In our previous studies, we have recognized the subgenotype A1 (*Afro-Asian* subgenotype) as the predominant subgenotype of HBV genotype A circulating in the sera/plasma of our study population and that the occurrence of G145R mutation therein was sporadic[12-14]. In sharp contrast, we documented the confined and exclusive existence of HBV subgenotype A2 with the potent "immune escape" mutation G145R within the peripheral blood leukocytes (PBL), across the study population, irrespective of the HBV genotype/subgenotype circulating in the serum/plasma of the respective individual[2]. G145R is the mutation signifying Glycine to Arginine substitution at amino acid residue 145 in the major hydrophilic loop (MHL), a B-cell epitope of the hepatitis B surface antigen (HBsAg), which provides a strong immune escape property. These observations strongly signify that viral mutants with G145R does have an explicit replicative advantage within the PBLs, that are exposed to strong anti-HBs immunity and that this mutation emerges specifically in the perspective of subgenotype A2, but not in subgenotype A1. Moreover, all the subgenotype specific nucleotide substitutions in the MHL encoding region of A1 (505^{C}, 514^{C}, 616^{A} and 619^{T}) and A2 (505^{T}, 514^{A}, 616^{G} and 619^{C}) are

Table 1 Comparison of nucleotides at phylogenetically informative sites of subgenotypes A1 and A2 in GenBank sequences and in sequences isolated from our study population

Nucleotide Position of the HBV genome[1]	Base present in reference GenBank sequences			Base in genotype A1 sequences isolated from serum/ plasma[2]	Base in genotype A2 sequences isolated from PBL[2]
	Aa/A1 (Asia)	Aa/A1 (South Africa)	Ae/A2 (Europe/ United States)		
505	c	t	t	c	t
514	c	c	a	c	a
616	a	a	g	a	g
619	t	t	c	t	c

[1]Nucleotide positions indicate distance from the unique EcoRI site in the HBV genome; [2]Sequences isolated from our study population. HBV: Hepatitis B virus; PBL: Peripheral blood leukocyte.

synonymous in nature, which aptly rules out the possibility that the predilection of the subgenotype A2 in the PBL is due to the subgenotype specific epitopic difference in the HBsAg[2]. Taken together, the above observations led us to hypothesize that the subgenotype specific nucleotide substitutions might modulate the base pairing of the A2 specific pgRNA in a way, which favours the emergence of G145R.

In the present work, we compared the changes in the base pairing of the pgRNA due to subgenotype A1 and A2 specific substitutions in the MHL encoding region. Based on the RNA secondary structure predictions, we demonstrate that the selection and emergence of G145R within HBV subgenotype A2 sequences in the PBL compartment occurs due to the differential base pairing characteristics in the subgenotype A2 specific pgRNA.

MATERIALS AND METHODS

Sequences for analysis

HBV surface gene sequences, corresponding to the nucleotide 341 to 660 of the HBV genome (nucleotide position counted from the unique EcoRI site in the HBV genome), that code for the epitopic MHL for both subgenotype A1 sequences (isolated from serum/ plasma) and subgenotype A2 sequences (isolated from PBL), obtained during our previous studies were used in this analysis[2,14]. Using alignment of these sequences along with other reference GenBank HBV sequences[15], subgenotype A1 and A2 specific nucleotide substitutions (summarised in the Table 1) were determined earlier[2]. These nucleotide differences were consequently used for studying the alterations in the subgenotype specific base-pairing and folding of the pgRNA.

For subgenotype A1 and A2 specific pgRNA secondary structure predictions, template sequences were generated separately by editing two well defined full length sequences, namely-GenBank accession number DQ315784 (India) for subgenotype A1 and GenBank accession number AJ309370 (France) for subgenotype A2, respectively, following the method described previously for generation of full length pgRNA sequence[16]. The pgRNA templates so generated were unpolyadenylated and included the terminal redundancy. These two sequences served as the base sequences for prediction of secondary structures, to which nucleotide substitutions observed in the MHL encoding and flanking regions of serum associated A1 and PBL associated A2 (as mentioned in the previous section) were substituted respectively at appropriate nucleotide positions. Finally, these two template sequences (approximately 3.3 kb) were subjected to RNA secondary structure prediction and comparison.

Prediction of RNA structure

For prediction of the secondary structures, the pgRNA sequences generated as stated above were submitted to the Vienna RNA secondary structure server[17,18]. The server predicts the minimum free energy *(mfe)* secondary structures for single RNA sequences using an algorithm proposed by Zuker and Stiegler, and also calculates the equilibrium base-pairing probabilities by means of partition function (*pf*) algorithm proposed by McCaskill[19,20]. Apart from the *mfe* and *pf*, the server also provides a centroid structure, which indicates the reliability of the predictions, while the dot-plot which provides information on base-pairing probabilities of all the possible predicted structures[17,21]. All the secondary structure predictions were performed for a temperature of 37 ℃, keeping all the other parameters to default[22]. Visualization, annotation and analysis of the *mfe* structures were performed using the RNAshapes program[23]. As the present study was focused on the genetic variability of the HBV genome encoding the MHL region of the surface gene, we restricted our detailed analysis of base pairing pattern to the secondary structure of the part of pgRNA, corresponding to the MHL encoding sequence.

RESULTS

Changes in the nucleotide base pairing of the predicted secondary structure of pgRNA

The gross structural features of the subgenotype A1 and A2 specific pgRNA were found to be entirely distinct (Figure 1). The difference in the pgRNA structure was also evident from the mountain plots showing the mfe, pf, centroid, entropy and the dot plot of the two subgenotypes. The difference in other features are summarised in the Table 2. Detailed scrutiny of the pgRNA secondary structures corresponding to the MHL encoding genetic regions, revealed entirely distinct pgRNA secondary structures with discrete intramolecular base pairing patterns due to the subgenotype specific and variations between A1 and A2 sequences (Figures 2 and 3). Interestingly, when we focussed on the base pairing of the nucleotides encoding the MHL region, we noted that in subgenotype A1 specific pgRNA, nucleotide 358^{U} base paired with 1738^{A} and nucleotide 587^{G} base paired with 607^{C} (Figure 2).

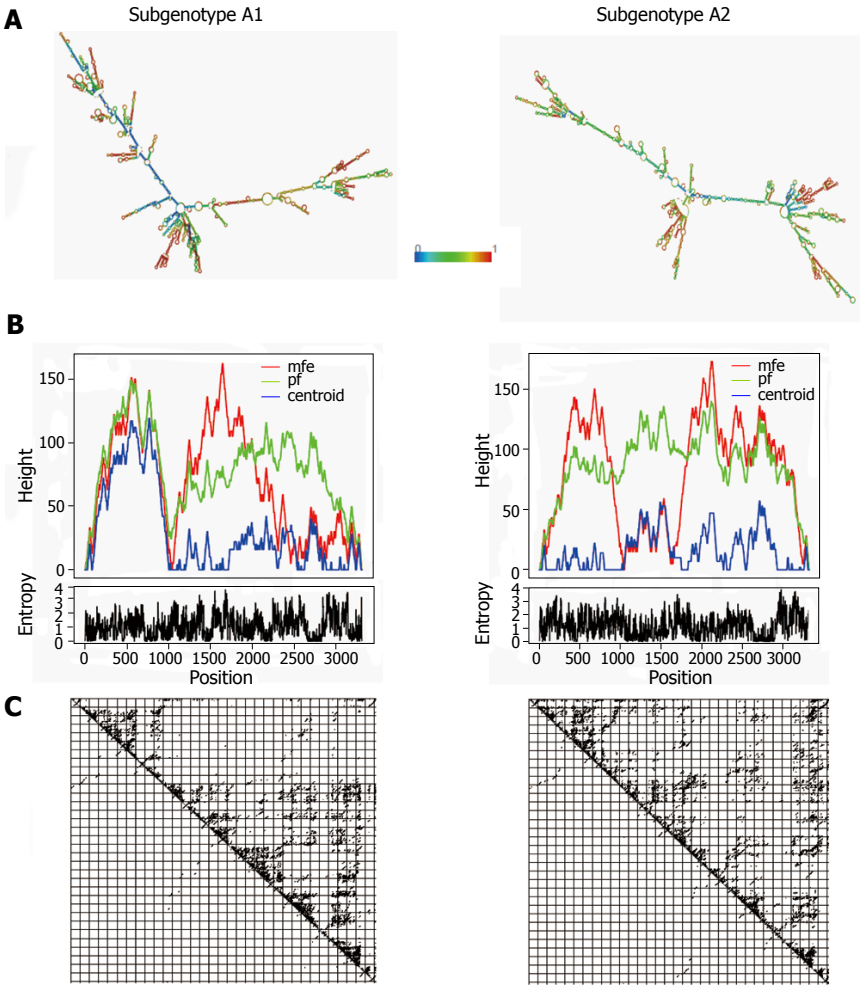

Figure 1 Comparative diagram showing the differences between different aspects of the predicted secondary structures of the pgRNA, specific for HBV subgenotype A1 and subgenotype A2. A: Predicted minimum free energy (mfe) structures, coloured by base-pairing probabilities (according to the rainbow scale shown in the middle, denoting base pair probabilities from 0 to 1). Colour of the unpaired regions denotes the probability of being unpaired; B: Mountain plot representing the mfe structure (red line), the thermodynamic ensemble of RNA structures (green line), and the centroid structure (blue line). Positional entropy for each position is presented below the mountain plot; C: Dot-plot showing the base-pairing probabilities of the two predictions.

Table 2 Comparison of the thermodynamic characteristics of the minimum free energy secondary structure predictions for subgenotypes A1 and A2 pgRNA

Features	Subgenotype A1	Subgenotype A2
Minimum free energy of the optimal secondary structure	-1052.10 kcal/mol	-1049.50 kcal/mol
Free energy of the thermodynamic ensemble	-1099.56 kcal/mol	-1098.93 kcal/mol
Minimum free energy of the centroid secondary structure	-722.20 kcal/mol	-679.21 kcal/mol
Ensemble diversity	863.25	954.99

However in sharp contrast, in subgenotype A2 specific pgRNA, nucleotide 358^{U} was opposite to nucleotide 588^{G}, while 587^{G} was opposite to 359^{U}, hence precluding correct base pairing and thereby less stability of the stem structure. When the nucleotides at 358^{U} and 587^{G} were replaced with 358^{C} and 587^{A} respectively (as observed specifically in the PBL associated A2 sequences), these nucleotides base paired correctly with 588^{G} and 359^{U}, respectively (Figure 3), forming a correctly paired stem-loop structure, hence stabilizing the local conformation. Nevertheless, the effects of other substitutions were not as influential as these two changes. The exclusive detection of subgenotype A2 sequences with the abovementioned substitutions in the PBL clearly suggest the selective advantage of the pgRNA with 358^{C} and 587^{A}, and in turn the importance of G145R immune escape mutation in the PBL compartment.

