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AIM AND SCOPE

World Journal of Hepatology (*World J Hepatol*, *WJH*, online ISSN 1948-5182, DOI: 10.4254), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJH covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology 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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EDITORIAL

All-oral interferon-free treatments: The end of hepatitis C virus story, the dream and the reality

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Abstract

The year 2014 marked the beginning of the end of the interferon era and the triumph of the all-oral interferon-free regimens for treatment of hepatitis C virus (HCV) infection. These innovative therapies are safe and yield a cure rate of over 90%. The scientific hepatology community is euphoric about the possibility of elimination and even eradication of HCV infection. However, the current high cost of the new all-oral regimens allows access to treatment only for a restricted number of HCV-infected patients. In addition, many other conditions such as modality of access and delivery of care, inadequate knowledge of HCV epidemiology and political commitments to be undertaken, hamper the fulfillment of the dream to eliminate the virus. Since, such conditions are not impossible to overcome, a global urgent effort must be made to allow a widespread access to the new treatments which will permit in the next years to avoid million of HCV-related deaths.

Key words: Sofosbuvir; Simeprevir; New-oral hepatitis C virus treatments; Paritaprevir; Dasabuvir; Daclatasvir; Ledipasvir; Ombitasvir

Core tip: It is begun the era of all-oral direct-acting antiviral drugs for hepatitis C virus (HCV) treatment allowing interferon-free therapeutic regimens. These regimens are safe and yield cure rate greater than 90%. The therapeutic success has posed the basis for HCV elimination, although, confirmation are waiting from real world. However, many conditions hinder the fulfillment of the dream. These conditions are the excessive cost of drugs, the access and delivery of care, the epidemiology of HCV and the political commitments. Thus, an urgent effort must be done to make accessible on large-scale all-oral anti-HCV therapies allowing saving millions of lives.

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MILESTONES THAT MARK HEPATITIS C VIRUS THERAPEUTIC EVOLUTION

In the years 2014-2015 the scientific hepatology community virtually laid the milestone that could mark the beginning of the end of the hepatitis C virus (HCV) story. The milestone marked the advent of the first new oral drugs approved for HCV treatment, namely sofosbuvir and simeprevir, which were quickly followed by several others allowing interferon (IFN)-free therapeutic regimens. These innovative therapies, which yield a cure rate of over 90%, have been approved in many regions of the world, including Europe and the United States. However, success did not come overnight but was the result of a step-by-step escalation in both understanding the molecular mechanisms of HCV replication and in the therapeutic approaches which started at the end of the 1980s, before the discovery of HCV, with IFN alpha affording a response rate of less than 15%[1]. The addition of ribavirin to IFN increased the success rate to about 30%, and then treatment moved into the era of the long-acting pegylated IFNs (pegIFNs). PegIFN plus ribavirin was the standard therapy for about 10 years during which the overall therapeutic success increased to 50%[2]. However, IFN-based treatment was associated with a significant number of severe side effects that limited its use considerably. Many other factors conspired to limit the therapeutic response, further hampering the use of IFN. These included host factors, such as age, body mass index, level of liver fibrosis and steatosis, insulin resistance, ethnicity, the genetic background such as the polymorphism of interleukin-28B, and viral factors, namely HCV genotype and HCV RNA levels. Furthermore, IFN-based regimens required a long therapeutic course, which reduced the adherence to treatment.

The year 2011 marked the beginning of the era of oral direct-acting antiviral drugs (DAAs). The scenery was complex with the first-generation DAAs, the NS3-A4 protease inhibitors boceprevir and telaprevir, which were approved in combination with pegIFNs and ribavirin for HCV genotype 1 treatment[3]. Although this triple therapy afforded a higher sustained virological response (SVR) rate than pegIFN plus ribavirin, the treatment was short-lived because of the dependence on IFN due to the low barrier to resistance of these first-generation DAAs. Moreover, the use of these DAAs was aggravated by a high rate of serious adverse events and by the scanty possibility to treat patients with advanced liver disease. Fortunately, the research moved fast and new oral HCV DAAs were devised, several of which had proved highly effective in a combined regimen, and finally approved for use in clinical practice (Table 1). Several other DAAs are currently in the drug development pipeline. Figure 1 shows the crucial milestones in the chronic hepatitis C treatment evolution, from IFN-based regimens to all-oral IFN-free combinations.

The first therapeutic strategy with the new DAAs approved in 2014 was pegIFN plus ribavirin plus sofosbuvir, a nucleoside analogue acting as a false substrate for HCV to stop viral RNA synthesis. Sofosbuvir is active against all HCV genotypes and has a high barrier to resistance. A phase Ⅲ trial[4] using the triple regimen showed an SVR of 89% and 96% for HCV genotype 1 and 4, respectively, and a phase Ⅱ trial[5] in treatment-experienced patients showed an SVR of 96% and 83% in genotype 2 and 3, respectively.

In the same year, based on the data of phase Ⅲ studies[6,7], another triple regimen with pegIFN plus ribavirin and simeprevir, a first-generation NS3-4A protease inhibitor, was approved for the treatment of HCV genotype 1 and 4. The two trials reported an SVR of 80% and showed that in HCV genotype 1a carrying the mutation Q80K in the NS3 protease sequence the response rate was significantly impaired.

The year 2014 also marked the beginning of the end of the IFN era and the triumph of the all-oral IFN-free regimens, which were highly effective and safe and afforded treatment also for patients with advanced cirrhosis. Phase Ⅲ studies[8,9] showed that the combination sofosbuvir plus ribavirin for 12 wk for HCV genotype 2 achieved an SVR from 88% to 100% depending on whether the patients were treatment-naïve or -experienced and on the absence or presence of cirrhosis, and for 24 wk for genotype 3, achieving an SVR from 60% to 94%, with the poorest results in therapy-experienced cirrhotic patients. The treatment of genotype 3 can be shortened to 12 wk by adding pegIFN to sofosbuvir and ribavirin. A phase Ⅲ trial[10] showed that the combination sofosbuvir plus daclatasvir, a "pan-genotypic" first-generation NS5A inhibitor, taken for 12 wk produced an SVR in genotype 3-infected patients of 90% for previously untreated patients and 86% for prior non-responders. Thus, at present, this combination seems to be the best option for the treatment of genotype 3 infection. The combination sofosbivir plus daclatasvir also showed good results in HCV genotype 1, 2, 4, 5 and 6.

The regimen sofosbuvir plus simeprevir has been approved for treatment of HCV genotypes 1 and 4. A phase Ⅱ study[11] showed an SVR of 93%-96% in treatment-naïve and -experienced patients treated for 12 wk. For HCV genotype 1a the Q80K substitution did not seem to influence the response rate of the sofosbuvir and simeprevir combination.

In early 2015 the combination of sofosbuvir and ledipasvir, a first-generation NS5A inhibitor, in a single pill to be administered once a day was available for the treatment of HCV genotypes 1, 3 and 4. The SVR after 12 wk of treatment was as high as 99%[12,13]. Importantly, it was shown that the treatment could be shortened to 8 wk with an SVR of 94%[14]. Patients with genotypes 5 and 6 have also been treated successfully.

The first IFN-free triple combination approved for HCV genotype 1 was ritonavir-boosted paritaprevir and ombitasvir in one pill, and dasabuvir. Several phase Ⅲ studies[15-18] showed an SVR from 95% to 100%. High SVR rates were also reported for patients with HCV

Table 1 Direct-acting antiviral agents approved for the treatment of hepatitis C virus in interferon-sparing regimens

DAAs	Class	Generation	Activity
Simeprevir	NS3-4A	Second-wave, first generation	Genotypes 1 and 4
Paritaprevir	NS3-4A	Second-wave, first generation	Genotypes 1 and 4
Sofosbuvir	Nucleoside/nucleotide analogues	Nucleotide analogue	Pangenotype
Dasabuvir	Non-nucleoside inhibitors of the HCV RNA-dependent RNA polymerase	Palm domain Ⅰ inhibitor	Genotype 1
Daclatasvir	NS5A inhibitors	First-generation	Pangenotype
Ledipasvir	NS5A inhibitors	First-generation	Genotypes 1, 3, 4, 5, and 6
Ombitasvir	NS5A inhibitors	First-generation	Genotypes 1 and 4

DAAs: Direct-acting antiviral agents; HCV: Hepatitis C virus.

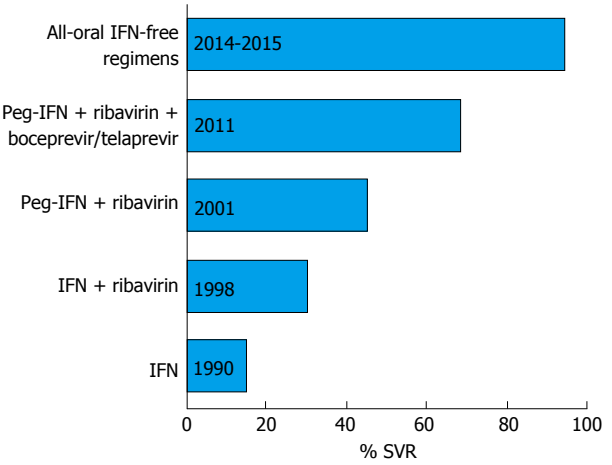

Figure 1 Milestones in the chronic hepatitis C treatment evolution, from interferon-based regimens to all-oral interferon-free combinations, and the respective sustained virologic response rates. IFN: Interferon; SVR: Sustained virologic response.

genotype 4 without adding dasabuvir.

All the above-mentioned data are awaiting solid confirmation from the real world. The early real-life data presented seem to confirm the trial results, albeit with a slightly lower SVR[19]. Further studies are necessary to evaluate the effect of treatment in patients with advanced liver disease as well as on the development of hepatocellular carcinoma (HCC). To this regard, preliminary studies seem to indicate that cirrhotic patients should undergo a close follow-up after HCV clearance by DAAs due to the residual possibility of developing HCC. In addition, considering the widespread use, the possibility to shorten the treatment duration should be evaluated.

Overall, the data on safety seem to indicate good tolerability for all of the above combinations. Moreover, it seems that there are no absolute contra-indications to the use of these approved DAAs. However, it is necessary to be prudent with the use of sofosbuvir for patients with severe renal impairment. In addition, the triple combination of ritonavir-boosted paritaprevir, ombitasvir, and dasabuvir has not yet been adequately evaluated in patients with Child-Pugh B decompensated cirrhosis and is contraindicated in patients with Child-Pugh C decompensated cirrhosis. Furthermore, the pharmacokinetics and safety of simeprevir for decompensated cirrhosis have not yet been fully evaluated, and it induces increased bilirubin levels in about 10% of cases. At present, considering the scanty data on the safety of DAAs in advanced liver cirrhosis, they must be used with extreme caution in this subgroup of patients. Moreover, if DAAs are administered to patients taking other drugs, it is mandatory that the drug-to-drug interaction be taken into account[20].

CRITICAL POINTS THAT HAMPER THE FULFILMENT OF THE HCV ELIMINATION DREAM

On the basis of the solid data from the trials on the efficacy and safety of all-oral INF-free combinations, the scientific community is euphoric about the elimination and even eradication of chronic hepatitis C. Therefore, now that we have a cure, the question is: In the real world, can we meet the conditions to make the dream come true? At the present, considering that HCV infection and its treatment are tremendously complex situations, the answer cannot be directly affirmative because of the many conditions, not impossible to overcome, that hamper the fulfillment of the dream. These conditions are linked to the elevated cost of drugs, to the access and delivery of care, to the epidemiology of HCV and to the political commitments to be undertaken.

The current high cost of the new all-oral regimen allows access to treatment only for a restricted number of HCV-infected patients; at present, many countries have chosen to treat only patients with advanced liver diseases. The high treatment costs can only be justified by the need to recuperate the research and development costs of the drugs; it has been estimated that the predicted manufacturing costs of HCV DAAs range from United States $100 to $270 per person for a 12-wk treatment course[21]. Thus, action strategies should be evaluated with the aim to reducing the price of the new drugs and allowing extensive access to the

new treatments not only in high- but also in middle- and low-income countries, where the highest prevalence of HCV infection is concentrated. By extending access to treatment, a double advantage can be achieved: First, it will enable the drug companies to recover their costs sooner, and second, it will allow substantial individual and public health benefits by reducing both the hepatic and extrahepatic HCV-associated pathologies and the spread of HCV infection worldwide. Global negotiations should be undertaken to include state authorities, drug companies, healthcare authorities and the scientific communities to reduce the cost of treatment, thereby extending access to more patients and preventing the spread of HCV infection. In this respect, the policies adopted by Egypt and India have obtained a price reduction that will allow widespread treatment of HCV-infected patients. Alternatively, it will be necessary to wait 15 years for generic drugs, the time required for drug patents to expire. If this should occur, the question that everyone should ask themselves is: Is it ethical to let someone die of hepatitis C when effective treatment is available but is inaccessible due to the high cost, especially since we know that treatments can be produced at a very low cost?

At present, of the estimated 150-200 million HCV-infected subjects worldwide, only the tip of the iceberg is really known. Consequently, if we presume today that the drug costs could be reduced, we would be able to treat only the prevalent cases of HCV. To achieve the elimination of HCV infection another two conditions have to be considered, the cases that are unaware of their HCV status and the incident cases. As a substantial number of countries do not have a screening program, it is critical to start adequate screening programs to identify subjects to be treated. Furthermore, the spread of HCV infection must be taken in hand to identify incident cases. Therefore, marginalized populations, drug users and intra-family spread of the infection must be monitored. To this regard, the current costs for both the epidemiological and pre-treatment screening is high. To ensure widespread treatment with the new HCV DAAs it is necessary to reduce the costs of diagnostic tests and, perhaps, determine only the HCV genotype to detect viremic subjects and decide the best therapeutic approach.

A further critical point is the delivery of treatment. At present, due to the high cost and the complexity of therapy, treatment is confined to specialist care centers to administer the drugs. However, for several reasons, these centers are not accessible to all infected subjects and in order to implement a successful HCV elimination program, non-specialists in primary care settings could deliver treatment. Due to the complexity of treatment, to be sure that an effective regimen is delivered, specialist supervision in the primary care should be given. Accordingly, Project ECHO could be a model for the management of HCV[22,23] where the decisions on treatment could be made using communication technologies.

CONCLUSION

The fantastic race for effective treatment for HCV has reached the final straight and the finishing line that marks the fulfillment of the dream can be clearly seen. Although the reality has exceeded all expectations, the Healthcare Authorities do not appear to be ready to take on the challenge for victory. One analysis suggested that it is possible to achieve the elimination of HCV by 2030 if screening programs to identify the HCV-infected populations are implemented and if active management with new oral anti-HCV therapies is made accessible on a large scale[24,25]. However, time is running out and there is an urgent need to reduce liver transplantation, considering that HCV infection is the leading cause of liver transplants, and to save millions of lives. To this regard, it is important to underscore that the current HCV-related death rate ranges from 350000 to 500000 people per year[26]. Furthermore, a recent analysis showed that HCV-related morbidity and mortality are expected to increase in the next 15 years[25], and it is estimated that among the current chronically HCV-infected population with no liver cirrhosis, the HCV-related deaths will peak in 2030-2035[27]. Now that we are in the final straight of the extraordinary race for effective therapy, a concerted effort must be made by the state authorities to press for a reduction in the cost of the drugs and by the scientific communities to improve the knowledge on HCV epidemiology and to plan strategies for the access and delivery of therapies. Widespread access to the new treatments will allow the dream to become reality.