DISCUSSION

In this study, we present interesting observations about the possible mechanism of compartment specific selection of immune escape HBV mutants. Based on our previous studies done on serum/plasma isolated HBV genotypes, we have documented the predominance of at least three distinct HBV genotypes in our study population, namely genotype D (most abundant) followed by genotypes C and A[13,14]. However, when we investigated the paired HBV sequences isolated from

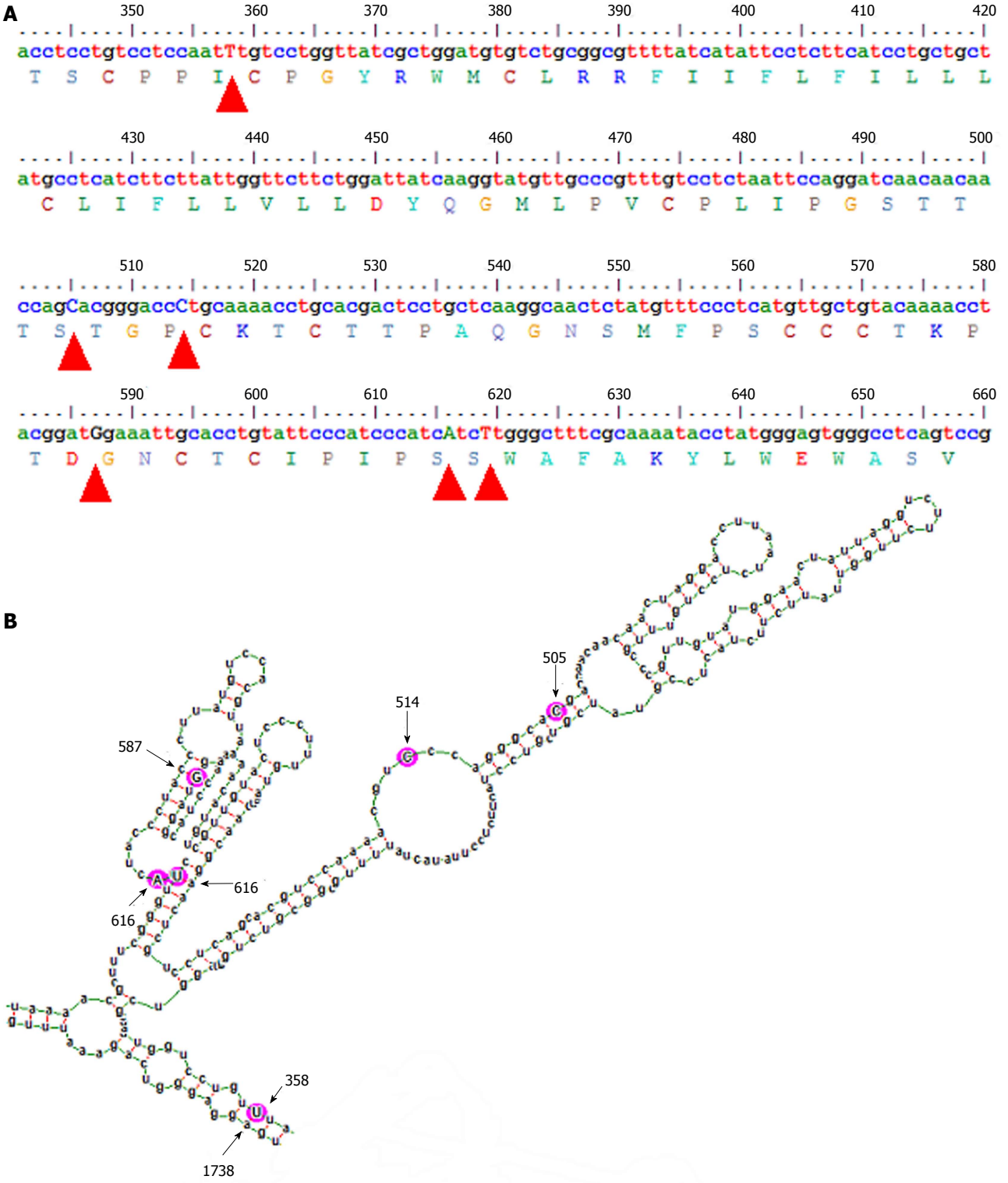

Figure 2 Diagram showing the genetic variability and the part of the predicted mfe structure, corresponding to the genetic region encoding the major hydrophilic loop of subgenotype A1. A: Consensus nucleotide sequence (corresponding to positions 341 to 660 of the HBV genome) and predicted amino acid sequence (corresponding to residues 63 to 168 of the HBsAg). Subgenotype A1 specific nucleotides (505^{C}, 514^{C}, 616^{A} and 619^{T}) and two variable nucleotides (358^{T} and 587^{G}) are indicated by arrowheads; B: Detailed base pairing pattern of the mfe pgRNA structure, specific for subgenotype A1. Aforementioned variable sites are encircled by pink circles and indicated by arrows and numbers correspond to their nucleotide position in the HBV genome. HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen.

serum/plasma and the PBLs, we surprisingly observed the exclusive preponderance of the genotype A in the PBL, irrespective of the HBV genotypes circulating in the serum/plasma of any given individual[2]. More interestingly, the genotype A sequences isolated from the PBL was found to be markedly distinct from that of genotype A sequences isolated from serum/plasma, in terms of subgenotype and specific nucleotide sub-

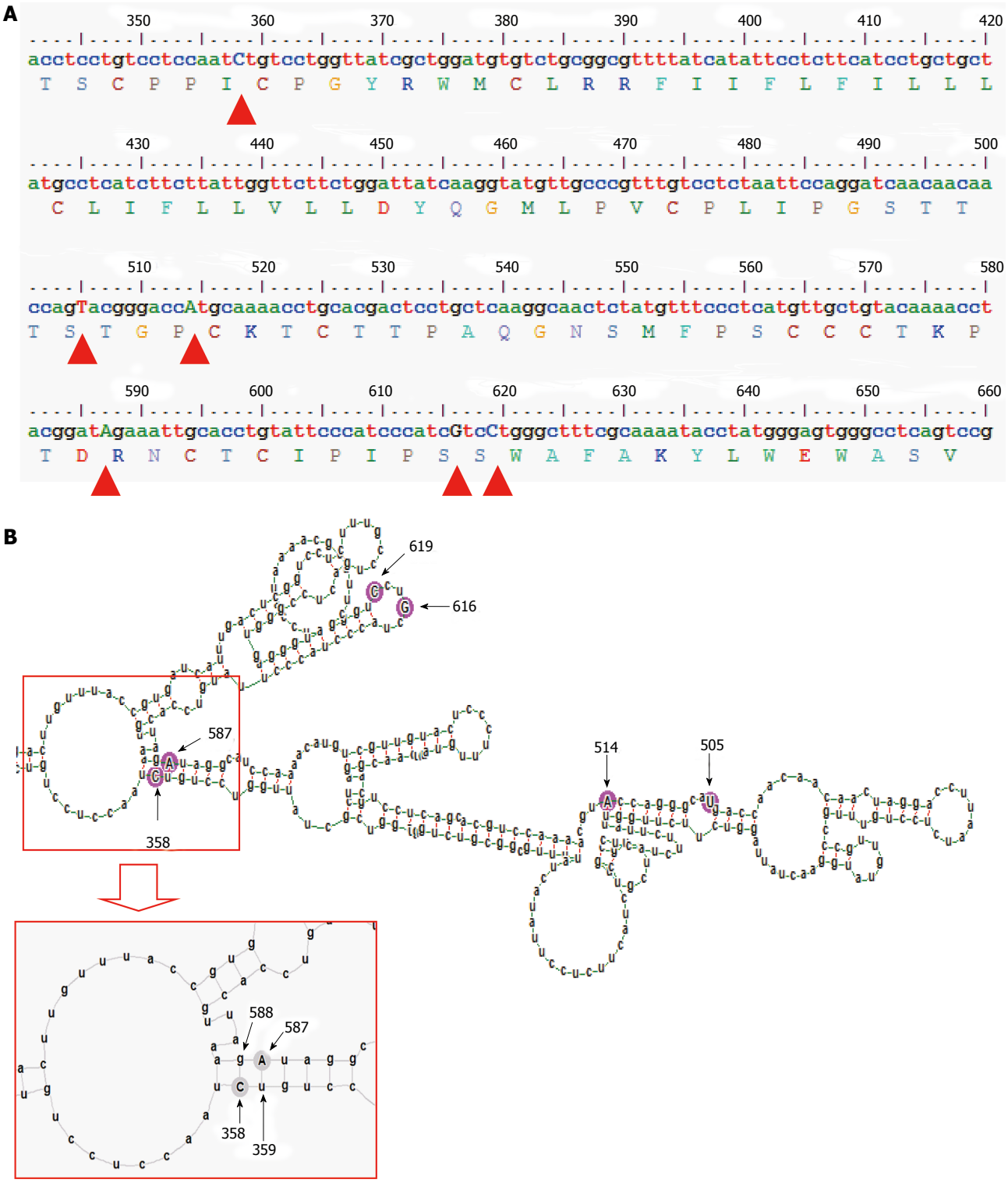

Figure 3 Diagram showing the genetic variability and the part of the predicted mfe structure, corresponding to the genetic region encoding the major hydrophilic loop of subgenotype A2. A: Consensus nucleotide sequence (corresponding to positions 341 to 660 of the HBV genome) and predicted amino acid sequence (corresponding to residues 63 to 168 of the HBsAg). Subgenotype A2 specific nucleotides (505^{T}, 514^{A}, 616^{G} and 619^{C}) and two co-evolving nucleotides (358^{C} and 587^{A}) are indicated by arrowheads; B: Detailed base pairing pattern of the mfe pgRNA structure, specific for subgenotype A2. Aforementioned variable sites are encircled by pink circles and indicated by arrows and numbers correspond to their nucleotide position in the HBV genome. Part of the mfe structure is amplified in the inset for better visualization of the base pairing. HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen.

stitution patterns. More precisely, subgenotype A1 of HBV genotype A was prevalent in the serum/plasma while in sharp contrast; subgenotype A2 was solely isolated from the PBL[2].

In the present study, we sought to examine the selective advantage of subgenotype A2 in the PBL compartment with the help of advance computational prediction and analysis programs. We focussed our

analysis on the examination of nucleotide sequences encoding the dominant B-cell epitope (MHL) of the HBV surface antigen, since in a number of other viruses, analogous genetic regions (epitope regions of the envelope protein) have been shown to undergo faster evolution to facilitate the emergence of compartment specific immune-escape variants[2]. Interestingly, when we compared serum/plasma circulating subgenotype A1 sequences with PBL confined subgenotype A2 sequences, we found that only four subgenotype specific nucleotide substitutions differentiate both sequences[2]. However, all these four subgenotype specific nucleotide substitutions in the MHL encoding region were found to be synonymous in nature (*i.e.*, the sequence of amino acids in the MHL remains same between subgenotypes A1 and A2), suggestive of the fact that MHL epitope diversity might not be directly relevant to the selection of subgenotype A2 over subgenotype A1 in PBL. On the other hand, in addition to these four subgenotype specific nucleotide substitutions, two additional nucleotide substitutions (358^{C} and 587^{A}) were evident with PBL associated A2 sequences, across the study population, which we have earlier shown to be co-evolving in the PBL[2]. Interestingly, we further noted that nucleotide substitution 358^{C} was also synonymous, while substitution 587^{A} was non-synonymous and translated into the potent immune escape G145R mutation of HBsAg. Earlier studies have demonstrated that by virtue of its definite advantages, G145R mutation helps HBV to dynamically evade anti-HBs specific immune response, thereby ensuring viral persistence in anatomical compartments, which are exposed to strong anti-HBs immunity[2]. The association of these five synonymous nucleotide substitutions and a potent immune escape mutation with PBL associated Ae/A2 sequences led us to hypothesize that the advantageous 587^{A} (G145R) might be selected at the pgRNA base pairing level and to verify this hypothesis, this comparative study was undertaken.

On comparison of the pgRNA secondary structures, we observed that the invariable association of subgenotype A2 with the selection of nucleotide 587^{A} (causing G145R) most possibly occur in the context of genotype A2 specific altered pgRNA base pairing patterns. Fascinatingly, we observed that substitution of a uracil (U, corresponding to Thymidine, T in DNA sequence) to cytosine (C) at position 358 altogether changed the local base pairing pattern of the pgRNA (358^{C} paired with nucleotide 588^{G} instead of the normal pairing with 1738^{A} in subgenotype A1). In the context of this altered base pairing, a single nucleotide change (U to C) at 359 was found to stabilize the stem structures by pairing with the wild type 587^{G}, just opposite to it. However, the nucleotide at position 359 encodes a Cysteine residue at amino acid position 69 of HBsAg, which is extremely essential for the generation of subviral 20 nm HBsAg particles, and thus, any non-synonymous substitution at this position is most likely to be detrimental for the virus persistence[24]. Therefore, based on the predicted secondary structures, we hypothesized that, instead of selecting an altered nucleotide at this exceptionally essential position (359), a compensatory alteration of a single nucleotide (G to A) at position 587 is expected to serve dual purpose, firstly it may help stabilize the stem structure (by pairing with the highly conserved $359^{T/U}$) and secondly it results in the emergence of a potent immune escape G145R mutation, both of which appears to be highly advantageous for the virus.

The HBV polymerase lacking proof reading function has been implicated in the generation of random mutations and generation of "quasi-species", of which the genomes (viral DNA) or pregenomes (pgRNA) having mutations useful for escaping the immune response of the host are gradually selected and subsequently become the prevalent viral population[25]. Apart from viral polymerase induced random mutations, host PBL associated APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide) family of cellular cytidine deaminases have also been shown to induce hypermutation (G to A mutations) in the HBV genome, which has been suggested to cause genetic diversification and consequently selective evolution among the divergent genomes[26-28]. Nevertheless, the finding of selective predominance of the point mutation leading to G145R in the present study is highly significant in the context of PBL, since PBLs are exposed to strong anti-HBs humoral immune response and HBV variants with G145R are capable of strongly neutralizing this immune response, without any compromise in the replicative competence, thereby ensuring viral perseverance[29,30]. Whatever is the source of genetic diversification, in the present work we describe a probable mechanism of RNA folding, through which divergent viral genomes/pregenomes having favourable mutations are selected for propagation.

We acknowledge that the RNA folding predictions are based on statistical/mathematical algorithms and the biological relevance of these predictions are based on their corroboration with the biological data. Interestingly, the results of the present RNA folding predictions beautifully elucidate the observed co-evolution of the mutations at positions 358 and 587, which supports the biological relevance of the observed predictions. The results of the present study further implies that, certain HBV mutations are selected at the subgenomic RNA level (as they are synonymous at the protein level), which may significantly alter the base pairing of the pgRNA, which in turn may hasten the selection of mutations at other sites. Interestingly, the mechanism suggested in this work is very much similar to the mechanism described for HBV genotype specific selection of the most widely studied HBV precore mutation (1896^{A}), which emerges to stabilize the stem-loop structure of the epsilon "ε" signal of pgRNA[31]. Altogether the present study, support the findings of Kidd-Ljunggren *et al*[16], that demonstrate the implications of genotype specific differences in the pgRNA secondary structures in the emergence of genotype specific variations in the HBV genome.

In conclusion, our results based on the predicted RNA secondary structures suggest the role of HBV genotype/subgenotype specific base pairing patterns of the pgRNA in selection/emergence of advantageous mutations. Furthermore, the observed association of a potent immune escape mutation with a particular HBV subgenotype, confined in a specific anatomic compartment indicate the possible mechanism of genotype/subgenotype specific compartmentalization of HBV, which may have important implications in extrahepatic maintenance and transmission of HBV through hitherto unknown routes.

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COMMENTS

Background

In our previous study we have observed highly compartment specific prevalence of hepatitis B virus (HBV) genotype A. In particular, HBV subgenotype A1 was detected in serum/plasma isolates, while HBV subgenotype A2 was predominant in the peripheral blood leukocytes (PBLs). Apart from subgenotype specific differences, G145R - a potent immune escape mutation was specifically observed in the PBL related subgenotype A2 isolates. The authors undertook this study to understand the possible mechanism of the compartment specific distribution of HBV subgenotypes and immune escape mutation.

Research frontiers

Compartment specific evolution and emergence of HBV mutations is a poorly understood area. A few recent studies, including the authors have provided evidences, indicating that HBV independently evolves in different anatomical compartments, depending upon the immune selection pressure on that compartment.

Innovations and breakthroughs

Using computational prediction methods, the authors show that the PBL specific emergence of G145R occurs in the context of HBV subgenotype A2, due to altered base-pairing patterns, as compared to the HBV subgenotype A1.

Applications

The findings of the present study are important in the long term persistence, evolution and transmission of different HBV genotypes/subgenotypes/mutations in different anatomical compartments. These findings have important implications in transmission of HBV.

Terminology

Compartmentalization: Compartmentalization is the process of compartment specific infection, evolution and persistence of viral variants (genotypes/subgenotypes) in different anatomically distinct sites. Due to difference in the immune selection pressure, viral variants with alterations advantageous under the given immune pressure are gradually selected, leading to their divergence from the circulating strains. Compartmentalization has been well studied in human immunodeficiency virus, hepatitis C virus, Epstein-Barr virus, *etc.* in comparison, studies on HBV compartmentalization are scanty and the mechanisms of emergence of mutations is poorly understood.

Peer-review

This study has shown that HBV subtype A1 and A2 have entirely different pgRNA secondary structure, which may explain compartment specific selection and preponderance of specific HBV subgenotype with unique mutational pattern. This study has novel findings.

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SYSTEMATIC REVIEWS

Geographic integration of hepatitis C virus: A global threat

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Author contributions: Daw MA designed the study, extracted the data, drafted and finalized the manuscript; El-Bouzedi AA, Ahmed MO, Dau AA, Agnan MM and Drah AM analyzed the data and contributed to the drafting of the data; all the authors read and approved the final manuscript.

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Data sharing statement: We confirm that the data is available from the corresponding author who will provide a permanent, citable and open access home for the dataset.

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Abstract

AIM

To assess hepatitis C virus (HCV) geographic integration, evaluate the spatial and temporal evolution of HCV worldwide and propose how to diminish its burden.

METHODS

A literature search of published articles was performed using PubMed, MEDLINE and other related databases up to December 2015. A critical data assessment and analysis regarding the epidemiological integration of HCV was carried out using the meta-analysis method.

RESULTS

The data indicated that HCV has been integrated immensely over time and through various geographical regions worldwide. The history of HCV goes back to 1535 but between 1935 and 1965 it exhibited a rapid, exponential spread. This integration is clearly seen in the geo-epidemiology and phylogeography of HCV. HCV integration can be mirrored either as intra-continental or trans-continental. Migration, drug trafficking and HCV co-infection, together with other potential risk factors, have acted as a vehicle for this integration. Evidence shows that the geographic integration of HCV has been important in the global and regional distribution of HCV.

CONCLUSION
HCV geographic integration is clearly evident and this should be reflected in the prevention and treatment of this ongoing pandemic.

Key words: Geo-epidemiology; Integration; Hepatitis C virus genotypes; Geography; Phylogeography

Core tip: Geographic integration of hepatitis C virus (HCV) is a newly described epidemiological phenomenon that is illustrated for the first time in this review article. The global burden of HCV infection has surpassed expectations and HCV genotypes are no longer restricted to certain countries or regions. All countries and their citizens are at a higher risk of HCV infection. HCV integration can be either intra-continental or trans-continental. Globalization, immigration and drug trafficking, in addition to the traditional HCV transmission factors, have acted as vectors for the geographical integration of HCV. International efforts and new strategies that go beyond borders should be combined to tackle this global threat.

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INTRODUCTION

Infection with hepatitis C virus (HCV) is a global public health threat that affects millions of individuals worldwide. In recent years, HCV has become one of the most important viruses. The global epidemic of HCV is distributed unevenly, with a high disease burden in low income regions and more than one-third of the estimated worldwide burden in the Western Pacific region. Approximately 32.2 million people have chronic HCV infection in Southeast Asia alone, Sub-Saharan Africa accounts for almost one-fifth of worldwide infections and over six million people are infected in Latin America[1].

Globally, about 27% of cirrhosis cases and 25% of hepatic cellular carcinoma cases are attributable to HCV[2]. Based on death certificate analyses, it has been estimated that there were about 3500 HCV-related deaths in France in 2001 and 15000 in the United States in 2007. In Egypt, there were an estimated 7379 HCV-related deaths in 1999 and the number is expected to more than double by 2020[3]. HCV-infected individuals have a 2.4 times higher risk of all-cause mortality compared to the non-infected population, 26.5 times the risk of liver-related mortality, and 1.8 times the risk of non-liver-related mortality[4].

Studies have reported an upsurge in the prevalence of HCV infection, particularly in developing countries and in some European regions. Southern provinces in Greece, Italy, France and Spain have reported higher levels of HCV infection (2%-7%) than in the North African nation of Libya[5]. Certain geographical spots in the Netherlands and Germany have a higher rate of HCV infection (7%) than other regions in the same countries[6].

The predisposing risk factors and modes of transmission of HCV have evolved in various ways in different parts of the world and this could have major implications for prevention programs[7]. However, many questions concerning the roles of risk factors and lifestyles that might be associated with the spread of HCV in different regions remain unanswered. Like some other important infectious diseases, HCV infection has been correlated with geographical, historical, social, economic and even political factors. Globalization and worldwide integration have added new epidemiological concepts that are clearly reflected in the prevalence of HCV worldwide. Neither HCV genotypes nor risk of exposure can be easily confined to certain regions or countries. Immigration, massive population displacement, unsettled conflicts and drug trafficking have aggravated the status of HCV infection and made it difficult to obtain a clear picture of HCV spread over the world. The objective of this review was to assess the worldwide geographical integration of HCV and its global evolution and to use the assessment to propose strategies to intervene in its spread.

MATERIALS AND METHODS

This study was conducted in four stages: (1) identification of the literature on HCV integration; (2) selection of relevant studies; (3) extraction of data; and (4) data sensitivity analysis. This review was designed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement[8].

Search strategy and literature review

Relevant studies were identified by searching PubMed, Scopus, Google Scholar and other databases using the following key terms: HCV, HCV integration, history, geography, epidemiology, phylogeography and evolutionary analysis. Some articles were also found by checking the lists of references in published papers. No language or time restrictions were applied.

Publication selection criteria

All identified abstracts were reviewed by two co-authors independently (Daw MA and El-Bouzedi AA or Ahmed MO/Agnan MM and Drah AM). They were considered eligible for full-text review if they provided accurate information on the geo-epidemiology of HCV and the distribution of genotypes. Data on evolutionary analyses were also included to estimate the dates of HCV origin and the temporal rates of virus spread from West Africa and China to Europe and North America.

Data abstraction and quality assessment

The data were independently abstracted by two co-authors (Daw MA with El-Bouzedi AA or Dau AA and Agnan MM or Drah AM). The studies were assessed using standardized data collection forms. The collected data include publication details, type of study and information on HCV transmission, the vectors involved, phylogenetic analysis, historical follow-up and evaluation criteria of the spread of HCV. If the evaluation results were controversial, a consensus among the authors was reached.

Data analysis

Data on the spatial spread of HCV was combined with the phylogenetic and epidemiological information to understand the dispersal of the virus worldwide. Furthermore, sensitivity analysis was conducted and the consistency of the data search results was evaluated.

Statistical analysis

A statistical review of the study was performed by a biomedical statistician.

RESULTS

Literature categorization and analysis of the publications

The publications were identified primarily by online searches. Unrelated studies were excluded based on the title or the abstract. The search results were categorized according to the historical, geographical, epidemiological and clinical parameters. Each of these fields was analyzed in the context of HCV integration.

History of HCV integration: Historical estimates have speculated about divergence time, distribution patterns and epidemic behaviors of HCV. Evolutionary analyses of the HCV genome have shed light on its epidemic history and transmission. Studies combining demographics with phylogeographic and molecular clock analyses have demonstrated the global dissemination of HCV[9,10]. The origin and evolution of HCV may date back to centuries ago in ancient China. An *et al*[11] recently estimated that the common ancestor of Chinese HCV variants (6 g and 6 w subtypes) isolated in Hainan Island dates back to between the sixth and ninth century. The authors speculated that the ancestors of a particular group of Austronesian-descended aborigines might have carried the earliest HCV-6 strains when they sailed to and settled in the Indochina peninsula in Southeast Asia, where HCV-6 is now indigenous.

Viral phylodynamic analysis indicates that HCV was disseminated in Africa before the rise of global travel and modern medicine[12]. It has been estimated that the most recent common ancestor of the CAR HCV-4 strains existed in the sixteenth century[13]. CAR HCV-4 strains spread rapidly and exponentially from 1935 to 1965, at about the same time as in Cameroon and Gabon. There is also epidemiological evidence of a wave of infection in Western countries during 1945-1965[14].