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REVIEW

Monoclonal antibodies: Principles and applications of immmunodiagnosis and immunotherapy for hepatitis C virus

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Abstract

Hepatitis C virus (HCV) is a major health problem worldwide. Early detection of the infection will help better management of the infected cases. The monoclonal antibodies (mAb) of mice are predominantly used for the immunodiagnosis of several viral, bacterial, and parasitic antigens. Serological detection of HCV antigens and antibodies provide simple and rapid methods of detection but lack sensitivity specially in the window phase between the infection and antibody development. Human mAb are used in the immunotherapy of several blood malignancies, such as lymphoma and leukemia, as well as for autoimmune diseases. In this review article, we will discuss methods of mouse and human monoclonal antibody production. We will demonstrate the role of mouse mAb in the detection of HCV antigens as rapid and sensitive immunodiagnostic assays for the detection of HCV, which is a major health problem throughout the world, particularly in Egypt. We will discuss the value of HCV-neutralizing antibodies and their roles in the immunotherapy of HCV infections and in HCV vaccine development. We will also discuss the different mechanisms by which the virus escape the effect of neutralizing mAb. Finally, we will discuss available and new trends to produce antibodies, such as egg yolk-based antibodies (IgY), production in transgenic plants, and the synthetic antibody mimics approach.

Key words: Hepatitis C virus; Monoclonal antibodies; Immunodiagnosis; Immunotherapy

Core tip: The monoclonal antibodies (mAb) of mice are predominantly used for the immunodiagnosis of several viral, bacterial, and parasitic antigens. Human mAb are used in the immunotherapy of several blood malignancies, such as lymphoma and leukemia, as well as for autoimmune diseases. In this review, we discuss methods of mouse and human monoclonal antibody production. We will demonstrate the role of mouse mAb in the detection of hepatitis C virus (HCV) antigens as rapid and sensitive immunodiagnostic assays. We will also discuss the role of HCV-neutralizing antibodies in the immunotherapy of HCV infections and in HCV vaccine development.

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INTRODUCTION

Monoclonal antibodies (mAb or moAb) are monospecific antibodies that have the ability to bind to the same epitope[1]. These antibodies are made by homogeneous hybrid cells (B cells) that are each clones of the same origin parent cell. Polyclonal antibodies, on the other hand, are made of several different immune cells (B cells). Hybridomas are hybrid cell lines that are generated through the fusion of an antibody-producing B cell with a myeloma (B cell cancer) cell. Myeloma cells are characterized by the ability to grow in tissue cultures and the absence of antibody chain synthesis. All antibodies produced by these hybrid cells (using hybridoma technology) are of a single specificity, and therefore mAb. The establishment of cell lines producing mAb was first reported by Köhler *et al*[2] in 1975.

The hepatitis C virus (HCV) is a major health problem worldwide. According to the estimates of the World Health Organization, this virus infects more than 180 million people across the world (representing 2%-3% of the world's total population)[3,4]. HCV (genotype 4) is one of the major health issues in Egypt, infecting 22% of the country's general population[3,5-7]. HCV is a small-enveloped, single-stranded RNA virus belonging to the family Flaviviridae. The genome encodes a single polyprotein that is co- and post-translationally processed into four structural and six non-structural proteins[4,8]. This is done by different cellular- and viral-encoded proteases. The envelope glycoproteins E1 and E2 are two structural proteins located on the surface of the HCV, and hence play a crucial role in HCV entry into hepatocytes. Their presence on the surface of the virus makes these two proteins, particularly E2, a major target for HCV antibody neutralization and interaction with host cellular receptors[3,8].

PRODUCTION OF MOUSE MAB

Mouse mAb by hybridoma technology

In 1975, Köhler *et al*[2] developed a hybridoma method for the production of mAb. The persistence of antibody-producing cells *via* their fusion with tumor cells may be an obvious procedure today, but at the time, this procedure was regarded as a key innovation that would allow for the unlimited yield of a specific antibody molecule. In 1984, Köhler *et al*[2] were awarded the Nobel Prize in Physiology or Medicine "for theories concerning the specificity in development and control of the immune system and the discovery of the principle for production of mAb. While mAb are now long-established as vital research products, their therapeutic use requires further development, particularly in terms of the humanization of mouse antibodies and recombinant productivity protocols. Several hundreds of mAb are currently under evaluation for the treatment of a broad range of conditions and use within a variety of therapeutics on the market[9]. The principle production of mouse mAb by hybridoma is shown in Figure 1. The different types and applications of mAb as diagnostic and therapeutic applications are presented in the Figure 2.

Hybridoma cell production has conventionally been performed *via* cell fusion between spleen cells (B cell source) and myeloma cell lines by chemical fusion techniques using for example polyethylene glycol (PEG). A recent publication by Kandušer *et al*[10] in 2014, however, describes another technique for cell fusion based on electrofusion. This technique is superior to the PEG method due to its high fusion efficiency. Kato *et al*[11] have stated yet another technique that involves CpG oligodeoxynucleotide (CpG ODN) for cell activation prior to electrofusion. Kato *et al*[11] reported that CpG ODN stimulation not only increases fusion efficiency but also the number of antibody-producing cells, leading to an increased number of positive clones obtained.

Rat and rabbit mAb can be produced by the hybridoma technology using rat and rabbit spleen cells, respectively. A recent study[12] generated rat hybridoma clones *via* the cell fusion of immunized rat spleen cells with mouse myeloma SP2/0 cells and screened the generated antibodies using recombinant mouse CXCL4 and rhCXCL4. This study concluded that the CXCL4 signaling pathway is a potential therapeutic target in numerous diseases including cancer. In addition, Zhang *et al*[13] used rabbit hybridoma to produce highly sensitive rabbit mAb targeting an emerging cell surface in mesothelioma and other solid tumors (Mesothelin). They concluded that the generated rabbit mAb may be promising candidates for monitoring and treating mesothelioma and other mesothelin-expressing cancers.

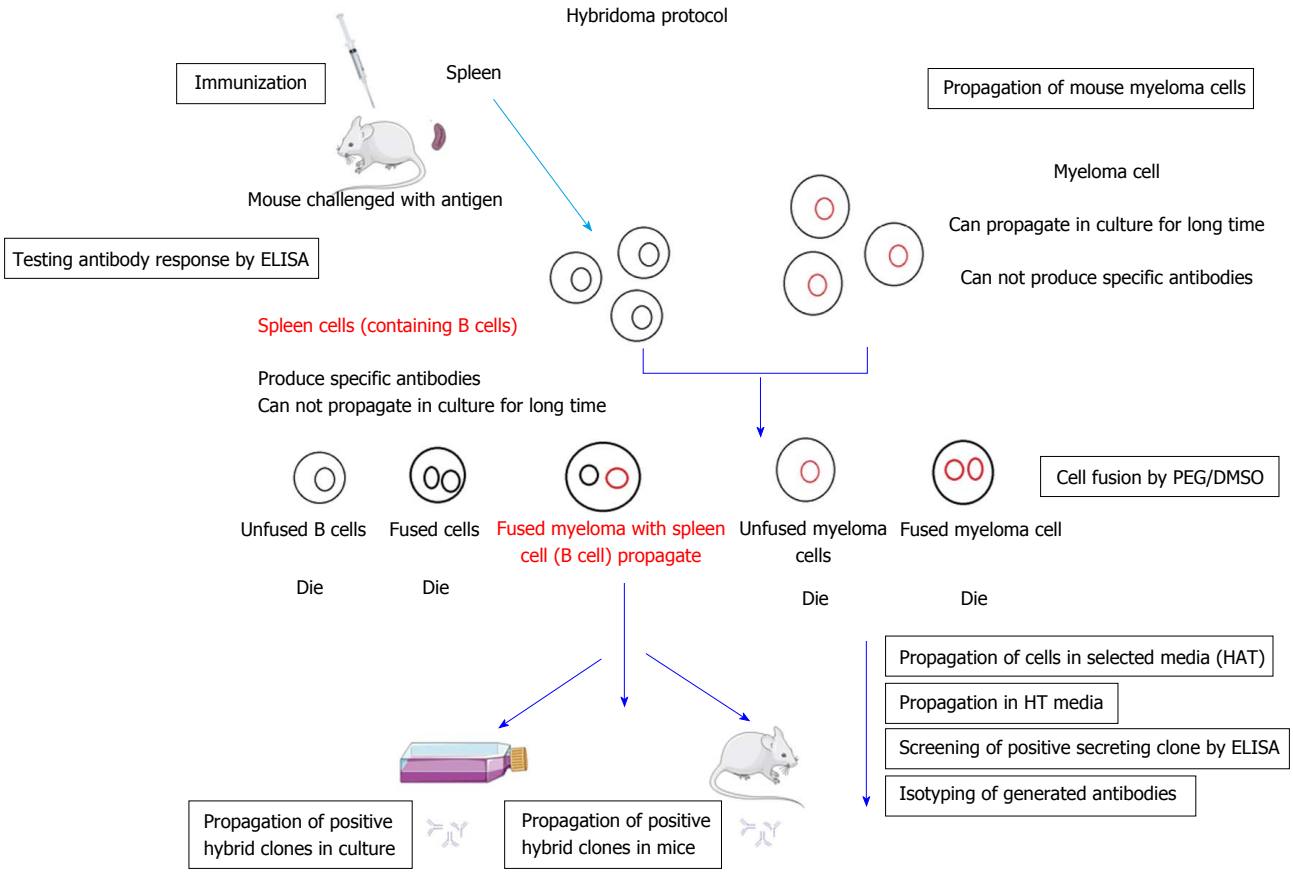

Figure 1 Diagrammatic procedure of the production of mouse monoclonal antibodies by hybridoma technology. ELISA: Enzyme-linked immunosorbent assay; PEG: Polyethylene glycol; DMSO: Dimethyl sulfoxide; HAT: Hypoxanthine-aminopterin-thymidine; HT: Hypoxanthine thymidine.

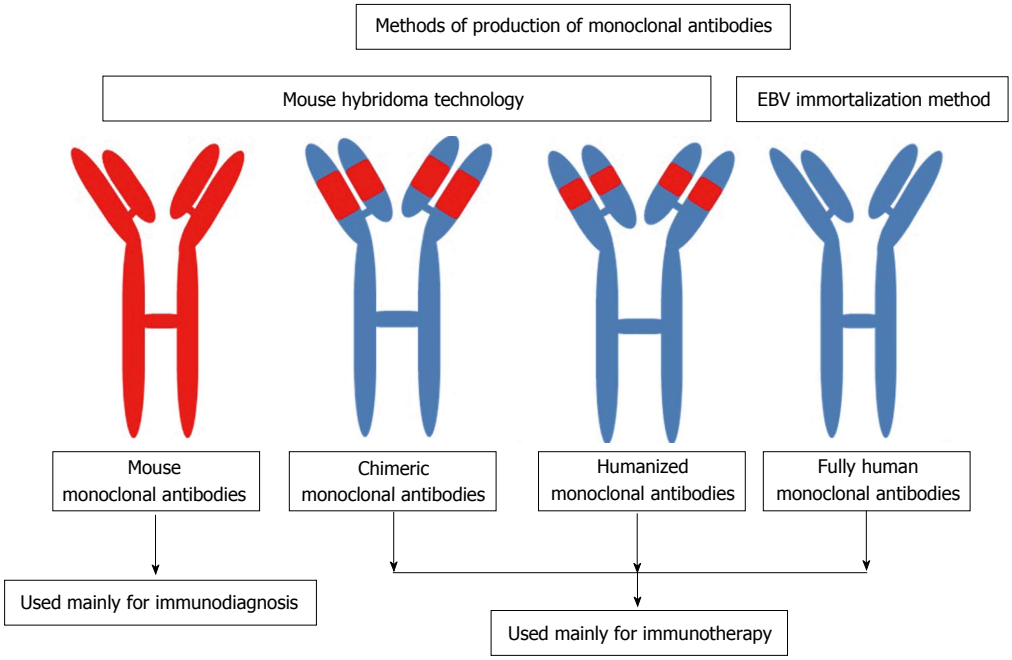

Figure 2 Diagrammatic presentations showing the applications of mouse and human monoclonal antibodies and their methods of production. EBV: Epstein-Barr virus.

PRODUCTION OF FULLY HUMAN MAB

There are several methods for the production of human mAb, such as phase display, transgenic mice, humanized mouse mAb, and immortalization by Epstein-Barr virus (EBV). In this review, we focus on the production

of fully human mAb by EBV immortalization. Human mAb (hMAb) provide novel ways for probing the B cell repertoire of various health and disease issues. Several difficulties have been encountered in the development of the hMAb, including cell line instability, low levels of specific antibody secretion, and poor cloning potency, particularly when using lymphoblastoid cells[14]. Martin *et al*[15] reported that the immortalization of B lymphocytes by EBV is a time consuming methodology for antibody production. EBV infects B cells *via* their CD21 receptors, which then transforms the B cells into lymphoblastoid cell lines that produce antibodies, representing the humoral immune response *in vivo*. Based on the type of parent cell, the generated antibodies target either an infective agent or a tumor cell, which makes them suitable therapeutic candidates against these diseases.

Compared to other antibody manufacturing techniques, the immortalization of B cells stands as the best technique, as the generation of fully human antibodies from the immortalized human B lymphocyte repertoire does not require immunization[16,17]. The first B lymphocyte immortalization experiment was performed by culturing B cells in the presence of a herpes virus obtained from a marmoset lymphocyte cell line B95-8[18,19]. Numerous changes to this procedure have since been tried, yet the immortalization and B lymphocyte rates remain inefficient. Several successful mAbs have been generated with this technique for use against different pathogens; Schieffelin *et al*[20] report that human mAb against dengue virus envelope were generated by the EBV transformation of B cells from patients after two years of naturally-acquired dengue virus infection. These antibodies were found to have completely different cross-reactivity, and neutralizing patterns. Schieffelin *et al*[20] and others[21,22] have used CpG2006 as stimulators for EBV immortalization. Furthermore, antibodies neutralizing the SARS corona virus and cytomegalovirus have been successfully created *via* the introduction of the polyclonal B lymphocyte activator CpG2006 into the B lymphocyte immortalization method and by B lymphocyte activation before EBV infection, respectively[21,22]. Moreover, Fraussen *et al*[23] report that seeding low B lymphocyte numbers per well serves to limit bias toward the advantageous outgrowth of fast-growing immortalized B cells including interleukin-2 (IL2) and CpG 2006.

Siemoneit *et al*[24] used herpes virus immortalization for the production of human organism antibodies to target HCV core macromolecules. In doing this, they revealed the establishment of two vegetative cell lines secreting human mAb to the 22-kD nucleocapsid macromolecule (core, p22) of HCV. Siemoneit *et al*[24] isolated B lymphocytes from an anti-HCV-positive donor and immortalized them by EBV infection. Two of the cell colonies were fused with the (mouse/human) heteromyeloma cell line K6H6/B5. The generated fused hybridomas produced antibodies of the IgG1/kappa (U1/F10) and the IgM/kappa (Ul/F11) isotype and were found to specifically react with the recombinant core macromolecule p22. Recently, Steinitz[25] reported that EBV has the *in vitro* ability to immortalize nearly all human B lymphocytes, which allows for the isolation of monoclonal cell lines that secrete specific human mAb.