It has been suggested that colonization by European countries played a major role in the spread of these HCV genotypes, predominantly to the Americas but also to former colonial territories in Asia and Africa[14]. Global dissemination of HCV-2 seems to have been facilitated by the slave trade across the Atlantic and by colonization. During the intense period of slave trade (1700-1850), HCV-2 was disseminated from what is now Ghana/Benin to the Caribbean. HCV-2 also found its way from the Dutch colonies of Indonesia and Surinam to the Netherlands with the migration of Javanese workers to the Netherlands[14].

Epidemiological integration of HCV: The prevalence of HCV varies widely, from as low as 0.1% in certain Scandinavian countries to 23% in some African countries (Figure 1). The prevalence rate is classified as low (< 2.5%), intermediate (2.5%-10%) or high (> 10%). According to this classification, regions of low endemicity include North America, Europe, Australia and the Far East. Intermediate prevalence regions include some Mediterranean countries, the Middle East, Africa and South America. High prevalence countries are Egypt, Cameroon, Burundi, Rwanda, Gabon and Guinea in Africa, Bolivia in South America, and Mongolia in Asia[15]. However, 60% of all infected people are in Asia, particularly in its southern and eastern regions. China used to be considered a relatively high endemic area (average seroprevalence of HCV 3.2% in the general population)[16]. In Taiwan, the prevalence was estimated at 5.5%; it was 2.0%-14.2% in towns on the main island and higher (2.3%-26.4%) on the Penghu islands[17]. The overall prevalence in South Korea and Hong Kong is low (0.6%-1.1%) and is higher among females[18,19]. Japan has one of the highest endemic rates of HCV infection[20]. The prevalence rate of viral hepatitis in Southeast Asia is higher, where over 11 million people are estimated to have HCV. The prevalence of HCV infection among Malaysian adults has been estimated at 2.5%. Similar results have been reported from Indonesia, Cambodia, Thailand and the Philippines. However, it is less than 1% in Laos, Myanmar and Singapore, whereas the highest prevalence (> 6%) has been reported in Vietnam[21]. In India and Afghanistan, HCV prevalence ranged from 0.5%-1.5%. However, in Pakistan, about 6% of the population is suspected of having HCV infection and in some regions the estimated rate reached 31.9%[22-24].

In the Persian-Arab regions of West Asia, there are considerable regional differences in the prevalence of HCV. In Iran, the prevalence of anti-HCV antibodies in the general population ranges from 0.2% to 6.25% but the overall average is < 1%, which classifies the country as having a low frequency[25,26]. HCV prevalence was higher in Iraq (2.3%), Jordan (3.5%) and the Gaza strip (2.2%) but moderate in Lebanon (1%) and Syria (1%). There are no national studies on the prevalence HCV in the Arabian Peninsula and Yemen. HCV is considered

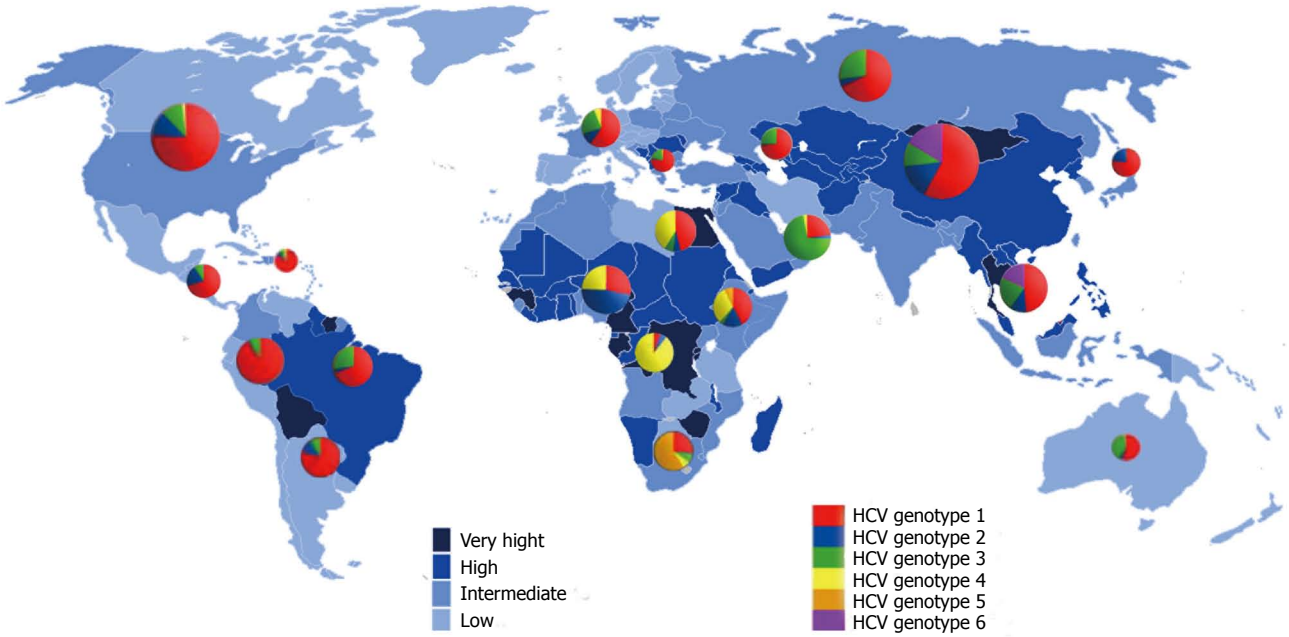

Figure 1 Overall prevalence of hepatitis C virus infections and the distribution of different hepatitis C virus genotypes worldwide. HCV: Hepatitis C virus.

endemic among the less populated countries, such as Qatar (6.3%), UAE (2.3%), Saudi Arabia (2%), Kuwait (1.8%) and Yemen (2.5%).

In Africa, > 28 million people have chronic HCV infection and future trends are difficult to predict[27]. Egypt has the highest prevalence in the world, with the prevalence rate estimated at 14.7% among people aged 15 to 59 years[1,28]. HCV has been well studied in the other North African countries, particularly in Libya. These countries are considered areas of low endemicity, with prevalence rates of 1%-1.5%[1,27]. In a recent study including 38 countries, seroprevalence was highest (after Egypt) in Central African countries, such as Gabon, Cameroon and Angola, and in some West African countries, such as Burkina Faso and Benin. The largest numbers of infected adults were in Nigeria, the Democratic Republic of Congo and Ethiopia[27].

In Latin America, it is estimated that 6.8 to 8.9 million adults are infected with HCV, of whom over 4 million are in Mexico and Brazil, which are the only South American countries that have carried out national population-based studies. The overall prevalence of HCV in Latin America is 1.5%; it varies from 0.1%-0.9% in Suriname, Chile, Peru, Venezuela, Panama and some other Latin American countries to 1.0%-3.4% in Brazil, Mexico and Argentina[29].

In Scandinavian countries, the spread of HCV infection started in the 1960s and peaked in the 1970s. Nosocomial and sexual transmission were minor routes and the main route was intravenous drug injection. The overall prevalence of HCV was 0.4%-0.6%, with higher rates among intravenous drug users and immigrants[30]. In central European countries, the prevalence of HCV was slightly higher than in the northern region. It varied from 0.6% in England, Luxemburg, Austria and Belgium to 1.5% in certain parts of Germany and Ireland[31]. High prevalence rates of HCV have been found in the southern European countries, particularly the southern regions of Spain, Portugal, Italy and Greece, which have the highest rates in Europe at 2.6%[32,33]. In Turkey, which is the European bridge to the oriental states, reported estimates range from 0.6% to 2.1%[34].

The HCV epidemic began later in Eastern European countries, particularly in the Czech Republic, Albania and Croatia. This delay is attributable to geographical barriers, limited immigration from neighboring endemic countries, and a delay in the increase in intravenous drug use[35]. The Czech Republic, Albania, Croatia, Estonia and Hungary are low-endemicity countries for HCV infection, where the prevalence rates range from 0.2% to 1%, but it was 1.4% in Latvia, Poland and Bulgaria. The highest prevalence was reported in Romania (3.3%), followed by Lithuania and Ukraine (2.3%). The Russian Federation and Baltic states have a high prevalence of HCV, ranging from 1.26% in the Republic of Belarus to 4.1% in some Russian states[36]. In the United States, Canada and Australia, the prevalence ranged from 0.61% to 1.8% and the number of people living with HCV is expected to continue to rise[37,38].

However, some studies indicated that there could be high rates of false positive results in HCV serological assays, as has been reported in Africa and China. In studies in Sub-Saharan Africa, such as in Uganda, Nigeria and the Republic of South Africa, the distinct variations in results were attributed to the wide variety of assays in use and to the different sample storage conditions[27,39]. Hence, future studies on HCV should give sufficient attention to testing strategies, sample handling and storage and include these details in the study reports.

Phylogenetic analysis of HCV genomes led to the

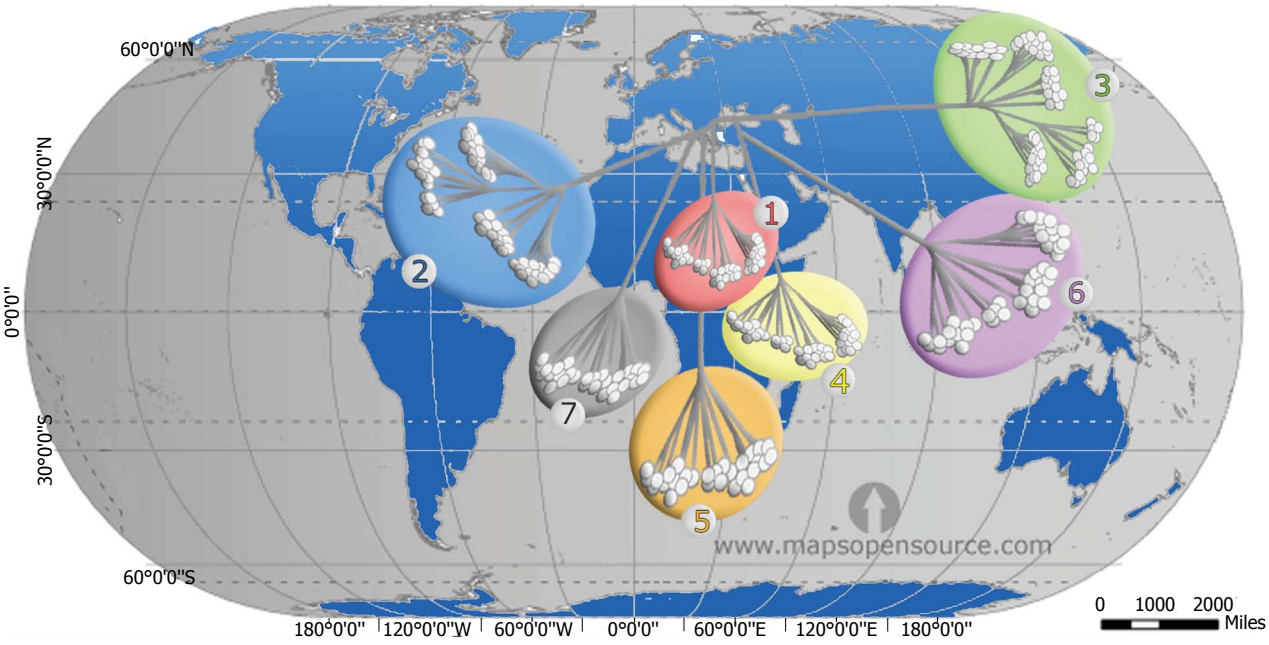

Figure 2 Geographical distributions of endemic pattern diversity of phylogenetic clades of hepatitis C virus (1-7).

development of a nomenclature for distinct virus types and subtypes, comprising seven recognized genotypes[1,7]. The genotypes differ at 30%-35% of nucleotide sites. The 7 genotypes are sub-classified into 67 subtypes plus 20 provisional subtypes. For strains of the same subtype, nucleotide differences are less than 15%[40,41]. However, while some genotypes are ubiquitous, others are found only in specific regions, where they exhibit a high diversity of subtypes (Figure 2). This distribution pattern and the antigenic and biological differences between the HCV types point to long periods of endemic infection during which there was no significant exchange with types from other regions[42].

Globally, genotype 1 accounts for more HCV infections (46.2%) than any other single genotype. Subtypes 1a and 1b account for 90% of all genotype 1 strains, at a ratio of 1:2 respectively. Over one-third of infections with genotype 1 are in East Asia. The next most common genotype is 3, which accounts for 30.1% of cases and is found mainly in southern Asia and in regions of Scandinavia. Genotypes 2, 4 and 6 are responsible for most of the remaining cases of HCV worldwide (9.1%, 8.3% and 5.4% of cases, respectively)[43]. Genotype 2 is predominant in West Africa, genotype 4 in Central and North Africa, and genotype 6 in Southeast Asia. Genotype 5 is responsible for < 1% of all HCV cases worldwide and is found mainly in South Africa. The more recently identified genotype 7 was isolated from a Congolese immigrant in Canada[40,44].

In China, genotype 1 is predominant (69.6%) and type Ib is more prevalent than 2a, whereas genotypes 3b and 6 are seen mainly in the southern provinces[45]. In India, the most prevalent genotypes are 3 and 1 (64% and 28% of HCV infections, respectively); genotype 1b is responsible for 16% of all infections, genotype 4a accounts for the remaining infections and the least prevalent is genotype 5 (< 1%)[46]. In Japan, 70% of infections are due to genotype 1b and 20% are caused by genotype 2a; the remaining infections are caused by genotype 2b. HCV genotype 6 is more geographically restricted than genotypes 1-3. It is found in parts of East Asia (South China, Hong Kong, Taiwan and Macao) and Southeast Asia (Singapore, Vietnam, Thailand, Indonesia and Burma). There is no information on HCV genotypes in the highly populated countries of Bangladesh, Malaysia and North Korea[47].

In most European countries, the most prevalent HCV subtype is 1b, although subtype 1a is more prevalent among patients co-infected with human immunodeficiency virus (HIV). In North America, parts of South America, United Kingdom, Scandinavia and Australia, 1a is the most prevalent subtype[42]. In Greece, Poland and the Netherlands, subtype 3 is responsible for 30% of all cases, and in Russia and the Baltic States, subtypes 1b and 3a share dominance[48]. It is noteworthy that the first HCV recombinant, RF2k/1b, was initially identified in Russia but since then has also been identified in Ireland, Estonia, Uzbekistan and Cyprus[49].

In Africa, genotypes 1, 2 and 4 appear to be endemic in regions of West and Central Africa and in the Middle East-North African region. Genotype 5 is more prevalent in southern and eastern Sub-Saharan Africa. Genotype 4 is the most frequent cause of chronic hepatitis C in the Middle East, North Africa and Sub-Saharan Africa[50]. In Egypt, 90% of all HCV infections are caused by type 4[51]. The emergent genotype 7, which originated from Central Africa, is phylogenetically very similar to genotype 2 variants. The greatest

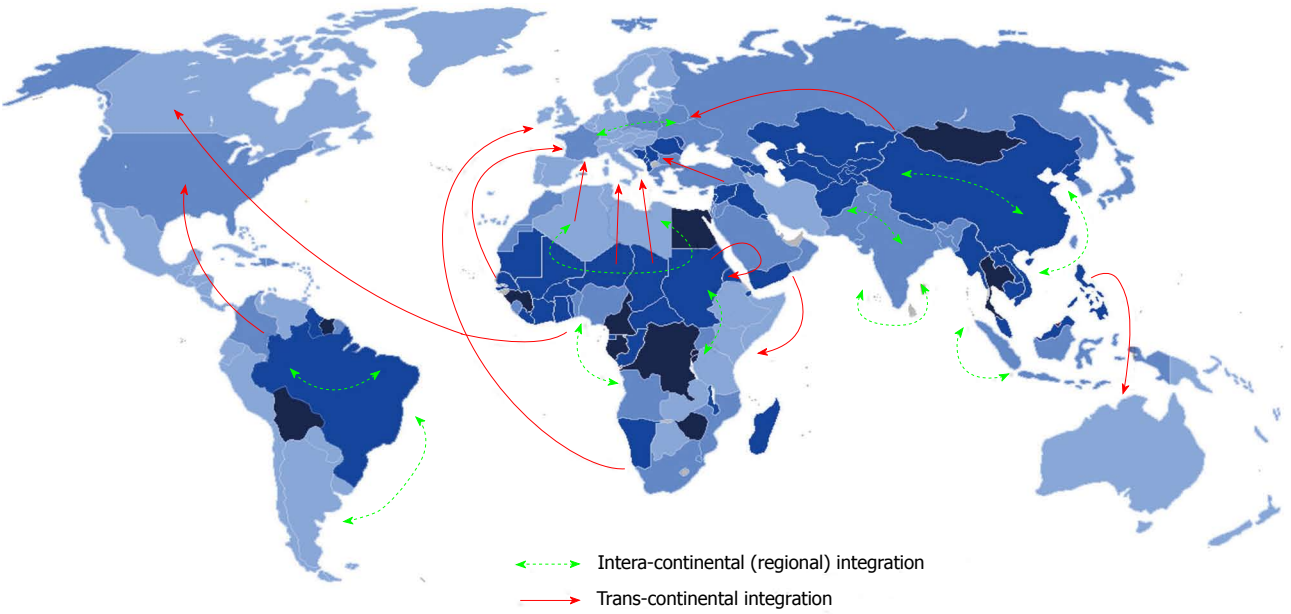

Figure 3 Plausible route of the integration of hepatitis C virus worldwide: The arrows indicate the probable route of spread (regional or transcontinental).

diversity of genotype 2 is observed in West Africa. It has been proposed that HCV genotype 2 originated from West Africa and then spread to the east[40]. Indeed, the finding of a new genotype further indicates the endemic nature of HCV in certain parts of Africa and again shows that HCV infection may have evolved from a common ancestor originating in this region of the world.

Geographic integration of HCV: The integration of HCV is influenced by host population size and density, the spatial distribution of the host, the frequency of contact between individuals, and other epidemiological factors. This is particularly evident in China and in many countries in Southeast Asia, as well as in Western Europe and Australia, possibly due to migration from Africa and/or Asia[45]. Endemic strains that are relatively rare have circulated for a long time in particular regions. Endemic strains of genotypes 1 and 2 are found mainly in West Africa, strains of genotype 3 in southern Asia, strains of genotype 4 in Central Africa and the Middle East, strains of genotypes 5 and 7 in southern Africa, and strains of genotype 6 in Southeast Asia. The global emergence of HCV has been either intra-continental or trans-continental (Figure 3). Nowadays, new strains of HCV have been reported in different regions and continents. They are due to surpass the commonly known strains resident in these places, particularly among high risk groups[52]. This evolving process of integration has played an important role in the spread of HCV and thus there is a need for developing specific strategies to combat the global spread of HCV infection.

Regional integration of HCV is seen in Africa and Asia. The endemic subtypes of HCV genotypes 1, 2 and 4, which are found mainly in geographically restricted areas of West Africa and Sub-Saharan Africa, are nowadays endemic in other parts of Africa. Egypt has the highest prevalence of HCV in the world (14.7%-32%) and has high rates of morbidity and mortality from chronic liver disease, cirrhosis and hepatocellular carcinoma. It is alarming that > 20% of Egyptian blood donors are seropositive for HCV. The dynamics of HCV in Egypt vary from one region to another. Desert areas have the lowest rates of anti-HCV positivity. The rates are higher in rural regions than in cities, and higher in the Nile Delta than in the Nile Valley[51]. Libya, neighboring Egypt, is considered an area of low endemicity for hepatitis C (1.2% average prevalence)[15]. The prevalence of HCV in Libya reaches its highest (1.6%) in the regions closer to Egypt and Sudan and lowest (0.2%) among other regions in the mid-coastal and western regions, where they resemble or are less than the rates in neighboring Tunisia[5]. The high prevalence rates in some parts of Libya could be due to its proximity to Sub-Saharan countries and the presence of large numbers of African immigrants. In the same way, the Albatnan region of Libya, which has a higher prevalence rate, borders Egypt, from where both legitimate and illegal workers come to Libya[5].

Most cases of HCV in Egypt are of genotype 4a. This strong homogeneity indicates epidemic spread of HCV. On the other hand, different genotypes were isolated from the Libyan population: Genotypes 1, 2, 3 and 4, as well as the newly emerged genotype 5. The prevalence of these genotypes in Libya varies from one region to another and is influenced by demographics and risk factors[53]. The dynamics of integration are applicable in Central Africa and East Africa, notably in Cameroon and Angola which have a high prevalence of HCV comparable to that in the Democratic Republic of Congo[54]. HCV seroprevalence was intermediate in the Horn of Africa: 2.7% in Ethiopia, 2.6% in Somalia and 0.3% in Djibouti[27]. In Southeast Asia, HCV genotype 6 integrated within northern countries, such

as Myanmar, Laos and Vietnam, while genotype 3 integrated in Thailand and Malaysia. In the island nations of Singapore, Indonesia and the Philippines, genotype 1 was the most prevalent. Similar integration dynamics have been observed in the Caribbean, India and the Baltic region[55].

The transcontinental integration of HCV is clearly mirrored between African and European countries, particularly around the Mediterranean regions of north African and southern European countries. The relatively high rates of HCV genotype 4 in southern Europe could be attributed to different factors: (1) the historic link between regions in southern Italy and Spain on the one hand and North Africa and the Middle East on the other hand; (2) the employment of multiple-use needles and glass syringes; and (3) the use of blood products that have not been tested for HCV. HCV genotype 4 seems to have recently spread from its endemic reservoir in Africa to southern Europe by immigrants. The prevalence rates of HCV type 4 have been rising in France, Italy, Greece and Spain[56]. In France, the prevalence of genotype 4 increased from 4% in 1990 to > 11% within one decade. In Europe, most patients infected with HCV genotype 4 are intravenous drug users or patients co-infected with HCV and HIV[57]. Recently, genotype 4 was shown to be the second most frequently detected genotype. One study identified genotype 4 in 23% of a large cohort of HIV-positive homosexual men from England, the Netherlands, France, Germany and Australia[58].

Another example of trans-continental integration is the existence of multiple types of "migrant clusters" of people who moved from West Africa to other regions of the world. These HCV types include the transfer of HCV-2e and 2f to Indonesia, HCV-2i to Morocco, France, Vietnam and Quebec, HCV-2j to Venezuela, HCV-2k to Martinique and France, HCV-2m to Vietnam, HCV-2r to Haiti and the Dominican Republic, and many unclassified type 2 lineages to Suriname[59]. The fact that genotype 2 is the most prevalent in West Africa, Europe, North America and parts of South America could reflect population dynamics resulting from the trans-Atlantic slave trade in the past and/or immigration, as illustrated by the identification of the new genotype 7 in Canada in an immigrant from Central Africa[40]. Figure 4 illustrates the integration dynamics of HCV genotypes all over the world. These trends clearly mirror the regional and global integration of HCV. Such a profile of interaction is associated with transmission and population dynamics of HCV and thus may reflect differences in when HCV infection occurred, which could influence the time of the peak burden of complications of HCV infection, such as cirrhosis and hepatocellular carcinoma. In view of these trends, regional differences in the prevalence of HCV genotypes and HCV epidemiology might have to be taken into consideration by tailoring prevention and treatment strategies to local needs.

Factors associated with HCV integration: HCV integration is a continuous dynamic phenomenon clearly influenced by population movements, demographic factors, clinical practice and personal behaviors in addition to the genetic entity of HCV. These global integration vectors play an important role in the spread of HCV worldwide. The outcomes of this integration coincide with the epidemiological evidence associated with immigration, trafficking and massive use of iatrogenic procedures and IDU, which escalated as early as the mid-twentieth century. The global prevalence of HCV is determined more by these social, behavioral and demographic factors than by genetic variation of the virus.