The method of immortalization by EBV allows for the production of human mAb of different classes (IgM, IgG, IgA, and IgE) from any individual. Human mAb produced by EBV immortalization resemble the supplies of antibody molecules derived from the lymphocytes of blood donors. Therefore, Steinitz[25] concluded that human mAb are promising reagents of passive immunization for several diseases, such as cancer, and viral or bacterial infections. In our recently published paper[26], we aimed to produce human cell lines by manufacturing neutralizing human mAb for use against the envelope E1/E2 macromolecule of HCV. For this, we used two methods for the EBV immortalization of $CD22^+$ cells taken from HCV-positive patients: (1) immortalization with 100% EBV only; and (2) immortalization by 30% EBV and CPG2006 with IL2 (Figure 3). Our results indicated that cell stimulators play a role in the production of antibodies, with immortalization by 100% EBV only producing large clones compared to immortalization by 30% EBV and CpG2006 with IL2. The antibody levels, as measured by enzyme-linked immunosorbent assay (ELISA), showed high optical density in cells immortalized with 30% EBV and stimulants CpG2006 and IL2. Our results also indicated that the immortalization of low B cell numbers by 30% EBV, CPG2006, and IL2 was introduced with high efficiency and reproducibility leading to immediate generation of single clones that produced mAb. The specificity of the generated mAbs was confirmed by screening them against linear synthetic peptides (as epitopes) derived from HCV E1 (a.a 315-323) and two synthetic peptides derived from HCV E2 (a.a 412-419 and a.a 517-531) using in-house, ELISA-based optimized assays, all of which were studied by several investigators[27-29]. Fifteen clones secreting human immunoglobulin G against HCV E1/E2 protein were isolated. The ELISA results showed that one antibody was binding to the E2 peptide (a.a 517-531), while two antibodies were binding to the HCV E2 peptide (a.a 412-419). The three generated antibodies (IgG3, one antibody, and IgG2, two antibodies) showed high neutralization activity against HCVpp. We therefore concluded that these antibodies may be useful for the passive immunotherapy of HCV infections, particularly for HCV-positive liver transplantation patients.

USING MOUSE MAB FOR THE DETECTION OF HCV ANTIGEN(S) AS DIAGNOSTIC MARKERS

The detection of anti-HCV antibodies involves a simple, inexpensive, and quick test, yet this test has a low sensitivity in the first six to eight weeks of infection, or given the presence of several clinical conditions, such as chronic immunosuppression or hemodialysis[30]. A recent study[31] has shown that the proteins of Core and

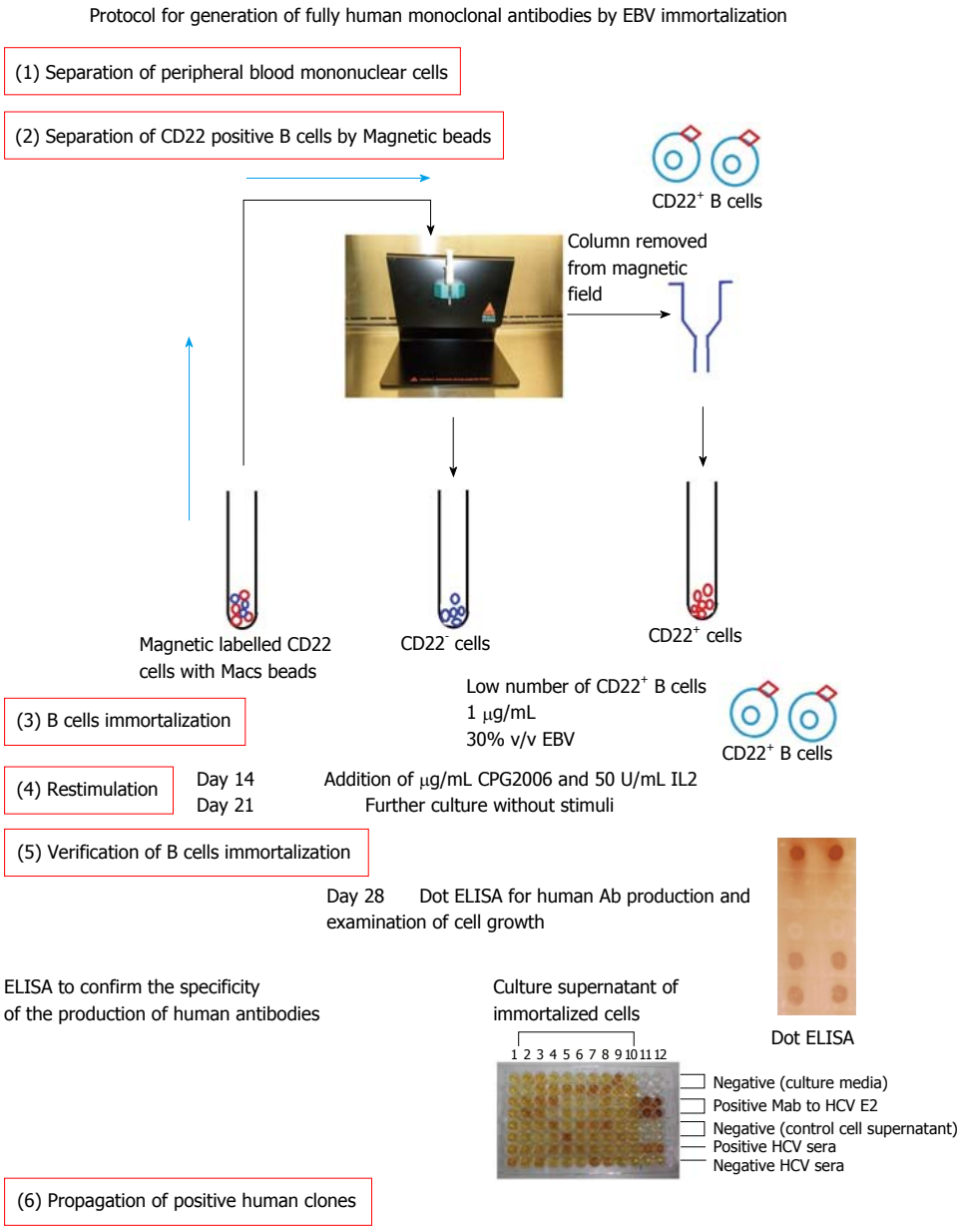

Figure 3 Generation of fully human monoclonal antibodies by Epstein-Barr virus immortalization. EBV: Epstein-Barr virus; HCV: Hepatitis C virus; IL2: Interleukin-2; ELISA: Enzyme-linked immunosorbent assay.

E2 genes within the antigenic regions of a local HCV-3a genotype can be used to develop highly sensitive, specific, and economical diagnostic assays for the detection of HCV infection. The recombinant antigen showed 100% reactivity against HCV-infected sera, with no cross reactivity in the HCV-negative sera. The authors therefore concluded that a mixture of Core and E2 antigens is potentially valuable for HCV-Ab detection[31].

The disadvantage of HCV diagnosis *via* the detection of HCV-Abs is the inability to distinguish between active and past infection. Given this, HCV-Ag detection is preferable, especially during the window phase of HCV infection. Recently, Florea *et al*[32] investigated the diagnosis of an HCV infection based on a HCV core Ag detection assay and found both a good correlation between the core Ag results with the HCV RNA viral load and very high specificity. Furthermore, Chevaliez *et al*[33] evaluated the clinical performance of the Architect HCV-Ag assay in terms of the detection and quantification of HCV core antigens in patients with chronic HCV genotype 1-6 infections. They concluded that the Architect HCV-Ag assay is highly specific, easy to perform, and represents a valuable screening, diagnostic, and monitoring tool.

Several investigators have demonstrated evidence of HCV antigens in liver tissue[34-37] serum samples[38,39] and plasma samples[40]. We reported the detection of HCV NS4 antigen in the sera of infected HCV patients using the Dot ELISA technique as a rapid assay[41]. The assay developed was able to detect the HCV target antigen in 95% of the serum samples from HCV-infected individuals with a specificity of 97% compared to the sera of uninfected individuals, using reverse transcription

polymerase chain reaction results as a reference. This antigen-detection-based method showed high positive predictive values (99%) and negative predictive values (90%). The added advantage of the assay is that it was able to detect HCV target antigens in the sera of patients during the window phase (negative for HCV-Ab and positive for HCV-RNA), as well as in the sera of both low and high HCV-RNA viral loads. The authors showed that their developed assay was highly sensitive and specific for HCV antigen detection, and that it could be applied for the mass screening of HCV infection.

In two reports[41,42], Attallah *et al*[41] describe their use of ELISA for the detection of HCV-NS4 and assessing the diagnostic performance of the assay in 883 chronic HCV patients. Taking quantitative HCV-RNA as a gold standard for HCV diagnosis, areas under the receiver operating characteristics (ROC) curves (AUC) were used to assess the diagnostic accuracy of ELISA for HCV-NS4. Attallah *et al*[42] identified HCV-NS4 to be at 27 kDa using Western blot. The areas under the ROC curves (AUC) in HCV-NS4 detection were 0.95 among patients with different pathological states of liver disease, with 0.93 for liver fibrosis, 0.95 for liver cirrhosis, and 0.98 for hepatocellular carcinoma (HCC). The mean ± SD (ng/mL) of HCV-NS4 in liver fibrosis was 94.2 ± 55.6, in liver cirrhosis was 99.3 ± 64.8, and in HCC was 124.9 ± 70.3. The authors therefore concluded that HCV-NS4 antigen detection using ELISA is a reliable test to confirm HCV infection. We established a hybrid cell line that produced mouse mAb targeting HCV E1 a.a 315-323[43]. We also produced mouse mAb targeting HCV E1/E2 and used them in the ELISA as a diagnostic assay for HCV infection (unpublished data). Our results showed a sensitivity of 80% and a specificity 96%.

HCV neutralizing antibodies

HCV cellular receptors are targets for HCV neutralization: Many HCV neutralizing antibodies target E1 and E2 HCV glycoproteins. However, an alternative strategy to preventing HCV entry may be achieved by targeting host receptors, such as CD81 and SR-BI. These antibodies block viral-host receptor interaction. A large number of broad spectrum, anti-HCV, host-targeting antivirals (HTAs) have been developed that trigger the innate immune system. Examples of these anti-HCV compounds include anti-SR-BI and toll-like receptor agonists[3,8]. The mechanism of action in this group of compounds involves the inhibition of certain cellular factors that are crucial to the HCV lifecycle. One of the main advantages of HTAs is that they act on host factors that are of a much lower rate of mutation[3]. Therefore, we are going to discuss primarily cellular receptors required for the attachment of HCV together with their involvement in the neutralization process.

CD81 receptor: The tetraspanin CD81 (26 kDa) is an integral membrane unglycosylated protein. CD81 is reported to possess several functions, such as signal transduction, cell activation, and cell adhesion. Moreover, in prior studies uding HCVpp and HCVcc systems, it has been confirmed that the CD81 receptor plays a major role in HCV cell entry[4]. A large number of broadly anti-HCV neutralizing antibodies block CD81 interaction with the HCV envelope glycoprotein E2. Indeed, it has been previously shown that anti-CD81 mAb inhibit the entry of both HCVcc and HCVpp into the Huh-7 cell line[44,45]. Resolving the crystal structure of CD81 complexed with E2 protein[44,45] has revealed that CD81 binds the HCV envelope glycoprotein E2 within certain specific amino acid residues (*i.e.,* 412-423, 432-447, 480-493, 528-535, and 544-551). K04, a recently generated anti-human CD81 monoclonal antibody, was shown to have a broad-spectrum antiviral action in the prevention and treatment of HCV infection[46].

Lipoprotein receptor scavenger receptor BI: The scavenger receptor class B type Ⅰ [scavenger receptor BI (SR-BI)] is highly expressed in hepatocytes. This receptor functions as a lipoprotein receptor that mediates cholesteryl ester selective uptake from high density lipoproteins[47]. The SR-BI receptor can bind both high density lipoproteins (HDL) and low density lipoproteins[8,47]. SR-BI has been previously shown to mediate interactions of E2-CD81. It has been suggested[45,48,49] that the SR-BI receptor interacts with HCV glycoprotein E2 hypervariable region 1 [hypervariable region-1 (HVR1), the first 27 amino acids in E2]. In line with this hypothesis, HVR1 deletion has been shown to inhibit E2-SR-BI interaction and to reduce HCV infectivity[50-52]. Indeed, it has been shown that HVR1 facilitates the interaction between HDL and SR-BI, which inhibits the neutralization of both HCVpp and HCVcc. HCV infection in cell cultures has also been shown to be inhibited with antibodies against SR-BI, confirming the crucial role of SR-BI in HCV cell entry[47,53,54]. Lastly, it was recently indicated that anti-SR-BI antibodies inhibit the HCV infection of different genotypes, both in cell cultures and humanized mice[55].

Other HCV receptors: Aside from SR-BI and CD81, other receptors including claudin-1 (CLDN1) and occludin (OCLN) compose the tight junction factors[56]. The tight junction multiprotein complex is comprised of four types of transmembrane proteins: Claudins, occludins, junction associated molecules, and the coxsackie virus B adenovirus receptors[57-59]. It is still unclear how the CLDN1 and OCLN inhibit HCV cell entry. Anti-claudin-1 antibodies neutralize HCV infectivity *via* inhibiting the interaction between CD81 and claudin-1 receptors, which is important to the viral entry process[58,59]. Occludin has been reported to co-precipitate with the HCV E2 glycoprotein. However, unlike CD81 and SR-BI and claudin-1 cellular receptors, no virus-specific neutralizing antibodies for occludin have been identified thus far[60].

HCV-NEUTRALIZING EPITOPES AND ANTIBODIES

The identification of the various mechanisms involved in immune protection is an important step in designing an HCV vaccine. The production of neutralizing antibodies by adaptive immune systems following vaccination has been prior demonstrated as a key strategy for protection against human viruses[61]. In the case of the HCV, a viral infection can persist even in the presence of a broadly neutralizing antibody, in many cases leading to chronic infection[44,62]; this can result in liver fibrosis, cirrhosis, and even eventually lead to hepatocellular carcinoma, causing death.

Anti-HCV antibodies can be targeted against structural and nonstructural protein epitopes (classified as either linear or conformational)[63,64]. The envelope glycoproteins E1 and E2 are considered major targets for neutralizing antibodies, as they are present on the surface of the HCV virus. Consequently, the development of an HCVpp system using unmodified HCV E1 and E2 envelope glycoproteins has allowed researchers to achieve remarkable progress in the study of neutralizing antibodies. However, little is understood so far as to the structure of the HCV envelope glycoproteins and how they interact with neutralizing antibodies[65].

Despite the fact that the HCV E1 glycoprotein is more conserved than E2, it has been proposed that E1 is of lower immunogenicity and hence a more difficult target for neutralizing antibodies[47,66]. However, two mAb against HCV glycoprotein E1 (IGH 505 and IGH 526) have been identified[27]. Both antibodies neutralize HCVpp bearing the E1 envelope glycoprotein of genotypes such as 1a, 1b, 4a, 5a, and 6a. These antibodies work within the region of E1 amino acids comprised of amino acids 313 to 327[27]. In addition to these two antibodies, the H-111 antibody has been reported to neutralize expressed E1 proteins from genotypes 1a, 1b, 2b, and 3a *via* binding to the 192YEVRNVSGVYH211 region of E1[67,68].

Anti-E2 human conformational-dependent HCV antibodies targeting E2 in HCVpp cell culture systems have been used to identify E2 epitopes. Due to such studies, three different regions of the E2 HCV glycoprotein E2 have been identified[50]. These regions include the E2 HVR1, the E2 HVR2, and the CD81 binding region of E2 and the C-terminus of HVR1[69]. The HCV glycoprotein HVR1 is a major target of neutralizing antibodies. This region is crucial for the virus, as it plays an important role in the HCV virus binding and entry process[50,70]. The physicochemical properties of the residues of HVR1 and its conformation are both highly conserved among various species despite the sequence variability of HVR1. It has been suggested that the sequence variability of E2 HVR1 is driven by the antibody selection of immune-escape variants[71]. Stable HVR1 sequences associated with resolved infection have also been reported, with HVR1 sequence change being suggested as one of the reasons for persistent HCV infection. This suggests that the most important target of antibody response to the HCV E2 glycoprotein is HVR1 (Table 1 for other examples)[72].