In 2013, 231 million people (3.2% of the world's population) migrated to new host nations. Migrants come mainly from developing countries in the south and migrate to the developed nations in North America and Western Europe. Migrants can be classified as immigrants, migrants and seasonal workers, refugees, asylum seekers, international students and others[60]. These newly emerged populations may suffer from infectious diseases usually more exotic or more prevalent in their own environment. These individuals come from regions in which HCV is endemic and thus pose a unique challenge to controlling the global prevalence of viral hepatitis[61]. A Canadian study from 2000 to 2007 compared immigrants with Canadian-born individuals and demonstrated high rates of HCV infection among immigrants. Compared to Canadian-born patients, immigrant patients were more likely to be female, non-white, older and to be infected with genotypes 4, 5 or 6[62]. United States studies on refugee populations found that the rates of HCV infection were up to 8% and only 1.8% among nationals[63].

One study estimated that 50% of HCV infections in the Netherlands are among immigrants, in whom the prevalence (2%) was tenfold higher than in the native population (0.2%)[61]. Likewise, the prevalence of chronic HCV in the United Kingdom among South Asians, and especially among migrants from Pakistan, may be as high as 2.7%, which is over fivefold higher than in the general population (0.5%)[64]. In Italy, the prevalence of HCV among Sub-Saharan refugees varied between 2.7% and 7.1%, while in Spain it was 12.5%, considerably higher than in the autochthonous population[65]. A recent study from Switzerland showed that the molecular epidemiology of HCV infection in low prevalence countries such as Switzerland is driven mainly by migration rather than by the distribution of virus genotypes in the native population[66].

The predominant HCV genotype in Middle Eastern and African countries is genotype 4. It is noteworthy that the prevalence of this genotype has been increasing in southern Europe, with region-specific increases in particular subtypes: 4a in Greece, 4d in Italy, 4c and 4d in Spain, and 4d in the Netherlands. The prevalence of genotype 4 among high-risk individuals in France increased from 15% of infections in 2003 to 22% in 2012. These changes in the trends of genotypes in these countries corresponds well with the increasing

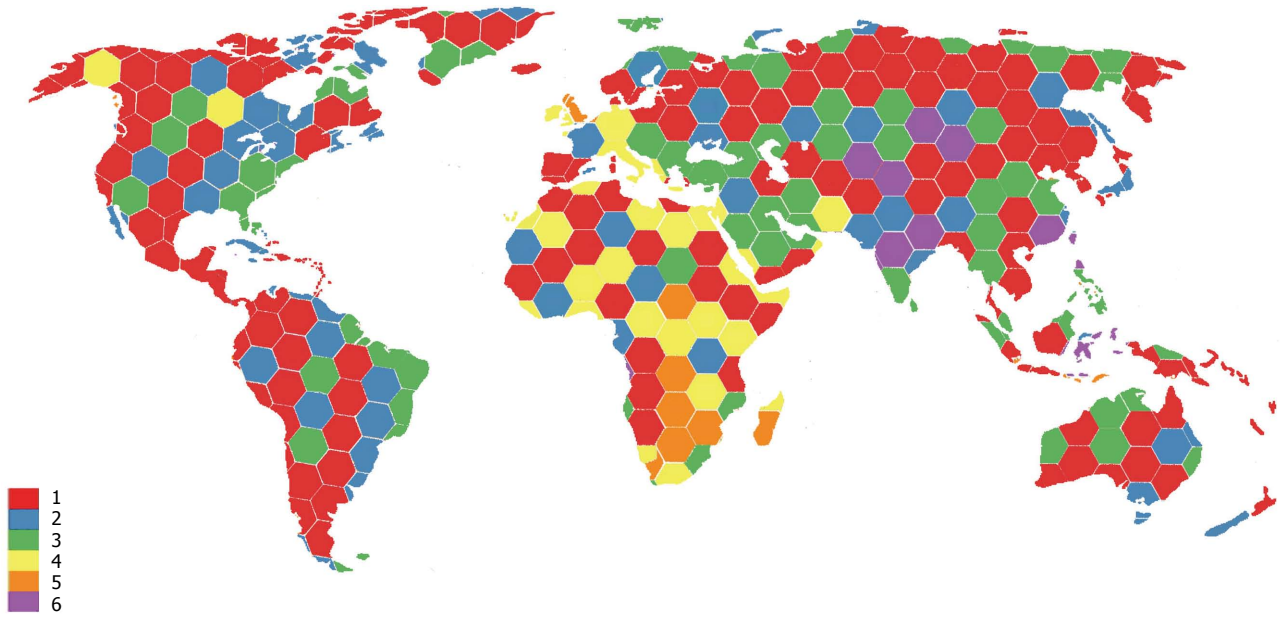

Figure 4 The integration complexity of hepatitis C virus worldwide: Virus genotypes mirroring the integration are shown in different colors.

numbers of migrants from Africa[64]. As the foreign-born populations expand, the integration dynamics of HCV will become globally imminent[67].

HCV transmission is closely associated with drug trafficking routes worldwide and HCV positivity is found among 15.6-98.7% of injection drug users. About 0.5% of the world's population injects drugs and of these about 6.8% are infected with HCV. Drug injection is the most common HCV transmission route and the main risk factor for acute and chronic hepatitis C (33.3% and 83.7%, respectively)[68].

The largest populations of people who inject drugs are found in China, Russia, the United States and Brazil, followed by Mexico, Pakistan and Thailand. People who inject drugs have a high seroprevalence of HCV in almost all European countries. This pattern is seen in Austria, Bulgaria, Cyprus, Greece and Romania and the rates are even higher in Latvia, Portugal, Turkey and Cyprus. The prevalence rate is < 30% only in the Czech Republic, Hungary and Slovenia. It is noteworthy that the rates of HCV seroprevalence have declined in Germany, France, the United Kingdom and Italy[69]. In most developing countries, transmission *via* drug injection is becoming more prevalent and replacing the iatrogenic and habitual transmission methods that have been reported for decades.

Intravenous drug use has become a predominant factor in the integration and transmission dynamics of HCV. This is clearly mirrored by the variations of the genotypes among these at-risk populations. The most commonly isolated genotypes worldwide from people who inject drugs are genotypes 1a and 3a. HCV subtype 3a is endemic in Southeast Asia and is spreading among intravenous drug users in the United States and Europe. An increase in the prevalence of genotypes 1a and 3a has been observed in Germany, France, Italy and Portugal. Mixed infections have been identified in some European countries: Italy (1b/3a), Germany (2a/3b) and Sweden (1a/1b)[70]. Eastern Europe (Russia and Estonia) and Central Asia have the largest drug epidemics globally, where the rapidly expanding HCV epidemic is associated with the injection of heroin and new synthetic or homemade drugs[71]. The frequency of genotype 3a is also rising in Eastern and Central European countries, as reported in Romania, Bulgaria, Poland and Serbia and Montenegro. It has been reported that people who use drugs in England are more likely to have genotype 3a in comparison to other risk groups, in whom genotype 1a is the most prevalent[71]. Thus, people who use drugs are important contributors to the spread of infections to the general population and drug trafficking is a key vector in the integration and diversification of HCV. This factor is boosted by other risk factors, such poverty, incarceration and HIV co-infection.

Worldwide, HCV and HIV are among the leading causes of death from infectious diseases[71]. Rates of co-infection with HCV and HIV range from 1.2% to 98.5% and co-infections are endemic, particularly in Asia and Africa[72]. In Europe and the United States, about one-fourth of HIV-infected individuals are co-infected with HCV. HCV infection outbreaks have been reported in HIV-positive men who have sex with men in North America, Europe and Asia. HCV co-infection reached up to 26.9% among heterosexuals compared with the HCV infection rate alone, which is reported to be 2.5%[1]. A cross-sectional study conducted between 2008 and 2010 in the Mazandaran province of Iran demonstrated that 33.8% of HIV-positive patients were co-infected with HCV and 25% were co-infected with both HBV and HCV[72]. The co-infection rates among intravenous drug users ranged between 58.2% and 91.6% and among commercial blood donors between 15.8% and

71.6%. These rates are higher than among those who become co-infected *via* sex (5.3%-20.0%). In Vietnam, between 89.8% and 98.5% of HIV-positive intravenous drug users are infected with HCV[73]. In China, 62.4% of HIV-infected individuals are seropositive for HCV. The co-infection rate is much lower in India (8.3%) and central, southeast and west African regions (4.9% to 8.5%) and much lower in North Africa (1.3%)[74].

Although HCV integration is clearly driven by migration, drug trafficking and HIV infection, there are many other additional factors: Health practices, hemodialysis, poverty, imprisonment and HBV/tuberculosis co-infections. A deeper understanding of HCV epidemiology should take into consideration all these factors. In developing countries, the routes of transmission of HCV are also found within medical care, such as the use of unsafe injections or improperly sterilized medical equipment, which are responsible for 40% of worldwide HCV infections, as well as the use of blood and blood products that have not been screened properly[1]. The prevalence of HCV infection in patients on maintenance hemodialysis reached 63% in the Arabian Peninsula (Kuwait, Saudi Arabia, Qatar and Yemen) and in China it was 41%. Moreover, hemodialysis patients who had blood transfusions were 5.65 times more likely to be infected with HCV than their counterparts who had no transfusions[1]. HCV co-infection among HBV-infected individuals ranged from 3% in Thailand to 22% in Japan, 23% in the United Kingdom and 30% in Spain[75]. HBV/HCV co-infection is more prevalent among the homeless, sexual assault victims and victims of intimate partner violence. Tuberculosis infection is also a major public health issue associated with HCV; it is responsible for more than a third of the opportunistic infections among drug users who are co-infected with HCV and HIV[75].

HCV infection can be acquired during travel for tourism or for medical treatment, particularly if the distant area is to the Indian subcontinent or Africa. Patients traveling abroad in general, and particularly if they are planning to have hemodialysis abroad, should be made aware of the risks and possibility of bringing new HCV genotypes to their homeland. Homosexuality is another prevailing factor for HCV transmission and outbreaks have been increasingly reported among men who have sex with men in Europe, Australia, Asia and the United States[76]. This is also emerging in developing countries, such as in the Arabian Peninsula, where such behavior is stigmatized. The spread of HCV may be influenced by habitual and social vulnerabilities between and within countries, urban and rural settings, and according to the burden of risk groups and economic status. This is clearly seen along the river Nile of Egypt and in China and Southeast Asian islands. Economic crises can influence HCV seroprevalence and the distribution of circulating genotypes. This was shown by outbreaks of HCV/HIV co-infections among intravenous drug users in Athens and Bucharest between 2011 and 2013, as both Greece and Bucharest have the highest unemployment rates among young people.

DISCUSSION

HCV is widely integrated, resulting in great heterogeneity both in the prevalence of infection and in the distribution of viral genotypes. This imposes a tremendous health burden globally in terms of morbidity and mortality. Hence, effective intervention requires a clear understanding of the dynamics of viral epidemics. Despite all scientific advances and the mounting knowledge of HCV, it remains "a hidden pandemic"[77]. HCV is considered to be endemic in developing countries, which have inadequate genotype data, and the largest populations of HCV-infected individuals are in Asia (accounting for 3.6% of the global population), followed by Africa (3.2% of the global population) and Latin America (1.4% of the global population)[15]. Most of these regions face many structural, cultural, societal and political obstacles in responding to this epidemic.

While preventive screening programs should be mandatory, 99 countries do not perform routine screening of blood donors for infectious agents that can be transmitted by transfusion. In other countries, the testing of blood donors for HIV, HBV and HCV is not consistent and attention is given mostly to HIV. Even where testing is done, there is preference for rapid assays with poor quality control and the result is lack of sensitivity. Testing based on nucleic acid is rare in countries with low or middle income due to lack of financial resources and skills. Even in Egypt, only 20% of the blood supply is tested by nucleic acid methods. Health authorities worldwide should give priority to this issue because any efforts to prevent or limit HCV transmission will not be effective unless the safety of blood and blood products is guaranteed. Moreover, most HCV infections are asymptomatic and in the absence of comprehensive, coordinated surveillance systems, the result is fragmented reporting and underestimation of the disease burden[1]. Improved surveillance is important not only for gaining a better understanding of the epidemiology of HCV but is also needed to identify population groups that should be targeted in prevention, testing and treatment programs.

Public healthcare systems, particularly in developing countries, should simplify service delivery to HCV-infected patients and specifically track progress to guarantee a high quality of services for both prevention and treatment[78]. People who inject drugs should have priority because prevalence estimates of HCV among this group is lacking in most countries. This is especially the case in countries with low or middle income, where data are scanty even for the general population[79]. Effective data collection and accurate reporting at the national level is the key in any program targeting HCV because it enables healthcare providers to implement policies targeting the populations that require the greatest attention.

Social and educational programs have to be promoted, particularly in countries that still ignore and stigmatize certain behaviors leading to HCV infection. All groups prone to HCV and associated co-infections should be included, whether they involve intravenous drug use, heroin sniffing or sexual promiscuity. Disseminating awareness of HCV infection in the general population can be beneficial in two ways. First, people who become aware exert pressure for provision of treatment. Second, promoting appropriate behavior helps to limit the risk of disease progression. Peer support has been proposed as one way to overcome these barriers. This is usually accompanied by screening as recommended by key United Nations agencies, such as the World Health Organization, United Nations Office on Drugs and Crime and Joint United Nations Program on HIV/AIDS. The high rates of HIV/HCV/TB co-infection in some settings indicate the need for another approach to increase access to HCV care, namely, to move towards an integrated policy resembling those used for HIV and tuberculosis[1,79].

A national action plan accompanied with national guidelines for the treatment of HCV infection should be advocated. Targeted populations should be provided with equal access to medical care, reliable supplies of medications and medical follow-up. Nowadays, it is possible to treat almost every person with HCV regardless of liver disease stage, viral genotype, past therapies and comorbidities. However, this approach imposes a heavy burden on the health system, particularly in developing countries where it is most needed. Research and operational projects supported by international funds should be established particularly in low-income countries[80].

Sensitivity and bias analysis

Sequential omission was performed for sensitivity analysis and we consider our data reliable. We observed no publication bias. The main limitation we noticed is the lack of specific data on certain aspects contributing to HCV integration, such as spatial and social factors associated with the global spread of HCV. Hence, further studies are needed to overcome such limitations.

Conclusion

From all that is described and discussed above, we conclude the following: HCV is an integrating dynamic threat and no country can be considered safe enough. Despite awareness of the spread of HCV infections, epidemiological data remain scarce. This study highlights the need for integrated cooperative actions at the local, regional and global levels if the spread and burden of hepatitis C virus are to be contained. Governments, the scientific community, industry and non-governmental organizations should develop a cooperation framework for combating HCV infection. Priority should be directed to help low or middle income countries to gain access to effective screening and medical care for HCV and associated infectious diseases.

ACKNOWLEDGMENTS

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COMMENTS

Background

Hepatitis C virus (HCV) infection is a major public health threat and its geo-epidemiology varies widely worldwide and over time. HCV genotypes and the risk of infection can be easily transmitted to any country and cannot be restricted to certain regions. Therefore, new evaluations of the epidemiology of HCV should be considered.

Research frontiers

Geographical integration is an epidemiological phenomenon that highlights the spread of HCV globally. Globalization, immigration and drug trafficking are important driving forces in this integration.

Innovations and breakthroughs

Worldwide HCV integration is a newly described phenomenon that can be either intra-continental or trans-continental.

Applications

Geographical integration should be taken into consideration in both prevention and treatment policies for HCV. National and international strategies should be designed on the basis of accurate analysis of HCV epidemiology.

Terminology

Intra-continental (regional) integration: The dynamic spread and dissemination of HCV genotypes within a certain region or continent is clearly evident in Africa and Asia; Trans-continental integration: The dynamic spread of HCV from one continent to another. This is clearly evident in the Mediterranean basin (Africa-Europe), Africa-America.

Peer-review

This is a very well-conceived, lucid, informative mini review that should be shared with the scientific community. The readers will gain valuable insight into the evolution of HCV at the global level.

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CASE REPORT

Spread of human immunodeficiency virus 1 among men who have sex with men is emerging as a genuine social concern and affecting the general populace - case reports from Eastern India

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Abstract

Human immunodeficiency virus (HIV) infection among men who have sex with men (MSM) has increased to a drastic proportion throughout India in the last couple of years due to a lack of productive identification and management framework. In apprehension of social disgrace these men attempt to live a normal hetero conjugal life and, in the process, act as a bridge in spreading the virus to their women partners. In this case report we have highlighted two cases which clearly distinguished the adequacy of HIV treatment among MSM when they are diagnosed during early or late phases of infection. An intensive and ample counseling to comprehend the psychology and sexual behavior of these men was found to be critically important in both the cases. Our study, which is actually the first of its kind, recorded and documented evidence of HIV infected MSM from Eastern India and renders a ray of hope among this marginally isolated group to comprehend the challenges and health risks faced by the MSM population. It also provides a format for the medical practitioners here in managing and treating related cases.

Key words: Human immunodeficiency virus; Men who have sex with men; Tuberculosis; Human cytomegalovirus

Core tip: The role of men having sex with men (MSMs) in the transmission and spread of the human immunodeficiency virus infection among the general population has been an active area of debate for the last few years. This case report highlights the grave weight of this issue from an Indian standpoint and describes the health risks and related treatment procedures concerning these men. Another major point touched by this article concerns those MSMs who in fear of social stigma and try to live a normal hetero conjugal life and in the process act as a bridge in spreading the virus to their women partners.

Chatterjee A, Sarkar A, Ansari S, Siddhanta S, Banerjee S, Sarkar R, Chakraborty N. Spread of human immunodeficiency virus 1 among men who have sex with men is emerging as a genuine social concern and affecting the general populace - case reports from Eastern India. *World J Virol* 2016; 5(4): 183-188 Available from: URL: http://www.wjgnet.com/2220-3249/full/v5/i4/183.htm DOI: http://dx.doi.org/10.5501/wjv.v5.i4.183

INTRODUCTION

Human immunodeficiency virus (HIV) infection among men who have sex with men (MSM) has been expanding drastically around the globe, especially in Asia. This worldwide pattern is being found in India, with the current evaluated HIV predominance among MSM extending somewhere around 7% and 16.5%[1]. This is an major cause of concern in light of recent HIV counteractive endeavours that have been drastically extended across the country, bringing up second thoughts about whether extra measures are required to capture the spread of HIV in this populace. In Mumbai, 12% of MSM looking for deliberate testing and medical advice were found to be HIV seropositive, while 18% of the MSM screened in Andhra Pradesh were observed to be infected[2]. In another study, it was discovered that close to 8% of the reported MSM's were seropositive[3].

Despite the fact that discoveries from the Independent Impact Assessment Study demonstrate that the National AIDS Control Program (NACP) has been consistently trying to end the HIV scourge in India over the period 2007-2012, current intervention measures for HIV transmission among MSM include single estimation modalities and thus fail to resolve the delicate problems associated with this socially marginalized group in India[4]. To deal with this issue, there is a requirement for far-reaching, multi-layered approaches that effectively counter the HIV aversion among these men. The idea concerning the sexual character of MSM in India can be fluctuating and fluid. Since people may change their self-perception with time and behaviours might be situational, crediting particular behaviours ascribed to these men is difficult and constraining. In the majority of these people, same-sex behaviour does not block having sex with women or taking part in conventional marriages. Thus the expression "MSM" is not used to depict someone's sexual identity but to identify his characteristic behavior.

An essential inconvenience in getting the related MSM information is that homosexuality is concealed in silence to a vast degree in India both on account of social standards and on the grounds that it is unlawful. Homosexuality in India was formally classified by lawful code Section 377 which, until recently, made sexual relations between two men a criminal offence. Endeavours are progressing to attempt legalization of homosexuality in India; however these have not been successful yet. In light of the fact that numerous MSM in India don't transparently share information on their sexual behaviour, this has brought about meager information about their sex conduct and its setting. Without this clear knowledge, it is hard to arrange successful MSM-related HIV counteractive action. With regards to this disproportionally abnormal state of HIV risk, it is critical to comprehend the socio-behavioural components that may worsen sexual danger among this population. MSM in India encounter different types of social and legal discrimination. It is this pervasive narrow-mindedness of society alongside the social pressure for men to take part in heterosexual marital relations that have driven numerous MSM to marry and have children. Numerous MSM participate in unprotected anal and vaginal sex with multiple male and female sexual partners. MSM in India may play a "connecting" role in the spread of HIV among the general population. Other studies have additionally discussed that disgrace and stigma contribute to the development of one's negative self-images and low self-regard, depression, expanded sexual risk behavior and/or diminished utilization of HIV prevention services. The silent riddle connected with institutional disgrace and separation may generate ideal conditions for the drastic acceleration of the AIDS epidemic. The social stigma among these people may arise from healthcare providers, employers and other administrative workers. These difficulties create genuine obstacles against successful HIV services procurement; segregation can hinder access to HIV and sexual healthcare administrations and relative prevention programs. A comprehensive understanding of the issues around disgrace and discrimination would help MSM cross the obstructions connected with disgrace concerning sexual risk, disclosure issues and access to human health services.

CASE REPORT

Case 1

A 40-year-old man suffering from severe diarrhoea, fever, drastic weight loss and nausea was admitted to the Department of General Medicine, Calcutta Medical College and Hospital, Eastern India. His HIV serostatus was ascertained by an ELISA (HIV ELISA, Rapid test)

and Western blot as recommended by the National Aids Control Organization (NACO), Ministry of Health and Family Welfare, Government of India. The fourth generation HIV detection test performed with the patient's blood confirmed severe HIV infection. HIV blood viral load was found to be > 1 × 10^5 copies/mL. $CD4^+$ T-cell count was 32 and $CD8^+$ T cell count was 516 (Ratio-0.06). The man was confirmed with a diagnosis of AIDS, the final stage of HIV infection. Upon rigorous interrogation the man confirmed that he was primarily diagnosed as HIV seropositive at another government hospital "a few years back" (no documentation) but he did not think of complying with the physician's advice or to follow up and lived a "normal" life. A thorough risk review and rigorous counselling revealed that the patient was homosexual and had sexual contacts (unprotected anal intercourse) with multiple men (probably the cause of HIV infection). But due to the fear of social stigma he never revealed his sexual preference or HIV status to anyone and continued to live a "straight" life with his wife as a heterosexual man. His wife was also diagnosed with HIV seropositivity.

Upon admission, the patient showed high fever with a significant evening rise, severe headache, rapid seizures, and drastic weight loss. The patient admitted to suffering from nausea, difficulty breathing, seizures and vomiting for quite a considerable time span. He also had a dry cough with mild expectoration and mild chest pain. The patient was mildly febrile with the presence of mild pallor, slightly anaemic, and had low blood pressure (106/58 mmHg) with a pulse rate of 110 beats/min. Clinical examination revealed acute lymphadenopathy.

On detailed examination, the man was found to have severe syphilis with chronic genital ulceration, acute pulmonary tuberculosis and human cytomegalovirus (HCMV) retinitis. HCMV IgM was detected in the patient' s blood, followed by confirmation of HCMV DNA by PCR detection. Real-time PCR estimated the viral load of HCMV to be 5.9 × 10^6 DNA copies/mL of serum. Indirect ophthalmoscopic examination revealed the presence of retinal haemorrhage with a hardened peripheral lesion, characteristic of HCMV retinitis. Cerebrospinal fluid culture, Mantoux test and sputum culture confirmed the presence of *Mycobacterium tuberculosis*. Test for cryptococcal capsular antigen turned out to be negative. Head CT scan of the patient revealed abnormal enhancement of the basal cisternae. Chest X-ray and bronchoscopy showed bilateral lung infiltration with non-specific diffused interstitial pneumonitis.