EXAMPLES OF HCV-NEUTRALIZING ANTIBODIES

Epitopes can be classified into two main groups (linear or conformational epitopes). Various viral epitopes that are targeted by neutralizing antibodies have been identified and characterized. Two human mAb, HCV1 and 95-2, have been identified as successfully neutralizing HCVpp belonging to different genotypes (*i.e.,* 1a, 1b, 2b, 3a, and 4a). In addition, a highly conserved linear epitope in E2 (amino acids 412 to 423) has been reported as recognized by HCV1 and 95-2 mAb[65]. Moreover, e137, a human monoclonal Fab, has been shown to bind to the HCV E2 glycoproteins of all HCV genotypes, with the exception of HCV genotype 5. Furthermore, it has been confirmed that this epitope interacts with highly conserved residues in all HCV genotypes, such as T416, W529, and D535[73]. CBH-5 has also shown itself to be capable of neutralizing all examined genotypes (genotypes 1-6). It was revealed that two of the amino acids comprising the epitope of CBH-5 are crucial for E2-CD81 interaction, which suggests direct competition between CBH-5 and CD81 to bind with the HCV E2 glycoprotein[74]. AP33 is another broadly neutralizing mouse monoclonal antibody that has been shown to neutralize all genotypes (*i.e.*, 1a, 1b, 2a, 2b, 3a, 4, 5, and 6). This antibody recognizes a highly conserved epitope in HCV glycoprotein E2 (amino acids 412 to 423)[75]. The high conservation of its epitope may have resulted in the broadly neutralizing activity of this antibody. Finally, AR3B displays a broadly neutralizing human antibody activity. AR3B neutralizing antibodies have been shown to protect against viremia in an infected mouse model[76].

NEUTRALIZING ANTIBODIES' MECHANISMS OF ACTION

The lack of cell culture-based assays to measure and quantify HCV activity has long hindered the study of the role of neutralizing antibodies in HCV infections. The mechanism of action in the antibody neutralization process still remains unclear. Several mechanisms through which neutralizing antibodies interfere with different stages of the HCV life cycle have been suggested[77,78], including immune aggregation and the blocking the attachment of the virion to the viral receptor which inhibits HCV infection. In addition to these mechanisms, neutralizing antibodies have been reported to interfere with other stages of the HCV life cycle following the binding process, such as the penetration of the virus through the cell membrane *via* host entry factors. Neutralizing antibodies may also prevent conformational changes important for the fusion of the virus to a host cell[77,78].

Table 1 Hepatitis C virus neutralizing antibodies

Neutralizing antibody	Epitope	Specificity	Escape mutants	Ref.
IGH505	E1 (313-326) linear	Cross-reactive	NA	[27]
IGH526	E1 (313-326) linear	Cross-reactive	NA	[27]
H-111	E1 (192-202) linear	Cross-reactive	NA	[68]
95-2	E2 (412-423) linear	Cross-reactive	NA	[65]
HCV-1	E2 (412-423) linear	Cross-reactive	NA	[65]
HC-1	E2 (523-540) conformational	Cross-reactive	No escape	[69,125]
3/11	E2 (412-423) linear	Cross-reactive	N415Y, N415D, N417S, G418D	[75]
3C7	E2 (396-407)	H strain	NA	[126]
AP33	E2 (412-423) linear	Cross-reactive	N415Y, N415D, N417S, G418D	[75]
CBH7	E2 conformational	Cross-reactive	NA	[74]
CBH5	E2 (523-540) conformational	Cross-reactive	NA	[68,127]
CBH2	E2 (425-447), E2 (523-540) conformational	Cross-reactive	D431G, A439E	[68,125,128]
CBH-8C	E2 conformational	Cross-reactive	NA	[129]
CBH-8E	E2 conformational	Cross-reactive	NA	[129]
CBH-11	E2 conformational	Cross-reactive	NA	[129]
CBH-17	E2 conformational	Cross-reactive	NA	[129]
CBH4B	E2 conformational	Cross-reactive	NA	[129]
CBH4D	E2 conformational	Cross-reactive	NA	[129]
CBH4G	E2 conformational	Cross-reactive	NA	[129]
9/27	E2 conformational	H strain	NA	[130]
A8	E2 (523-540) conformational	Cross-reactive	NA	[127]
J6.36	E2 Partially conformational	J6 strain	NA	[131]
HC-11	E2 (425-447) E2 (523-540) conformational	NA	L438F	[69,125]
Fab e20	E2 (523-540) conformational	Cross-reactive	NA	[132]
Fab e137	E2 (412-423) E2 (523-540) conformational	Cross-reactive	NA	[73]
1:7	E2 (523-540) conformational	Cross-reactive	NA	[127]
AR3A	E2 (394-424), E2 (437-447) E2 (523-540) conformational	Cross-reactive	NA	[76]
AR3B	E2 (394-424) E2 (437-447) E2(523-540) conformational	Cross-reactive	NA	[76]
AR3C	E2 (394-424), E2(437-447) E2 (523-540) conformational	Cross-reactive	NA	[76]
AR3D	E2 (394-424), E2 (437-447) E2 (523-540) conformational	Cross-reactive	NA	[76]
AP213	E2 (396-407) partially conformational	Gla strain	NA	[133]
H77.39	E2 (384-520) linear	Cross-reactive	NA	[131]
H35	E2-conformational	Poorly cross-reactive	NA	[134]
H48	E2-conformational	Poorly cross-reactive	NA	[134]
2/69a	E2 (436-443) linear	NA	NA	[135]
9/86a	E2 conformational	NA	NA	[130]
6/1a	E2 (464-471) linear	NA	NA	[130]
9/75	E2 (524-531) linear	NA	NA	[130]
6/53	E2 (544-551) linear	NA	NA	[130]
6/16	E2 (384-391) linear	NA	NA	[130]
1/39	E2 (432-443) linear	NA	NA	[130]
6/41a	E2 (480-493) linear	NA	NA	[130]
11/20c	E2 (436-447) linear	NA	NA	[130]
ALP98	E2 (644-651) linear	Cross-reactive	NA	[136]
ALP1	E2 (647-658) linear	NA	NA	[136]

NA: Not available.

VIRAL MECHANISMS FOR EVADING NEUTRALIZING ANTIBODIES

HCV has developed the following different mechanisms to evade neutralizing antibody responses[79]: (1) the association of HCV with low density lipoproteins[47]; (2) the association of HCV with certain glycans can play a role in shielding important neutralizing epitopes of E1 and E2 envelope glycoproteins[80]; (3) the production of interfering antibodies, which may interfere with anti-HCV neutralizing antibodies[81]; and (4) direct cell-to-cell transmission of the HCV virus[82]. HCV typically employs a combination of these mechanisms together at the same time.

Lipoproteins

It is reported that poorly infectious HCV particles linked to immunoglobulins have been separated from the higher density fractions of chronic HCV plasma samples, with highly infectious particles found in lower density

fractions, indicating the effect of lipoproteins on the infectivity of HCV[83]. The binding of HCV to lipoproteins may facilitate HCV uptake by liver cells[47,84]. This facilitated viral entry is mediated through interaction between the ApoB and SR-BI receptors, which enable HCV to escape recognition *via* different HCV neutralizing antibodies. This suggests that lipoproteins could possibly play a role in protection against antibody-mediated neutralization by masking the epitopes of the viral surface glycoproteins[85,86].

HCV genetic diversity

The high variability of the HCV genome provides the virus with an effective escape strategy from antibody neutralization[87,88]. HCV is classified into seven different genotypes, with each genotype including a number of subtypes that are roughly 25% different at the nucleotide level. The loss of the proofreading ability of HCV NS5B polymerase increases the mutation rate of the virus to one nucleotide per replication cycle[89]. This results in the evolution of HCV into many quasispecies with different though closely related nucleotide sequences even within the same patient, which may help the virus to escape from the human immune response[54,90]. HCV likely escapes the effects of the immune system because immune responses develop over weeks and HCV replicates on a timespan of days[91]. Interestingly, the sequence variation occurs mainly in the HCV E2 HVR1. HVR1 can remain with no change in its genome sequence for roughly two years given the absence of neutralizing antibodies. Given the virus's exposure to neutralizing antibodies, the HCV genomic RNA sequence undergoes several adaptive mutations[89].

Glycans

Glycans associated with HCV envelope proteins protect the virus from neutralization by various antibodies *via* shielding crucial epitopes, especially in the E2 glycoprotein. With eleven N-glycosylation sites, E2 is the most glycosylated protein of the E1-E2 heterodimer, whereas the E1 protein contains only four sites. These fifteen glycans in total were reported to play a role in the entry of HCV into host cells. Several glycans (E2N1, E2N2, E2N3, E2N4, E2N6, E2N8, E2N9, E2N10, an E2N11) on E2 were found to limit the accessibility of neutralizing epitopes on E2[92]. These nine glycosylation sites were found to be conserved across all genotypes[80], which may indicate their importance as an HCV escape mechanism[92].

Direct viral transmission from cell to cell

Direct transmission between cells helps HCV to evade both innate and adaptive immune systems. Cell-to-cell spread has been found to occur in other viral families, such as the herpes virus, retroviruses, and rhabdoviruses[93]. Direct cell-to-cell transmission is more efficient in spreading the virus in host, as it allows the virus to escape neutralizing antibodies. Furthermore, direct cell-to-cell transmission has been shown to be CD81 independent. Lastly, both CLDN1 and OCLDN cell receptors were reported to play a role in cell-to-cell transmission[94,95].

AVAILABLE AND NEW TRENDS IN ANTIBODY PRODUCTION

The high cost of production of antibodies by hybridoma or the humanization of mouse antibodies, page displays, or transgenic mice has led to the emergence of new trends to produce antibodies, such as egg-yolk-based antibody (IgY) production within transgenic plants and synthetic antibody mimics.

Egg yolk antibodies (IgY)

Providing passive immunity to chicks, IgY is passively transmitted to egg yolks to help safeguard a chick against infection until its own immune response can be developed. IgYs are functionally similar to IgG in mammals, and intensive research has been conducted on the utilization of IgY for passive immunization[96]. The transient activity of passive antibodies increases the need for large-scale production due to their frequent administration. Hen eggs are an excellent source of antibodies for passive immunization due to their being a non-invasive means of production and the large production capability of a chicken[97]. The IgY collected from egg yolks can yield eighteen times more antibodies than the serum obtained from rabbits without sacrificing the animal[98]. An average hen can lay roughly 325 eggs a year. Given that an egg can produce 60-150 mg of IgY[99], one hen can produce 20-40 g of antibodies a year, with 2%-10% of the antibodies being antigen specific[100].

Additional advantages of using IgYs to combat infections in the human body include the ease of isolating egg yolk antibodies and the absence of interaction accruing between IgYs and the Fc receptors of mammals, which can initiate an inflammatory reaction and fail to induce complement activation in mammals[101]. In addition, chicken antibodies produce a different antibody repertoire and identify different epitopes than the antibodies produced by mammals[102]. Furthermore, the large-scale industrial production of eggs makes the process of IgY production technically efficient. IgY has been used effectively against several human and veterinary viruses, such as the bovine rotavirus[103], infectious bursal disease virus[104], human influenza virus H1N1[105,106], rabies virus[107], bovine leukemia Virus[108], rabbit hemorrhagic disease virus[109], bovine respiratory syncytial virus[110], avian reovirus[111], norovirus[112], hepatitis A virus[113], and the white spot syndrome virus[114].

The use of genetic engineering technologies to produce chicken mAb (mIgYs) will enhance the utility of IgY antibodies[115]. The combination of the high specificity and homogeneity of the mAbs and the unique features of chicken antibodies provide additional features to mIgYs.

Very few studies have investigated the generation and utility of mIgYs with respect to prion immunogen[116]. The use of antibody engineering technologies facilitates the production of mIgY, while the phage display technique provides the features of a large, diverse library, as well as an efficient selection procedure[117].

Antibody expression in plants

The first trial of the production of antibodies in plants was carried out in 1989 in transgenic plants. Plant expression systems have several advantages in terms of the production of antibodies: First, these systems cost less compared to other conventional expression systems. Second, with regard to safety, they do not contain mammalian viruses or pathogens and do not produce endotoxins. Furthermore, as reported by Xu *et al*[118], the antibodies can be applied parenterally, topically, or orally. Castilho *et al*[119] and Olinger *et al*[120] report that, with antibody plant expression systems, it is possible to produce antibodies with desired glycoforms, and that glyco-engineered plants have a much higher degree of glycan homogeneity. In a recent review article by Virdi *et al*[121], they report that the production of antibodies in edible tissues would allow for oral passive immunization of the mucosal surface of the stomach. The use of technology together with the natural capacity of plant tissues to collect complex antibodies will enable in the enrichment of the antibody market. A review by Virdi *et al*[121] also showed the role of plants as an adaptable expression system for passive immunotherapy. Nianiou *et al*[122] showed the production of antibodies against HCV core gene in transgenic tobacco plants. The resultant HCV core antigen was found to be immunoreactive not only with polyclonal and mAb, but also with human sera positive for HCV-infected patients. Therefore, the authors prospected that the use of a plant-based HCV vaccine could be possible. Recently, Iranian scientists Mohammadzadeh *et al*[123] designed a highly codon-optimized HCV core protein gene for the construction of an effective plant expression system for the production of HCV core proteins with antigenic properties in an Iranian Jafarabadi-cultivar tobacco plant. The authors concluded that, through the use of a gene optimization strategy that uses vectors based on HCV and the suppression of plant-derived, gene-silencing effects, an effective expression system including the HCV core proteins of tobacco plants with antigenic immunogenic characteristics may be possible.

Synthetic antibody mimics

Recently, McEnaney *et al*[124] of the Yale University lab have crafted the first synthetic molecules (synthetic antibody mimics) that possess both the targeting abilities and functions of natural antibodies. The synthetic antibody mimics (SyAMs) attach themselves simultaneously to disease cells and immune fighting cells, performing a similar action to natural human antibodies. McEnaney *et al*[124] showed these molecules to be synthetic organic compounds that are approximately one-twentieth the size of natural antibodies. The authors report that their new SyAMs are thermally stable and can be administered orally. Furthermore, the authors report that the SyAMs have the potential to be used in treatments for cancer and other diseases, such as human immunodeficiency virus and various bacterial diseases[124]. We believe that these synthetic antibody mimics will open new areas of research and practice in the field of immunotherapy.

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MINIREVIEWS

Pathogenesis of hepatocarcinogenesis in non-cirrhotic nonalcoholic fatty liver disease: Potential mechanistic pathways

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Abstract

Although hepatocellular carcinoma (HCC) primarily arises in the background of liver cirrhosis, the development of HCC in nonalcoholic fatty liver disease (NAFLD) without cirrhosis is increasingly recognized. The pathogenesis of NAFLD associated non-cirrhotic HCC is distinct from that of cirrhotic HCC because the metabolic syndrome (MS) along with obesity and insulin resistance (IR) underlie several unique mechanisms that promote tumorigenesis. IR associated with MS, NAFLD, and type 2 diabetes mellitus lead to the release of multiple pro-inflammatory cytokines, including tumor necrosis factor alpha, interleukin-6, leptin and resistin, as well as decreased amounts of adiponectin. These processes favor the development of hepatic steatosis and inflammation within the liver, which precede HCC development. Nevertheless, further investigation is necessary to elucidate the determinants for development of HCC in patients with NAFLD in the absence of cirrhosis.

Key words: Nonalcoholic steatohepatitis; Hepatocellular carcinoma; Metabolic syndrome; Nonalcoholic fatty liver disease

Core tip: Although hepatocellular carcinoma (HCC) primarily arises in the background of liver cirrhosis, the

development of HCC in nonalcoholic fatty liver disease (NAFLD) without cirrhosis is increasingly recognized. The pathogenesis of NAFLD associated non-cirrhotic HCC is distinct from that of cirrhotic HCC because the metabolic syndrome along with obesity and insulin resistance underlie several unique mechanisms that promote tumorigenesis.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), obesity, type 2 diabetes mellitus (T2DM), and the metabolic syndrome (MS) are significant global health concerns that have been rising at alarming rates both in the Unites States and worldwide. Recent estimates suggest that, in the Unites States, the prevalence of obesity has risen to 33.8%, and the prevalence of T2DM among middle-aged adults has increased to 10.6%[1-3]. The epidemiology of NAFLD, the most common liver disorder in the Unites States and other industrialized countries, mirrors that of obesity and T2DM. Nonalcoholic steatohepatitis (NASH), a subset of NAFLD, can progress to cirrhosis and to hepatocellular carcinoma (HCC). A recently published prospective cohort study from the Unites States involving asymptomatic middle-aged patients determined the prevalence of NAFLD to be 46% and NASH 12.2%[2].

HEPATOCARCINOGENESIS IN NON-CIRRHOTIC NAFLD

Development of HCC in the setting of non-cirrhotic NAFLD is not common, but multiple studies have established obesity and T2DM - two major features of MS - as significant independent risk factors for HCC. Patients with cryptogenic cirrhosis, widely accepted as representing NASH, have a prevalence of T2DM and obesity similar to that of patients with NASH[4]. The typical non-cirrhotic NASH patient who presents with HCC is older, male, and meets criteria for one or more features of MS[5]. The majority of published data has been from Japanese studies, but similar observations have been made in studies from both Europe and South America[6-10]. The largest group of NAFLD associated non-cirrhotic HCC patients studied to date once again showed an older male predominance with more than half exhibiting features of MS. This cross-sectional study of 87 Japanese patients with a median age of 72 years found that male patients appeared to develop HCC at a less advanced stage of liver fibrosis when compared to their counterparts, with the prevalence of cirrhosis at diagnosis significantly lower in men compared to women (39% *vs* 79%, $P = 0.008$)[10]. However, an entirely Japanese cohort may not be generalizable to the United States. The only reported case series from the Unites States of HCC arising from non-cirrhotic NAFLD was published in 2008. Guzman *et al*[11] describes three patients with non-cirrhotic HCC in the setting of NAFLD; all had at least two features of MS with a mean body mass index (BMI) of 33.5. A French study analyzed 128 patients with HCC and found that in 31 patients (24%), features of MS were the only risk factor for HCC[8]. Compared to the group of patients who had an overt cause for underlying liver disease (chronic hepatitis B virus/hepatitis C virus, genetic hemochromatosis, auto-immune liver disease, alcohol), the MS group was older (mean age 67.4 *vs* 59.4, $P \leqslant 0.01$), had a higher BMI (29.7 *vs* 25, $P \leqslant 0.0001$), and less background liver fibrosis (F0-F2: 65% *vs* 26%, $P \leqslant 0.001$)[9]. Collectively, these data add to the growing evidence that HCC can occur in patients with non-cirrhotic patients with NAFLD, obesity, T2DM, or MS as risk factors. In addition, establishing the attributable risk of each component of MS for HCC requires further study. The pathogenesis of NAFLD associated non-cirrhotic HCC is distinct from that of cirrhotic HCC because MS and its' features of obesity and insulin resistance (IR) contain several unique mechanisms that help support a tumor-promoting environment. IR associated with MS, NAFLD, and T2DM lead to increased release of free fatty acids from adipocytes and the release of multiple pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), leptin and resistin. Decreased amounts of adiponectin are seen as well[5]. These processes favor the development of both hepatic steatosis and inflammation within the liver[5]. Increased levels of TNF-α lead to activation of nuclear factor κB (NF-κB), a regulator of immune and inflammatory responses resulting in inhibition of apoptosis[5].

Role of obesity in hepatocarcinogenesis

Obesity is characterized by a low-grade chronic inflammatory response that is associated with increased cancer death rates, especially in HCC[12]. Adipose tissue expansion promotes the release of the pro-inflammatory cytokines TNF-α and IL-6[13]. TNF-α stimulates pro-oncogenic pathways involving NF-κB, c-Jun amino acid-terminal kinase (JNK), mammalian target of rapamycin complex (mTOR) and extracellular signal-related kinases[14]. Additionally, IL-6 exhibits a major role in the inflammatory response and exerts tumor-promoting effects such as cell proliferation and anti-apoptosis[6]. In the setting of obesity, IL-6 levels are elevated. It has been shown that weight loss results in reduced levels of both IL-6 and TNF-α leading to a decreased inflammatory and potentially carcinogenic response[15]. A recent murine model demonstrated that obesity promoted malignant tumor growth in diethylnitrosamine-induced mice by enhancing the production of TNF-α

and IL-6. This resulted in hepatic inflammation and activation of the pro-oncogenic signal transducer and activator of transcription (STAT3) pathway[16]. The investigators proposed that obesity, elevated IL-6, and activated STAT3 alone do not cause HCC. Rather, chronic activation of the IL-6/STAT3 axis leads to an increased probability that hepatocytes with previously acquired oncogenic mutations from exposure to environmental and dietary carcinogens will continue their malignant transformation[16]. In the setting of a high fat diet, the activity of hepatic STAT3 increases and leads to accelerated liver tumor progression[17]. Sorafenib, an anti-HCC agent that exhibits anti-tumor cell progression and anti-angiogenesis effects, acts mainly by blocking VwsEGF/PDGF and has recently been shown to block the STAT3 pathway, providing further evidence of the importance of STAT3 in HCC tumorogenesis[18]. Adipokine imbalance *via* adipose tissue expansion plays a major role in obesity, diabetes and NAFLD[19]. This imbalance leads to increased levels of leptin, a potent inflammatory cytokine, and decreased levels of adiponectin, a potent anti-inflammatory cytokine. Low adiponectin levels precipitate a vicious cycle of ectopic fat accumulation and further IR. Low adiponectin levels may also be insufficient to suppress inflammatory signaling in Kupffer cells and other macrophages[19] or to activate adenosine monophosphate-active protein kinase (AMPK), a potent inhibitor of the mTOR oncogenic pathway[6]. Adiponectin has also been demonstrated to play a pivotal role in anti-angiogenesis and apoptosis while leptin has been shown to promote angiogenesis in animal models, further substantiating the role of low adiponectin and elevated leptin levels in tumor formation and growth[20].

Intrahepatic lipid accumulation, derived from either lipolysis or excess dietary lipid intake, followed by lipid peroxidation contributes to inflammation and ultimately hepatocarcinogenesis[21]. Peroxisome proliferator-activated receptor alpha (PPARα) upregulates fatty acid disposal in response to increased free fatty acid levels. However, in murine models, PPARα variants accelerate risk for hepatocarcinogenesis. Genetic variants affecting cell signaling (Akt, E-cadherin, β-catenin, ERK, MEK, MET, PI3K, Ras, Rat, mTOR and Wnt) as well as cell cycle regulation (p16, p53, INK4, cyclin's and cdk's) have all been implicated in HCC[21-23]. As differing classes of HCC have been observed in both humans and murine models, unique pathogenetic mechanisms linked to specific genetic variants may explain each class of HCC[22,23].

Role of insulin resistance in hepatocarcinogenesis

Obesity, T2DM, and NAFLD with its inherent activation of pro-inflammatory cytokines and lipotoxicity promote systemic and hepatic IR with resultant hyperinsulinemia. IR and hyperinsulinemia have been shown to upregulate the production of insulin-like growth factor-1 (IGF-1) and insulin receptor subtrate-1 (IRS-1). IGF-1 is a peptide hormone that stimulates cellular growth through proliferation and inhibition of apoptosis within the liver[24]. IGF-1 activity increases expression of the proto-oncogenes c-fos and c-Jun *in vitro* and activates mitogen activated protein kinases (MAPK), processes thought to contribute to HCC development[25]. Another important intracellular marker, JNK, is linked to obesity, IR, NASH, and HCC. JNK, a MAPK, is activated by IR, hyperinsulinemia, and obesity with an observed reduction of in JNK activity with weight loss[26]. JNK has also been shown to phosphorylate and activate IRS-1, which subsequently leads to the pathway that is responsible for obesity- induced IR[27]. Two main isoforms of JNK have been delineated in a mouse model: JNK1 appears to promote hepatic steatosis and inflammation, while JNK2 inhibits hepatocyte death[28]. The activity of JNK and its relationship to the development of carcinogenesis has been slowly elucidated by a growing number of studies. Approximately 70% of HCC tissues show positive immunostaining for phosphorylated JNK, suggesting a role in hepatocarcinogenesis[29].

Increasingly, reports are linking the treatment of IR and hyperinsulinemia in diabetic patients to reducing the risk of HCC[30]. Central to the effects of insulin-sensitizing therapy on the development of HCC is the interaction between AMPK, the tumor suppressor complexes TSC1 and TSC2, the mTOR oncogenic pathway, and autophagy. AMPK is increased in the setting of caloric restriction, starvation, and exercise and moderates cellular activities such as hepatic fatty acid and cholesterol biosynthesis[31]. AMPK activation also increases the expression of TSC1/TSC2 through phosphorylation, which in turn reduces the activity of the mTOR oncogenic pathway[32]. This reduced activity of the mTOR pathway leads to less uncontrolled cellular growth, proliferation, and survival and an increase in autophagy[33]. Autophagy is a cellular housekeeping strategy by which damaged proteins, organelles and invading microorganisms are removed by cellular auto-digestion[3,34]. Defects in this process have been shown to play a pivotal role in a wide array of diseases, including NAFLD and HCC, where autophagy exhibits anti-tumor and anti-inflammatory properties[35]. In the setting of obesity and IR, AMPK is inhibited, leading to an increase in the activity of the oncogenic mTOR pathway as well as a decrease in autophagy[36]. Increased mTOR activity leads to unabated cellular proliferation while decreased autophagy results in decreased removal of damaged mitochondria, enhanced oxidative stress, and activation of the JNK pathway, leading to inflammation and a tumor-promoting environment[34]. However, the reason why only a small fraction of patients with risk factors develop non-cirrhotic HCC remains elusive.

CONCLUSION

The pathogenesis of NAFLD associated non-cirrhotic HCC is distinct from that of cirrhotic HCC because the MS along with obesity and IR underlie several unique mechanisms that promote tumorigenesis. IR associated with MS, NAFLD, and T2DM lead to the release of

multiple pro-inflammatory cytokines, including tumor necrosis factor alpha, IL-6, leptin and resistin, as well as decreased amounts of adiponectin. These processes favor the development of hepatic steatosis and inflammation within the liver, which precede HCC development.

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MINIREVIEWS

Hepatitis delta virus: Making the point from virus isolation up to 2014

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Abstract

Chronic infection with hepatitis delta virus (HDV) has lately regained clinical importance because of the recent evidence of increasing prevalence in several European countries, due to immigration from highly endemic areas. HDV requires the mandatory presence of hepatitis B virus (HBV) for propagation to hepatocytes. It is transmitted by the same routes of HBV and it can be acquired either by co-infection (simultaneous transmission of the two viruses) or super-infection (acquisition of HDV by an already chronic carrier of HBV). As a consequence, every HBV carrier is potentially at risk for HDV superinfection. Since the clinical course of superinfection can be severe, early diagnosis of HDV infection is necessary.

Key words: Hepatitis delta virus; Epidemiology; Natural history; Treatment

Core tip: The re-appearance of hepatitis delta virus infection in developed Countries has risen new interest on one of the most severe forms of viral hepatitis in humans. The lack of research on the subject for about two decades is responsible for the present unavailability of specific and efficient antiviral treatments against hepatitis delta virus. This review focuses on what is known up today and what still needs to be done.

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INTRODUCTION

Hepatitis delta virus (HDV) is the etiological agent of one of the most severe forms of viral hepatitis in humans. Discovered and isolated in Italy in the mid-70s, the virus was demonstrated to be endemic worldwide, with prevalence rates varying greatly in different geographical areas, regardless of the prevalence of hepatitis B

virus (HBV), whose presence is mandatory for HDV propagation[1-3]. Clinical expression is wide and although sometimes it follows a benign course, more often the disease is clinically important with rapid progression to cirrhosis, liver decompensation and death, as compared to HBV monoinfection[4,5]. Several studies published since HDV discovery have provided further knowledge into biology, pathogenesis, epidemiology, and natural history of HDV infection, however the mechanism responsible for such a severe disease is still under debate. In this review we will focus on some of the most peculiar aspects of HDV infection, giving emphasis to the improvements made since virus discovery on the subjects of epidemiology, clinical course and natural history of the disease but also underlying what still needs to be done.

EPIDEMIOLOGY

HDV is a small defective RNA virus that requires the mandatory presence of HBV for its propagation to hepatocytes but not for viral replication[2]. The virus was discovered and isolated by Rizzetto *et al*[1] in the mid-70s, while investigating a group of chronic HBV infected patients with a severe disease. The existence of a new antigen-antibody system was shown and it was observed in patients with a severe course of HBV infection, only. Later on, the virus was demonstrated to be an RNA molecule of low molecular weight, encapsidated by the three HBV envelope proteins known as large, medium and small hepatitis B surface antigen (HBsAg)[6]. It was named the delta agent[7].

Epidemiological studies carried out in the 1980s demonstrated that the virus was present worldwide with prevalence varying widely in different areas of the world, regardless of the prevalence of HBV infection[3]. Today we know that about 350 million people in the world are considered to be chronic carriers of HBV infection and about 5% of the HBV carriers are believed to have HDV infection, leading to an estimated global burden of about 15-20 million cases of chronic HDV infection in the world. However, these data could be largely underestimated for several reasons. Data on HDV prevalence are still missing from large areas of the world, not exclusively clustered in countries with low socio-economic development (Figure 1). Moreover, a systematic screening for anti-HDV in HBV carriers is still not performed. For sure, main areas of prevalence are the Mediterranean basin, the Middle East, Central and Northern Asia, West and Central Africa, the Amazonian basin, Northern South America and the Asia-Pacific region (Figure 1). Mongolia is a country with particularly high prevalence of HDV, with about one third of chronic hepatitis being attributable to HDV[8].

The prevalence of HDV infection has significantly changed over the last three decades in different areas of the world. Indeed, mainly in Southern Europe[9-11], Taiwan[12] and Turkey[13] the prevalence has significantly declined mainly due to mass vaccination campaign against HBV, systematic screening of blood and blood products, amelioration of safety procedures against blood borne infections in healthcare workers, use of disposable needles and medical devices, improved socioeconomic conditions and protection against sexually transmitted diseases. In Italy, the prevalence of anti-HDV in chronic HBsAg carriers was 24.7% in 1978-1981, 28% in 1987 but declined to 14% in 1992 and to 8.3% in 1997[9,11]. In Turkey the prevalence observed in chronic HBV carriers and cirrhosis between 1980 and 2005 were 20% and 32% respectively, with wide differences within the country, being Eastern and Southeastern regions of the country characterised by higher rates[14]. Conversely, countries like Pakistan and Iran have shown increasing prevalence over time and the presence of HDV infection has been discovered in new regions such as Russia, Northern India, Southern Albania, mainland China and some Pacific Islands[15]. However, the decreasing trend of HDV prevalence has come to a stop in several areas of the world, with stable rates of infection demonstrated in London[16], Germany[17,18] and Italy[10]. Surprisingly, the prevalence rates of HDV infection seem to be increasing in France[19]. The main reason for this recrudescence is the increasing immigration from Eastern Europe, Africa, Turkey and former Soviet Union. In the United States, few studies in the 70s'-80s' have reported prevalence of 3.8% in blood donors, 15% in HIV coinfection, 30% in developmentally disabled with chronic HBV, and up to 67% in injection drug users[20-23]. More recent data reported that 50% of injection drug users with chronic HBV infection were also HDV infected[24]. Finally, among 499 chronic HBV infected patients from Northern California, 8% tested HDV positive[25].

Hepatitis D is also prevalent in the Amazonian region of western Brazil, in Venezuela and Western Pacific populations[26,27].