No clear evidence of cerebral palsy was observed at neurological presentation but slight neck rigidity was ascertained. A complete blood profile analysis of the patient was recorded and is provided in Table 1. The patient showed severe cachexia and very poor nutritional index with a BMI of only 15.8.

After confirmation, the patient was immediately put under highly active anti retroviral therapy (HAART) therapy (zidovudine, lamivudine and nevirapine) for 2 mo. After 4 wk of treatment and a limited $CD4^+$ count increase (CD4-54), the patient was immediately started on a combination based antitubercular drug (ATD) therapy as Directly Observed Therapy Short-course (DOTS - Cat 1 regimen) along with dexamethasone as corticosteroid therapy (0.4 mg/kg per day) and pyridoxine 40 mg/d on a planned 4 wk regime. Along with the ATD, the patient was also administered valganciclovir tablet 450 mg once daily as maintenance therapy against HCMV infection. For treating genital syphilis the patient was given an intramuscular injection of benzathine penicillin G (2.4 million units) once daily for 2 wk. But even after another 2 wk treatment, the patient failed to show any significant improvements with regard to his health conditions. He suddenly developed severe respiratory distress, spasms and his fever relapsed. He also complained of a gradual dimness of vision and total visual blurring. He was put under ventilation and immediate respiratory support. After few days he succumbed to the veracity of the infections and died due to multi-organ failure.

Table 1 A detailed clinical blood profile analysis of the two patients before start of treatment

Factors	Patient 1	Patient 2
CD4 count	32	232
CD4:CD8 ratio	0.06	0.25
HIV viral load	> 1 × 10^5 copies/mL	50000 copies/mL
Haemoglobin	9.1 g%	9.5 g%
TC	7100 cells/mm^3	7000 cells/mm^3
Neutrophils	48%	52%
Lymphocyte	34%	24%
Eosinophil	12%	2%
Monocyte	12%	2%
Basophil	2%	1%
Platelets	270000/mL	200000/mL
Blood sugar (fasting)	117 mg/dL	109 mg/dL
Urea	48 mg/dL	38 mg/dL
Creatinine	1.1 mg/dL	2.1 mg/dL
Bilirubin	0.6 mg/dL	1.2 mg/dL
SGOT	129 IU/L	119 IU/L
SGPT	54 IU/L	47 IU/L
Alkaline phosphatase	225 IU/L	185 IU/L
Albumin	3.9 g/dL	2.9 g/dL
Globulin	3.7 g/dL	3.5 g/dL

SGOT: Serum glutamic-oxaloacetic transaminase; SGPT: Serum glutamic-pyruvate transaminase.

Case 2

A 21-year-old man suffering from fever and flu-like symptoms along with severe diarrhoea and abdominal cramping was admitted to the Department of General Medicine, Calcutta Medical College and Hospital, Eastern India. His HIV serostatus was found to be positive by performing HIV ELISA, Rapid test and Western Blot as recommended by the NACO, Ministry of Health and Family Welfare, Government of India. The fourth generation HIV detection test performed with the patient's blood confirmed HIV seropositivity. HIV blood viral load was found to be 50000 copies/mL. $CD4^+$ T-cell count was 232 and $CD8^+$ T cell count was

920 (ratio-0.25). The man was diagnosed with acute or latent HIV infection. Upon rigorous counselling the patient admitted to being a homosexual and involved in unprotected anal sex with multiple male partners for quite a few years. He had never been sexually involved with any women. Due to the fear of social isolation, he never revealed his sexual preference to anyone. On detailed examination, he was found to be infected with genital syphilis and oral ulceration. Upon admission, the patient showed high fever, severe headache, seizures, vomiting and severe diarrhoea. He also had a dry cough with no expectoration at all but complained of a mild pain on the right side of the chest. The patient was slightly anaemic, and had low blood pressure (115/68 mmHg) with a pulse rate of 92 beats/min.

No evidence of cerebral palsy or any other neurological involvement was ascertained. There was no sign of lymphadenopathy. A complete blood profile analysis of the patient was recorded and provided in Table 1. The patient showed poor nutritional index. Liver function was found to be highly deranged with elevated levels of both SGOT and SGPT. Hepatic cholestasis was adequately observed by USG of the abdomen.

After confirmed HIV diagnosis the patient was started on HAART (zidovudine, lamivudine and nevirapine). Nevivir (200 mg) and lamistar were administered daily for 8 wk. Septran DS and feronia were given orally daily for 2 wk. After 2 mo the patient showed significant signs of improvement with much-relaxed breathing and no abdominal cramping. Cholestasis was found to be resolving gradually. $CD4^+$ cell count increased to almost double (CD 4-454) and HIV load in the patient's blood decreased uniformly (< 10000 copies/mL). As he responded actively without any genotoxic side effects towards the treatment, the anti-retroviral therapy was carried on and he was kept under observation. The doctors clearly made him understand the implications of latent HIV infection and transmission and also discussed the importance of regular medication to keep the disease under control.

DISCUSSION

In the latest United Nations General Millennium development agenda on HIV/AIDS-Goal 6, it has been reported that the percentage of individuals living with HIV/AIDS globally has diminished by 40% up till the end of 2013. However, in a study corresponding to the MSM population in India who had undergone HIV testing in the past 12 mo at different survey locations across the nation, variable results ranging from 3%-67% were observed[5]. In 2009, 46.3% of MSM in Tamil Nadu had tested positive for HIV while the HIV pervasiveness in a study from Mumbai was 12.5% with 14% of these men reporting STD side effects[6]. Only 68% of the positively tested MSM returned to gather their test reports. The above information demonstrates the inability and failure of the health councils of India to legitimately comprehend the requirements and problems of this socially marginalized group as well as the dereliction in garnering their trust[7]. HIV infection among MSM has been increasing in an exponential manner throughout India in the last few years due to the absence of efficient identification and productive management systems[8]. Because of the United Nations sustainable developmental goals (Goal 3), a much higher percentage of HIV-infected people are receiving antiretroviral therapy now.

In trepidation of social stigma, these MSMs attempt to render a normal hetero marital life and in the process act as a bridge in spreading the virus to their women partners. In this case report, we have highlighted two cases which obviously distinguishes the adequacy of HIV treatment among MSM when they are diagnosed during early or late phases of infection. A thorough and ample counseling to understand the psychology and sexual behaviour of these men were found to be vital in both cases. With the advent of highly active antiretroviral therapy (HAART), which is a customized combination of different classes of retroviral medications that a physician prescribes based on patient's viral load, the particular strain of the virus, the $CD4^+$ cell count, and disease symptoms etc treating HIV has become much easier. There is a partial recuperation of the host immune framework portrayed by a significant rise in the number of $CD4^+$ T lymphocytes and this leads to a decrease in AIDS-related mortality.

Early identification and appropriate treatment are of most extreme significance to battle HIV infection. Be that as it may, in a developing nation like India, subjects having a place with a lower financial strata with no or poor educational foundation and no knowledge of HIV/AIDS, are mostly diagnosed late over the span of the disease, just like the instance of the first patient whose HIV status was recognized surprisingly when his $CD4^+$ T-cell count had gone down too low. The vast majority of the patients (like in our case) intentionally ignore the doctor's advice and do not follow up routinely. Thus due to the absence of updated information, awareness and the fear of transcendent social disgrace, a large fraction of subjects are diagnosed very late. Many of them remain reluctant to approach a doctor about their condition and look for medical guidance and consideration[9]. The most imperative reason for mortality in the case of patients who have low $CD4^+$ T-cell number (or have developed AIDS) is the advancement of a few end organ diseases (EODs). These EODs are caused by different opportunist infections (OIs) which have been left untreated as an after effect of late HIV diagnosis inciting a progressive failure of the immune framework[10].

This case report ideally documents the medical conditions and disease transmission history of two HIV-1 infected homosexual men from Eastern India. The first patient was diagnosed very late with his CD4 T cell count plunging to as low as 32. Due to his own negligence and in fear of social stigma he never disclosed his sexual preference or HIV status with anyone. Despite having

multiple male sexual partners he continued to live with his wife and had sexual relations with her. As a matter of fact, he transmitted the virus to his wife. When he was admitted to the hospital he was suffering from multiple infections (both bacterial and viral), lying almost at the verge of death. HAART would have helped him if he had sought restorative medical help earlier.

Whether the cause be the narrow mindset of society with respect to homosexuals, misconstruing the graveness of the issue by the patient or insufficient knowledge among the people about HIV, ultimately the truth is that the clinical improvement of the subject could have been accomplished only if he had been treated earlier. The second patient, a young homosexual man, somehow comprehended the gravity of the circumstances and got himself diagnosed in a timely manner with complete support from his family. He was lucky enough that the HAART responded actively for him. He survived and hopefully will be cautious enough not to transmit the virus to anyone else. To address this serious issue the government should immediately develop a nationwide programme to screen all MSM for HIV and enroll the positive HIV cases for intense treatment.

Our study, which is actually a documented evidence of HIV-infected MSM from Eastern India, provides a ray of hope among this marginally isolated group in India to understand the difficulties and health risks faced by the MSM population and provides a format for medical practitioners in dealing with and treating related cases.

COMMENTS

Case characteristics

Case 1: A 40-year-old male suffering from severe diarrhoea, fever, drastic weight loss and nausea was admitted for treatment; Case 2: A 21-year-old man suffering from fever and flu-like symptoms along with severe diarrhoea and abdominal cramping was admitted for treatment.

Clinical diagnosis

Case 1: The patient showed high fever with a significant evening rise, severe headache, rapid seizures, drastic weight loss, nausea, difficulty breathing and vomiting. He also had a dry cough with mild expectoration and mild chest pain. The patient was mildly febrile with presence of mild pallor, slightly anaemic, and had low blood pressure (106/58 mmHg) with a pulse rate of 110 beats/min. Clinical examination revealed acute lymphadenopathy; Case 2: The patient showed high fever, severe headache, seizures, vomiting and severe diarrhea. He also had a dry cough with no expectoration at all but complained of a mild pain on the right side of the chest. The patient was slightly anaemic, and had low blood pressure (115/68 mmHg) with a pulse rate of 92 beats/min.

Differential diagnosis

Both patients were diagnosed with severe human immunodeficiency virus (HIV) infection. The first patient was in the final stage characterized by acquired immune deficiency syndrome, infected by other opportunist pathogens like syphilis, tuberculosis and human cytomegalovirus (HCMV). The second patient was in an early stage of HIV seropositivityand mainly suffering from bacterial lung infection.

Laboratory diagnosis

All labs were within normal limits.

Pathological diagnosis

HIV1 infection was diagnosed in both the patients using standard protocol by PCR, ELISA, 4TH generation test suggested by National Aids Control Organization. HCMV and *M. tuberculosis* were identified by ELISA, culture and PCR.

Treatment

Highly active anti retroviral therapy was given to both the patients. Valganciclovir was given to treat HCMV infection. DOTS was administered to treat tuberculosis.

Related reports

The role of men having sex with men (MSMs) in the transmission and spread of the HIV infection among the general population has been an active area of debate for the last few years. The main aim of this study is to share the actual scenario in an economically poor resource setting concerning homosexual men who, in fear of social stigma, try to live a normal hetero conjugal life and in the process act as a bridge in spreading the virus to their women partners.

Term explanation

MSM are highly prone to develop HIV infection due to unprotected sex, but in fear of social boycott suppress their identity thereby act as vectors to spread the disease among other individuals.

Experiences and lessons

In this article, authors have discussed two cases which clearly distinguished the adequacy of HIV treatment among MSM when they are diagnosed during early or late phases of infection. Thorough counseling to understand the psychology and sexual behavior of these men was found to be very important in both the cases. In fear of social stigma these men try to render a normal hetero conjugal life and in the process act as a bridge in spreading the virus to their women partners.

Peer-review

The manuscript is well written and well presented. In this article the authors presented 2 cases, one MSM who did not take HIV infection seriously and died. The second case was a young MSM, starting treatment led to an improvement in the patient's condition. The paper is suited well for publication in the journal.

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