CLINICAL COURSE

The virus shares the same routes of transmission of HBV, therefore it is transmitted by parenteral route through exposure to infected blood or body fluids. Since a very small inoculum has been demonstrated to be sufficient for virus transmission[28], intravenous drug users are particularly at risk of infection[29]. Sexual transmission is possible particularly in people with high-risk sexual activity[30]. Finally, inapparent parenteral transmission has been reported as common in areas at high endemicity, where intrafamiliar spread of the infection is common.

HDV infection can be transmitted either as co-infection, *i.e.,* the simultaneous transmission of both HBV and HDV or as super-infection, *i.e.,* the acquisition of HDV by an already chronic carrier of HBV and the course of the infection varies greatly, accordingly. Indeed, during co-infection the host response to HBV is responsible for HDV persistence and viral clearance as observed in more than 95% of adult cases[31]. Acute HDV co-infection can be characterised by a more severe

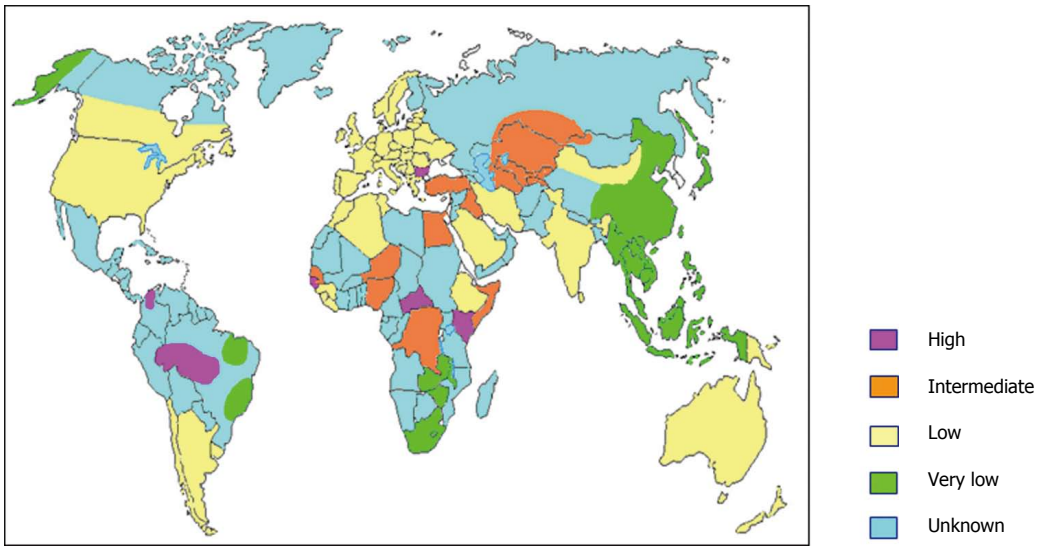

Figure 1 Estimated world epidemiology of hepatitis delta virus infection.

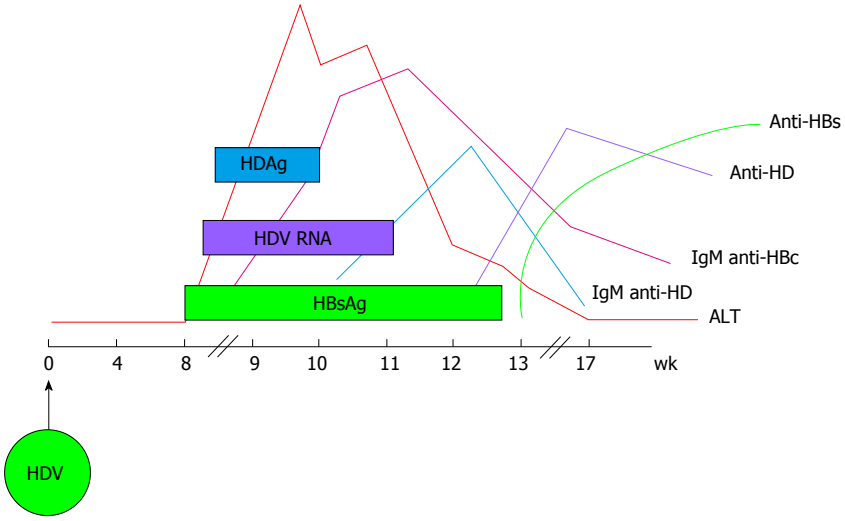

Figure 2 Typical serologic pattern of coinfection by hepatitis B virus and hepatitis delta virus. The ALT peak is characterised by a double phase being the first due to HBV replication and the second to HDV replication. IgM anti-HDV appear quickly, followed by seroconversion to IgG anti-HD. HBV infection in this phase is revealed by the presence of IgM anti-HBc and HBV viremia. The self-limiting coinfection is transient and persistence of serum IgG anti-HD is the only marker of past HDV infection. HDV: Hepatitis delta virus; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferases.

course than acute HBV mono-infection, often resulting in acute liver failure. HDV super-infection of an individual already chronic carrier of HBV, results in chronic HDV infection in the vast majority of cases[32]. It can present as an acute hepatitis in a previously unknown HBV chronic carrier and it is often misdiagnosed as an acute HBV or as a worsening of chronic HBV infection. The typical course of HDV coinfection is characterised by the appearance of serum HBsAg and HDAg during the increase of alanine aminotransferases (ALT), followed by HDV viremia (Figure 2). The ALT peak during coinfection is typically characterised by a double phase: The first is due to HBV replication while the second is related to HDV replication. IgM anti-HDV appear quickly, followed by seroconversion to IgG anti-HD. HBV infection in this phase is revealed by the presence of IgM anti-HBc and HBV viremia. The self-limiting coinfection is transient and persistence of serum IgG anti-HD is a marker of past infection. The typical course of HDV superinfection is characterised by a very rapid appearance and very high levels of HDV viremia and serum HDAg (Figure 3). The more rapid course of superinfection compared to coinfection is due to the chronic carriage of HBsAg. This situation entails that HDV infects for the first time an organism in which the mechanism of HBsAg production is already set and perfectly functioning, giving rise to the assembly of new HDV virions much rapidly than when the two viruses infect together for the first time. Severe acute hepatitis characterised by ALT elevation follows the peak of viremia and is coincidental with IgM anti-HD rise. Markers of HBV infection are usually inhibited with IgM anti-HBc and HBV DNA typically testing negative. Superinfection's progress is chronicity in more than 70% of cases.

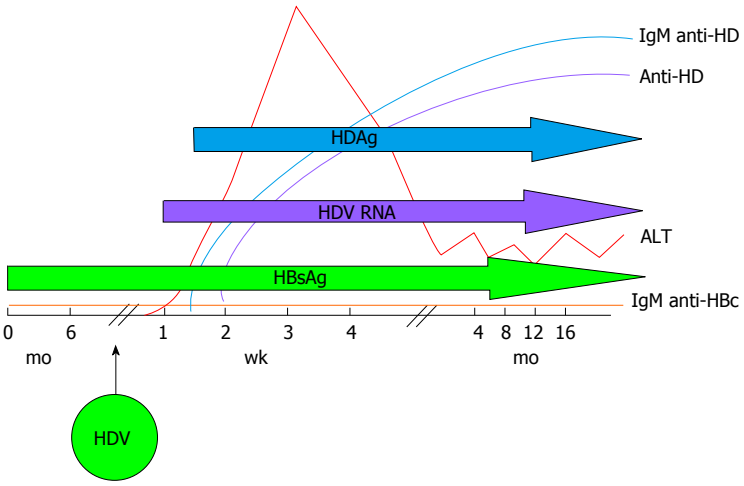

Figure 3 Typical serologic pattern of hepatitis delta virus superinfection: Severe acute hepatitis is characterised by alanine aminotransferases elevation that follows the peak of hepatitis delta virus viremia and is coincidental with IgM anti-HD rise. Markers of HBV infection are usually inhibited. IgM anti-HBc and HBV DNA typically test negative. HDV: Hepatitis delta virus; HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferases.

NATURAL HISTORY

The state of chronic carrier of HBV, represents the ideal environment for the maintenance of HDV replication. As a consequence, the chronic form of hepatitis Delta is, in the vast majority of cases, the sequela of an acute superinfection. Despite this knowledge, the clinical course of chronic HDV infection may be characterised by highly variable patterns ranging from mild, slowly progressive disease to aggressive disease characterised by a severe course that may lead to the fearsome form of acute on chronic liver failure and death in a few weeks, resolvable with liver transplantation, only. Epidemiological studies on genotypes distribution have demonstrated that different clinical courses may rely upon the presence of genotypes more virulent than others. Indeed, progressive forms of chronic HDV infection have been reported from Far East and South America, where genotypes 2 and 3, respectively, have been described and associated to rapidly progressive diseases. The distribution of genotype 1 is worldwide and the disease caused by this virus may have a variable course. Genotype 4, typically isolated in the Far East is usually characterised by a mild form of hepatitis. Finally, genotypes 5-8 have been described in West and Central Africa but there are not enough epidemiological data coming from these areas allowing conclusions on the clinical course characterised by these last viral types[33]. The different clinical course determined by genotypes may depend by different virion assembly as demonstrated by *in vitro* studies. These studies have demonstrated a higher assembly efficiency for genotype 1 as compared to genotype 2[34]. Supporting these findings are data demonstrating RNA replication fitness as low as 100-fold in a clone of genotype 2 from Taiwan as compared to a clone of genotype 1 from Italy[35]. Finally it has been suggested that HBV genotypes also may affect the clinical course as well as disease severity of chronic HDV infection. Indeed HBV genotype C appears to be associated to a worse clinical outcome while HBV genotype A appears to be able to determine lower HDV viral load[36].

As a general concept, cirrhosis occurs within few years from HDV infection in about two thirds of cases, and there is a three-fold higher risk of progression to cirrhosis as compared to patients with chronic HBV monoinfection only. Our recent publication on the natural history of 299 patients followed-up for a median of 28 years indicated the occurrence of cirrhosis at an annual rate of 4%[4]. However, the occurrence of cirrhosis may be characterised by a stable clinical state as well as progression to liver decompensation and development of hepatocellular carcinoma (HCC). Early reports on the natural history of chronic HDV infection indicated that the occurrence of HCC was quite unusual in this setting. Nevertheless, a more recent report on 200 Child-Pugh A class cirrhotic patients followed for 6.6 years, indicated that HDV infected patients had a 3.2-fold increased risk of developing HCC compared to the risk of HBV monoinfected patients[5]. Furthermore, the analysis of data on the risk of developing liver decompensation indicated again a 2.2-fold increased risk for chronic HDV infection with regard to chronic HBV monoinfected[5]. We demonstrated that in our experience clinical decompensation was the dominant complication of HDV cirrhotic patients with an annual incidence rate of 2.7% and that persistent HDV replication was associated with the risk of developing liver decompensation[4]. Finally, our data showed that persistent HDV replication was the only predictor of liver-related mortality[4].

The pathogenetic mechanisms of HDV-related liver disease is still a matter of controversy. Indeed, although conclusive evidence supporting a direct correlation between levels of HDV replication and progression to higher levels of liver disease are still weak, our group has recently demonstrated that HDV RNA levels above 600.000 copies/mL are discriminating for cirrhosis development[37]. On the other hand, in spite of data

available indicating that hepatitis D is mostly an immune-mediated disease, the observation of unusual histologic findings in fulminant hepatitis induced by HDV genotype 3 leaves room to the hypothesis of a direct cytopathic mechanism[38].

Whatever the mechanism, the optimal therapy against HDV should take into account the different pathogenetic hypothesis combining an immuno-stimulating role able to enhance the immunity against HDV and an anti-viral role, able to reduce and possibly eradicate the virus, leading to a long lasting control of the infection.

TREATMENT

Treatment of chronic HDV infection is difficult. The ideal drug would be able of inducing HDV clearance but also HBV clearance. Several studies have been published over time regarding treatment of chronic HDV infection with recombinant interferon (IFN)[39]. The indications emerging from these studies were that IFN is efficient in reducing transaminases but not in HDV RNA clearance. Better results were obtained with higher doses (9MU thrice weekly) and for longer time (12 mo)[40]. Subsequent studies have investigated the use of nucleos(t)ide analogues. Lamivudine alone did not emerge as effective at reducing HDV RNA concentrations and did not increase sustained virological response when combined with IFN[41]. Famciclovir and Ribavirin alone or in combination with IFN did not improve the virological response[42,43]. Entecavir was demonstrated to reduce ALT and HBV DNA in a small subset of patients but not HDV viremia[44]. More recently, several reports have investigated the use of pegylated interferon for chronic HDV infection, showing higher rates of virological response as compared to standard IFN, but still quite disappointing (17%-43%)[45-47]. Finally, the addition of adefovir or tenofovir to pegylated IFN did not improve the virological response rates[48].

Attempts on looking for new antiviral compounds are currently ongoing. Since prenilation of large-HDAg is essential for viral assembly and secretion and in mouse models prenylation inhibitors have demonstrated interesting results in terms of inhibition of assembly and release of HDV with consequently a rapid clearance of HDV RNA in serum, these compounds are under evaluation in humans as well[49]. Moreover, myristoylated synthetic peptides specific for the N-terminal region of the pre-S1 domain of HBsAg are able to inhibit viral attachment and HDV infectivity. These peptides could represent another therapeutic opportunity.

CONCLUSION

Ever since virus discovery more than three decades ago, several important studies have shed light on viral structure, mechanisms of viral replication, viral heterogeneity, clinical course and natural history of HDV infection. The declining prevalence observed mainly in the areas of initial endemicity like Europe, has significantly diverted the interest and research efforts from HDV in the belief that it was a vanishing disease. The awareness of massive immigration from areas of very high HDV prevalence has recently brought new attention on one of the most severe forms of viral hepatitis in humans. Physicians in their daily practice should keep in mind that any chronic carrier of HBV is potentially at risk of HDV superinfection and an efficient treatment against HDV is still unavailable.

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MINIREVIEWS

Liver transplantation for cholangiocarcinoma: Current status and new insights

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Abstract

Cholangiocarcinoma is a malignant tumor of the biliary system that can be classified into intrahepatic (iCCA), perihiliar (phCCA) and distal. Initial experiences with orthotopic liver transplantation (OLT) for patients with iCCA and phCCA had very poor results and this treatment strategy was abandoned. In the last decade, thanks to a strict selection process and a neoadjuvant chemoradiation protocol, the results of OLT for patients with non-resectable phCCA have been shown to be excellent and this strategy has been extended worldwide in selected transplant centers. Intrahepatic cholangiocarcinoma is a growing disease in most countries and can be diagnosed both in cirrhotic and in non-cirrhotic livers. Even though OLT is contraindicated in most centers, recent investigations analyzing patients that were transplanted with a misdiagnosis of HCC and were found to have an iCCA have shown encouraging results. There is some information suggesting that patients with early stages of the disease could benefit from OLT. In this review we analyze the current state-of-the-art of OLT for cholangiocarcinoma as well as the new insights and future perspectives.

Key words: Orthotopic liver transplantation; Perihiliar cholangiocarcinoma; Intrahepatic cholangiocarcinoma

Core tip: Cholangiocarcinoma is a malignant tumor of the biliary system. Perihilar cholangiocarcinoma is an accepted indication for orthotopic liver transplantation (OLT) in some centers under a strict selection process and after neoadjuvant chemoradiation. Intrahepatic cholangiocarcinoma is a formal contraindication for LT in most centers worldwide due to the poor reported results. Nevertheless, there is some novel research showing that the results of OLT in early stages of this disease may not be as bad and could potentially be accepted as an indication for transplant. In this review we will analyze the current state-of-the-art of liver transplantation for cholangiocarcinoma.

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INTRODUCTION

Cholangiocarcinoma (CCA) is a malignant tumor of the biliary system that represents approximately 10% of all hepatobiliary malignancies, standing as the second most common primary hepatic tumor of the liver after hepatocellular carcinoma (HCC)[1-3]. Depending on the anatomic location, it is classified into three subtypes: Intrahepatic CCA (iCCA), perihiliar CCA (phCCA) and distal CCA[4].

Surgical treatment is the only curative option for all subtypes[4]. Radical resection offers 5-year survival ranging between 25%-45%[2]. Unfortunately, most tumors are diagnosed at an advanced stage and the resectability rate is low. Many patients are not candidates for surgical excision due to the extent and location of the tumor or due to the underlying liver disease. In these patients, orthotopic liver transplantation (OLT) would appear a possible alternative of treatment[5].

Liver grafts are a scarce source. In these regards, it is important to take into account the maximum benefit of OLT offered to recipients who are included in the waiting list[6]. There are many authors who consider that long-term survival of recipients after OLT for a specific condition must reach the results of all other accepted indications[5,7,8]. Nevertheless, a 5-year survival after OLT over 50% has been considered acceptable[6] and it is currently the accepted survival for patients undergoing OLT for malignancies (mostly HCC) in most centers worldwide.

The aim of this paper is to review the role of OLT in the management of CCA and to describe the most recent advances in knowledge and the ongoing research in the field.

PHCCA

phCCA is an uncommon and aggressive malignancy of the biliary tract whose incidence is increasing[9,10]. It represents about two-thirds of all cases of CCA[5] and can be defined as a tumor that involves or is in close vicinity to the bile duct confluence[11]. Although no specific etiologic factor can be found in most patients, an association between long-standing biliary inflammation and development of CCA has been observed[9]. The risk factors for the development of CCA include primary sclerosing cholangitis (PSC), with a prevalence of phCCA ranging between 5% to 15%, choledocal cyst disease, hepatolithiasis and infection with certain parasites[9,10,12-15].

The existence of different nomenclatures and the lack of a reliable staging system have created problems to compare the management and outcomes of phCCA. The term phCCA was initially introduced by the John Hopkins group, and most recently adopted by the American Joint Committee on Cancer[16]. Fortunately, nowadays, this term is widely employed by the surgical community worldwide, making it easier to study outcomes of patients diagnosed with these tumors[11,16,17].

The results of nonsurgical therapies for phCCA have been disappointing and most of the patients survive less than 1 year after diagnosis[9,18]. The most important prognostic factor is to achieve a complete resection at the time of surgery, but this is only achieved in 25%-40% of the cases[5,19-21]. The current 5-year survival rate after surgery, even in select cases, rarely exceeds 40%[20,22-24]. Moreover, currently, no effective neoadjuvant or adjuvant therapy is available for ameliorating the outcomes of liver resection[25].

For tumors that are locally unresectable due to the invasion of major vessels, bilobar tumor involvement or insufficient hepatic reserve, a total hepatectomy with regional lymphadenectomy followed by an OLT could be a good alternative. This approach achieves a wide resection margin and the treatment of the underlying disease[26].

The early experience before 2005 with OLT in phCCA was disappointing. The first series reported 5-year survivals ranging from 18% to 25%[9,27-31]. However, these figures were refuted when two American groups developed a new concept that improved the outcomes of OLT for these tumors. The University of Nebraska introduced the routine use of neoadjuvant therapy prior to OLT[32] and this new approach was posteriorly adopted and redefined by the Mayo Clinic group[33]. Before this year, some authors had already suggested that the use of neoadjuvant therapy enhanced the outcomes of OLT in phCCA[3,10,34-36]. The Mayo Clinic group reported the inclusion of 71 patients in the transplant treatment protocol and 38 underwent OLT. One-, 3- and 5-year survival rates were 92%, 82% and 82% after OLT. Once recurrence and survival rates were analyzed, they found better outcomes in transplanted patients compared to patients undergoing resection[33]. The Mayo Clinic protocol involves careful selection of patients with unresectable *de novo* phCCA or phCCA in the setting of PSC without intrahepatic or extrahepatic metastases. Positive lymph nodes are an absolute contraindication. Criteria for anatomical unresectability include bilateral segmental ductal extension, encasement of the main portal vein, unilateral segmental ductal extension with contralateral vascular encasement and unilateral atrophy with contralateral segmental ductal or vascular involvement. There are no longitudinal limits for bile duct involvement[37]. A pancreaticoduodenectomy combined with OLT is justified to reach a R0 resection. The upper limit of tumor size is 3 cm when a mass is visible on cross sectional imaging studies. Patients initially receive external-beam radiation (45 Gy in 30 fractions, 1.5 Gy twice daily) and continuous infusion of 5-flurouracil administered over 3 wk. Brachytherapy (20 Gy at 1 cm in approximately 20-25 h) is administered 2 wk following completion of external beam radiation therapy.

After that, patients are treated with oral capecitabine, administered until the time of transplantation. An exploratory laparotomy is performed to exclude metastatic disease in all patients. Staging laparotomies are performed as patients come close to being on the top of the waiting list for deceased donor liver transplantation or the day before in the setting of live donor liver transplantation[10,33,37].

The Mayo Clinic group also published an update to their series with the aim of identifying prognostic factors. They found that older recipient age, prior cholecystectomy, CA-19.9 more than 100 at the time of OLT, visible mass on cross-sectional imaging and prolonged waiting times were related with worse prognosis[34]. This group attributes their success to both patient selection and neoadjuvant treatment. Currently, 10-20 patients are enrolled in the neoadjuvant therapy and OLT transplantation per year in this center[37].

The survival for transplanted patients with phCCA arising in the setting of PSC is better than for patients with *de novo* phCCA. It could be explained due to close follow-up in PSC patients, making an earlier diagnosis compared to patients with *de novo* CCA[38,39]. The same authors observed that pretreatment pathological confirmation was not associated with a statistically significantly higher risk for recurrence after OLT and they concluded that pathological confirmation before therapy is desirable, but it should not be a requirement for enrolling into their protocol[39].

Encouraged by the Mayo Clinic outcomes, in 2009, the United Network of Organ Sharing/Organ Procurement and Transplantation Network approved the allocation of a standard Model of End-stage Liver Disease (MELD) exception score for patients with phCCA who completed a standardized neoadjuvant therapy protocol[40,41]. Due to the lack of data, the MELD score was set to equal the current standard assigned score for HCC[40].

Other studies have confirmed the good outcomes of OLT for phCCA following this protocol. Darwish Murad *et al*[40] presented a multicenter study including 12 large-volume centers in the United States. Centers with three or more cases performed between 1993 and 2010 were included. They found that patients with phCCA who were treated with neoadjuvant therapy followed by OLT had a 65% 5-year disease-free survival and the intention-to-treat 5-year survival was 53%. The drop-out rate after 3.5 mo of treatment was 11.5%. Forty-three patients (20%) developed recurrence after OLT. This figure is very low compared with recurrence in patients who were transplanted without the use of any neoadjuvant protocol, which ranged from 53% to 84%. They concluded that this therapy was highly effective and that the MELD exception was appropriate[40].

The use of a multimodality oncologic approach including neoadjuvant chemo radiotherapy with subsequent OLT achieves excellent results for patients with localized, regional lymph node-negative phCCA. Patient survival after OLT is comparable to the results of OLT for other causes. OLT for phCCA should be considered an option in patients diagnosed of an un-resectable phCCA, in centers where the pre-transplant treatment of these patients is optimal. One of the main challenges of this protocol is to determine what patients are unresectable as this can differ between centers.

ICCA

The incidence of iCCA or peripheral CCA is increasing globally[5,17], and this tumor is responsible for 0%-20% of deaths related to an hepatobiliary malignancies[2,41]. In United States, 5000 new cases of iCCA are diagnosed each year[2].

Recent publications have suggested a strong association between the development of iCCA, hepatitis B and C and metabolic syndrome[5,42,43]. Hepatitis C virus (HCV), whose incidence is still increasing, is an etiological factor for hepatitis and cirrhosis and it has been clearly identified as one of the main risk factors for the development of HCC[42]. Different studies have found an increased prevalence of HCV in patients diagnosed of iCCA. Other publications also suggest that hepatic cirrhosis is one of the main risk factors for the development of iCCA as it is for HCC[42-46]. Hepatocytes and cholangiocytes share progenitor cells, therefore some authors have postulated that HCV could induce carcinogenesis in both cell types by the same mechanism[42].

HCC represents the most common primary tumor encountered in the liver and its incidence is also growing in Western countries[47]. The increased incidence of iCCA and its association with the presence of cirrhosis makes necessary to accurately differentiate between both tumors, as their treatment options and prognosis differ significantly[44]. However, diagnosis is particularly complex in cirrhotic patients; the distinction between high-grade dysplastic nodules, iCCA and HCC can pose a challenge[48]. When dynamic imaging studies (contrast enhanced computed tomography or magnetic resonance imaging) show an intrahepatic lesion in a cirrhotic liver, with atypical features of HCC, a tumor biopsy should be the next diagnostic step. The problem is that a biopsy is not always feasible due to coagulopathy or refractory ascites and does not always provide a reliable diagnosis[49,50].

Surgical treatment with hepatic resection and R0 margins is the only potential curative option[2,51], but this goal is achieved in less than 30% of patients as many of them are not candidates for resection at the time of presentation[4,20]. Following surgical resection, the median disease-free survival is around 26 mo and the reported rates of recurrence range around 60%-65%[52-54]. There are some cases where resection is not feasible due to the presence of decompensated cirrhosis or significant portal hypertension. Also, the proximity or involvement of main vascular structures of the liver may preclude surgical treatment. It is in such cases where OLT may become an alternative to surgical resection[7].

Table 1 Patient survival and tumor-free survival in patients with intrahepatic cholangiocarcinoma and mixed hepatocellular-cholangiocarcinoma

Ref.	*n*	No. of iCCA/HCC-CC	Patient survival (%)			Tumor-free survival (%)		
			1-yr	3-yr	5-yr	1-yr	3-yr	5-yr
O'Grady *et al*[76]	13	13 iCCA	38	10	10	-	-	-
Yokoyama *et al*[77]	2	2 iCCA	50	0	-	-	-	-
Pichlmayr *et al*[78]	18	18 iCCA	13.9	0	-	-	-	-
Pichlmayr *et al*[78]	22	22 iCCA	20.8	0	-	-	-	-
Casavilla *et al*[55]	20	20 iCCA	70	29	18	67	31	31
Shimoda *et al*[63]	16	8 iCCA 8 HCC-CC	62	39	-	70	35	-
Robles *et al*[27]	23	23 iCCa	77	65	42	68	45	27
Ghali *et al*[56]	10	9 iCCA 1 HCC-CC	-	30	-	-	-	-
Fu *et al*[66]	11	11 iCCA	50.5	50.5	-	51.9	51.9	-
Sapisochin *et al*[64]	14	6 iCCA 8 HCC-CC	79	66	47	60	50	30
Vallin *et al*[65]	10	10 iCCA	80	60	24	40	50	50
Sapisochin *et al*[68]	29	29 iCCA	79	61	45	89	71	71
	⩽ 2 cm	8 iCCA	100	73	73	0	0	0
	Multiple or single > 2 cm	21 iCCA	71	43	43	74	58	58
Facciuto *et al*[71]	32	16 iCCA 16 HCC-CC	71	-	57	62	-	44

iCCA: Intrahepatic cholangiocarcinoma; HCC-CC: Hepatocellular-cholangiocarcinoma.

iCCA as an indication for OLT is still highly controversial. OLT seems a promising treatment as it provides both a wider surgical margin and a potential cure for the underlying liver disease[55]. Nevertheless, most of the publications regarding OLT and iCCA have shown high tumor recurrence rates and poor long-term survival[5,27,55-59]. The main cause of death following OLT for iCCA is tumor recurrence, occurring in a range between 60%-90% of the patients[54,58,60,61]. These poor outcomes have also been described in patients with subtypes of iCCA such as mixed hepatocellular-cholangiocarcinomas (HCC-CC)[5]. It is important to address though, that most of these studies are single-center experiences, with a small number of patients, without differentiation between iCCA and phCCA and including patients both with and without liver cirrhosis. As with HCC, the presence of an iCCA on a cirrhotic liver may have a different behaviour than its development on a healthy liver and the results after OLT may also be different[62].

The results published until late 2000, showed a 5-year actuarial survival that ranges between 10%-18% (Table 1).

Robles *et al*[27] published a multicentre retrospective study in 2004 in which 23 transplanted patients with iCCA were analyzed, finding a 5-year survival of 42%, and a recurrence rate of 35%. The mean time between transplantation and recurrence was 22 mo. Ghali *et al*[56], in a retrospective study that aimed to review the outcomes after OLT in recipients found to have an incidental iCCA in their explanted native liver, showed that the long-term survival rates were not better than those seen in patients with known iCCA. We reported, in collaboration with the University of California, San Francisco, a study comparing patients that were transplanted due to HCC, but were found to have a pathological diagnosis of iCCA or HCC-CC, with a group of patients with pathological diagnosis of HCC. The incidence of iCCA and HCC-CC previously undiagnosed was 3.3%. It was observed that these tumors were associated with bad prognosis and high recurrence rate after OLT, finding a significant difference with those patients with HCC[63,64]. In all of the previous studies, probably due to the number of patients included, no subgroups could be made according to different tumor sizes or numbers.

Due to the poor results of OLT for iCCA, many authors have proposed to determine the tumor factors responsible for recurrence. Different factors such as vascular or lymphatic invasion and size or number of lesions[1,27,55,58] may need to be considered for strict patient selection. Recent studies have shown encouraging results that could potentially change the management of patients with iCCA on cirrhotic livers. Along these lines, Vallin *et al*[65], knowing that small iCCA might be undiagnosed or misdiagnosed as HCC in the context of liver cirrhosis, tried to determine the prevalence and clinical impact of undetected iCCA in liver explants of adult cirrhotic patients undergoing OLT. They identified iCCA in 10 patients (1%), being 4 of them less than 2 cm. Post-transplant tumor recurrence of the whole cohort was observed in 5 patients (50%) and all of them died. The authors couldn't determine if tumor size was associated with recurrence[65]. Fu *et al*[66] reported a retrospective study, evaluating 11 patients who, in absence of lymph node, vascular or bile duct involvement, underwent OLT and whose 3-year disease-free survival rate was 52% and recurrence rate was 45%. They reported a 4-year survival for selected patients of 50%.

In 2014, we coordinated a Spanish multicenter effort with the participation of 16 Spanish groups and published some novel results. The first part of the study aimed to evaluate the outcome of cirrhotic patients with HCC-CC or iCCA on pathological examination after OLT for HCC. This group of patients was compared to a control group of patients with HCC. The total number of patients with both tumors was 42, being the largest series published to date. A subdivision was made, according to the size and number of tumors, following the Barcelona Clinic Liver Cancer staging classification. The tumors were classified in single tumors $\leqslant$ 2 cm and multinodular or uninodular > 2 cm. One of the most salient results of that study was that there were no significant differences in the actuarial survival between patients in both the study and the control groups. Contrary, those patients with multinodular or larger tumors had a worst survival when compared to similar HCCs[67-69].

In a subsequent study, the risk factors for iCCA recurrence after OLT in cirrhotic patients were analyzed in 29 patients whose explanted liver showed an iCCA (both misdiagnosed as an HCC in preoperative imaging or incidental tumor). The main objective of this research was to analyze if there was a subgroup of patients with iCCA in which the results of OLT, in terms of survival, were acceptable. A subgroup of patients with single $\leqslant$ 2 cm (described as "very early" iCCA) was identified. Patients in this group did not present tumor recurrence and the 1-, 3- and 5-year survival was 100%, 73% and 73%, respectively, with a median survival of 52 mo[68]. This was the first study to report that patients with "very early" iCCA can have an acceptable survival after transplant and this may "open" a new indication for OLT for these patients. Nevertheless, the number of patients analyzed in that study was very limited and these outcome will need to be validated in new studies[4,68,70]. On-going research in the field is being conducted to validate the previous results of this experience but ultimately a prospective study will need to be performed to ensure these good results and to be able to include patients with "early or very early" iCCA in the waiting list for OLT. In our opinion, as occurred with OLT for HCC, the identification of a subgroup of patients with iCCa at initial phases will expand the indication for transplantation in these cases.

Subsequently, Facciuto *et al*[71] published a retrospective study where they identified 32 patients with cirrhosis and intrahepatic bile duct tumor on explant specimen. This series showed that patients with iCCA within Milan criteria[72] had a 5-year tumor recurrence rate of 10% and a 5-year survival rate of 78%, comparable with patients with HCC within Milan criteria. The conclusion of this paper suggests that patients with iCCA within Milan criteria may be able to achieve acceptable long-term post-OLT survival. Furthermore, in a review published by the Mayo Clinic group in 2013, they proposed that, despite the high rate of recurrence reported for these patients, OLT could be considered as an option of treatment in patients with very small iCCA ($\leqslant$ 2 cm) in the context of cirrhosis[52].

According to all these data, LT for "very early" or "early" iCCA may be an option for cirrhotic patients in the future, but further research must be conducted. If the results of these new investigations confirm the good expected outcome, this could potentially become a new indication for LT for a growing disease.

In the International Liver Cancer Association guidelines, the committee suggested that future studies should focus on standardized selection criteria for giving neoadjuvant chemotherapy for patients with iCCA who could be considered candidates for OLT[73]. In spite of the disappointing outcomes of the studies that analyze the role of neoadjuvant therapy in combination with OLT for these tumors[49,69], taking into account the benefits of neoadjuvant therapy for early phCCA, future clinical trials should evaluate the use of combining neoadjuvant therapy with OLT for iCCA[45,73]. The guidelines published in 2014 by Bridgewater *et al*[73] for the diagnosis and management of iCCA affirm that OLT for iCCA or HCC-CC should only be offered in centers with designed clinical research protocols employing adjuvant or neoadjuvant therapy and that futures studies should focus on standardized selection criteria plus adjuvant and/or neoadjuvant therapies with OLT as definitive therapy for iCCA.

The group from the University of California, Los Angeles, has been working on a neoadjuvant protocol for patients with iCCA. They administered chemotherapy alone or combined with radiation before and/or after surgical treatment. They reviewed a series of 40 patients who underwent OLT for locally advanced iCCA and phCCA (26 iCCA and 14 phCCA). The overall 5-year disease recurrence-free survival after OLT was 29% and the recurrence rate was 38%. The shortcoming of this score relies on the low number of cases, mixing different types of tumors and the lack of external validation[5,74]. Indeed this strategy for the management of iCCA looks very promising but future investigation needs to be conducted. With advances in stereotactic body radiation for the treatment of hepatic malignancies this therapy can play an important role in this strategy in the future[75]. Preoperative chemoradiation may be more applicable in patients with large iCCA developing in non-cirrhotic livers than in those patients with cirrhosis that present with a "very early" iCCA but this will need to be assessed in the future.

CONCLUSION

In conclusion, OLT for selected patients with non-resectable phCCA is an established strategy with good results when a strict protocol is applied. Transplantation for iCCA in cirrhotic patients is still very controversial but may be a good option in a highly selective group of patients with small unresectable tumors. Future investigations in the field may confirm previous results and change the management of patients diagnosed

with this growing disease.

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MINIREVIEWS

Benefits of nucleos(t)ide analog treatments for hepatitis B virus-related cirrhosis

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Abstract

Chronic hepatitis B infection induces progressive liver disease. Before nucleos(t)ide analogs (NUCs) became established as a safe and effective treatment for hepatitis B, it was difficult to suppress the activity of the hepatitis B virus (HBV). Currently, many patients with hepatitis or cirrhosis associated with HBV are treated with NUCs for an extended period of time, and the effects, benefits, and limitations of these treatments have been apparent. This article reviews HBV-related cirrhosis, its natural course and survival, histological improvement after NUC treatments, treatment effects for decompensated cirrhosis, the incidence of hepatocellular carcinoma (HCC) after NUC treatments, and the efficacy of NUC treatments before and after the treatment of patients for HBV-related HCC. Of particular interest are the histological improvements, including regression of fibrosis, that have been achieved with NUC treatments. Liver function of patients with decompensated cirrhosis has significantly improved regardless of the type of NUC applied, and treatment with NUCs has reduced the incidence of HCC in cirrhotic patients. However, cirrhosis remains the strongest risk factor for HCC occurrence following NUC treatments, and the long-term cumulative incidence of HCC after NUC treatments remains high. When recurrence does occur, it is important to reconsider the treatment modality according to the degree of improved liver function that was achieved.

Key words: Hepatitis B; Nucleos(t)ide analogue; Liver cirrhosis; Lamivudine; Entecavir

Core tip: We presented the benefits of nucleos(t)ide analogs (NUCs) treatments for HBV-related cirrhosis in this article. NUC treatments have been found to improve inflammation and fibrosis in the liver of cirrhotic patients. Moreover, even in patients with decompensated cirrhosis, liver function has improved in many cases. Furthermore, although NUC treatments can reduce the incidence of hepatocellular carcinoma (HCC), rates of HCC remain high in patients with cirrhosis. NUC treatments have been found to improve liver function and the survival of patients with HCC. Improved liver function was also achieved by providing NUC treatments for hepatitis B virus-related HCC when recurrent tumors

developed. Therefore, it is important to select the most appropriate treatment method considering the alterations in liver function that may occur following NUC treatments.

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INTRODUCTION

An estimated 400 million people worldwide are chronically infected with hepatitis B virus (HBV)[1]. Chronic hepatitis B infection induces a progressive liver disease that can lead to cirrhosis and hepatocellular carcinoma (HCC)[2]. Prior to establishing the antiviral drug, lamivudine, as an effective treatment for hepatitis B[3], it was difficult to prevent disease progression. Lamivudine was the first nucleos(t)ide analog (NUC) to be extensively characterized and it inhibits DNA synthesis. Subsequently, it has been found to rapidly reduce serum levels of HBV and to reduce inflammation in the liver[3]. Moreover, several NUCs, including adefovir dipivoxil, telbivudine, entecavir, and tenofovir disoproxil fumarate, have been developed. Initially, the indication of these drugs was limited to patients with chronic hepatitis or compensated cirrhosis, although they have gradually been applied to the treatment of decompensated cirrhosis. In this review article, we describe the effects, benefits, and limitations of using NUCs to treat cirrhotic patients.

NATURAL COURSE OF HBV INFECTION AND CIRRHOSIS IN PATIENTS

Several studies have documented the natural history of chronic hepatitis B prior to the availability of NUCs (Table 1). For example, Weissberg *et al*[4] studied 379 histologically confirmed chronic hepatitis B patients and reported the following estimated 5-year survival rates: 97% for chronic persistent patients, 86% for chronic active hepatitis patients, and 55% for chronic active hepatitis patients with cirrhosis. Liaw *et al*[5] also reported the natural history of chronic HBV infection following the recent development of histologically confirmed cirrhosis in a cohort of 76 patients. The annual incidences of hepatic decompensation and HCC development were calculated to be 2.3% and 2.8%, respectively. Furthermore, Liaw *et al*[5] estimated the 5-year survival rate to be 80%. de Jongh *et al*[6] conducted a follow-up study of 98 hepatitis B surface antigen - positive cirrhosis patients with histopathologically confirmed cirrhosis. The reported survival probability was 71% after 5 years. For the 21 patients with decompensated cirrhosis, the survival probability was only 14%. Consistent with these previous studies, Realdi *et al*[7] found that the 5-year survival rate for 366 patients with histologically confirmed cirrhosis was 84%. With the exception of the study by Liaw *et al*[5], these studies analyzed the factors affect their prognosis using multivariate analysis: Patient age, total bilirubin levels, albumin levels, platelet counts, hepatitis B e antigen (HBeAg) positivity, ascites, spider nevi, and splenomegaly were reported as significant prognostic factors. Patient age and total bilirubin were the factors that were included in each of the studies. Overall, the 5-year survival rates for HBV-related cirrhosis reported in these studies ranged from 55% to 84%, which may be due to differences in lead time bias, study design, country, ethnicity, HBV genotype, and/or HBeAg positivity.

HISTOLOGICAL IMPROVEMENT IN PATIENTS WITH CIRRHOSIS

Histologically, fibrosis has improved with long-term treatment with NUCs[8-17]. In early studies, lamivudine was administered for a short period of time (*e.g.*, 6-12 mo)[3,8,9]. In 52%-95% of patients that received lamivudine treatment for at least one year, an improvement in necroinflammatory activity was observed [which was defined as at least a 2-point decrease in the histologic activity index (HAI) score][3,9-11]. In contrast, the rate of hepatic fibrosis improvement (defined as at least a 1-point decrease in the HAI fibrosis score) associated with short-term lamivudine treatments were not as high[3,9] and ranged from < 10% to 35%. Since then, long-term use of NUCs has led to improvements in liver fibrosis, even in cases of advanced fibrosis or cirrhosis[11,13,14]. For example, Chang *et al*[13] followed 57 hepatitis B patients that were treated with entecavir for 3-7 years (median, 6 years) and underwent repeated histological examinations. Improved Ishak fibrosis scores ($\geqslant$ a 1-point decrease) were reported for 88% of these patients. In addition, four patients with cirrhosis also demonstrated an improvement in their Ishak fibrosis scores (median decrease: 3 points, range: 1-4). Table 2 summarizes the primary studies that have described histological changes after an initial NUC treatment. Dienstag *et al*[11] reported that fibrosis improved (defined as a decrease in the HAI fibrosis score of at least 1-point) in 45.5% of patients treated with lamivudine (n = 11) after 1 year, and this rate increased to 72.7% after an additional 2 years of treatment. Marcellin *et al*[14] reported that 51% of patients with hepatitis B that were treated with tenofovir (n = 348) showed improvement in fibrosis (defined as a decreased in the Ishak fibrosis score of at least 1-point). In addition, fibrosis improved in 74% of patients with cirrhosis (n = 97) at 5 years. Taken together, these results demonstrate that long-term treatment with NUCs can potentially lead to histological improvements in patients with cirrhosis.

Table 1 Studies characterizing the natural history of hepatitis B-related cirrhosis

Ref.	Publication year	Country	Number of patients with cirrhosis	5-yr survival for patients with compensated cirrhosis (%)	Cause of death
Weissberg *et al*[4]	1984	United States	130	55	Liver failure (70.3%) Unrelated disease (18.9%) Unknown causes (10.8%)
Liaw *et al*[5]	1989	Taiwan	76	80	Hepatic failure or variceal bleeding (100%)
de Jongh *et al*[6]	1992	The Netherlands	98	71	Hepatocellular carcinoma (38.5%) Liver failure or fatal upper gastrointestinal bleeding (38.5%) Unrelated disease (23.1%)
Realdi *et al*[7]	1994	Italy	366	84	Liver failure (53.6%) Hepatocellular carcinoma (27.4%) Unrelated disease (19.0%)

Table 2 Rates of fibrosis improvement in patients with chronic hepatitis or cirrhosis treated with lamivudine, entecavir, or tenofovir

Ref.	Publication year	Nucleos(t)ide	No. of patients	Treatment duration	Cirrhosis percentage	Improvement ratio of fibrosis
Honkoop *et al*[8]	1997	LAM (25 mg, 100 mg, 300 mg)	13	6 mo	Not described	No difference in fibrosis was observed
Lai *et al*[3]	1998	LAM (25 mg, 100 mg, placebo)	358	52 wk	5%	25 mg (n = 72) 5%[1,4] 100 mg (n = 142) 2.5%[1,4] Placebo (n = 143) 0%[1,4]
Suzuki *et al*[9]	1999	LAM (100 mg)	20	52 wk	0%	All patients (n = 20) 35%[1]
Dienstag *et al*[10]	2003	LAM (100 mg, placebo)	63	1 yr + additional 2 yr	17%	Bridging fibrosis (HAI fibrosis score of 3; n = 19) 63% (1 yr + additional 2 yr)[2] Cirrhosis (HAI fibrosis score of 4; n = 11) 45.5% (1 yr)[2]; 72.7% (1 yr + additional 2 yr)[2]
Schiff *et al*[12]	2008	LAM (100 mg) Entecavir (0.5 mg)	245	48 wk	Not described[5]	ETV (n = 120) HBeAg+ 57%[3] HBeAg- 59%[3] LAM (n = 125) HBeAg+ 49%[3] HBeAg- 53%[3]
Chang *et al*[13]	2010	ETV (0.5 mg)	57	48 wk, long-term (range: 3-7 yr, median: 6 yr)	7%	All patients (n = 57) 32% (48 wk)[3] 88% (long-term)[3] Cirrhosis (n = 4) 100% (long-term)[3]
Marcellin *et al*[14]	2012	TDF	348	5 yr	28%	All patients (n = 348) 51% (5 yr)[3] Cirrhosis (n = 97) 74% (5 yr)[3]

[1]Fibrosis improvement was defined as an HAI fibrosis score decrease of at least 1-point; [2]Bridging fibrosis improvement was defined as achieving an HAI fibrosis score of 0 or 1. Cirrhosis improvement was defined as achieving an HAI fibrosis score of 0, 1, or 3; [3]Fibrosis improvement was defined as a decrease in the Ishak fibrosis score of at least 1-point; [4]Approximate value estimated from the published graph; [5]All patients had advanced fibrosis or cirrhosis (Ishak fibrosis scores of 4-6). LAM: Lamibudine; ETV: Entecavir; TDF: Tenofovir; HBeAg: Hepatitis B e antigen; HAI: Histologic activity index.

NUC TREATMENT FOR DECOMPENSATED CIRRHOSIS

Once lamivudine treatment was established as an effective and safe drug for the treatment of chronic hepatitis B or compensated cirrhosis[3,10], it was gradually applied to the treatment of decompensated cirrhosis[18-30]. The 1-year, 3-year, and 5-year survival probabilities for patients with decompensated cirrhosis without NUC treatment were: 70%-71%[6,31], 35%-40%[6,31] and 14%-35%[6,31,32], respectively. These survival rates increased dramatically following the use of NUC treatments to: 70%-94%, 63%-87%, and 55%-86%, respectively[18,23,26,29] (Table 3). The liver function of the latter patients also significantly improved regardless of the type of NUC administered[18-30]. In addition, NUC treatment led to a reduction in the Child-Pugh class or a decrease in the Child-Pugh score ($\geqslant$ 2-points or $\geqslant$ 3-points decrease), in a substantial number of cases[18-28,30]. However, there were a small number of patients that were treated with NUCs who progressed to death or required a liver transplant[18-30]. Studies that analyzed the determinants of early mortality in patients with decompensated cirrhosis B treated with NUCs found that poor baseline liver function was associated with poor prognosis[23,26,30]. Furthermore, most of the deaths occurred within 1 year after NUC treatment, and the most common causes of death were liver failure or complications from liver failure[23,26,30]. In work by Hyun *et al*[26], Child-Turcotte-Pugh scores at baseline and the

Table 3 Effects of treatment with nucleos(t)ide analogs in patients with decompensated cirrhosis

Ref.	Nucleos(t)ide	Patient number	Treatment duration	Improvement ratio of Child-Pugh score	Cumulative survival rate[3]
Villeneuve *et al*[18]	LAM (100 mg or 150 mg)	35 (CPB: 10, CPC: 25)	Mean: 19 mo	62.9% (22/35)[1]	1 yr: 78%[4] 2 yr: 63%[4]
Kapoor *et al*[19]	LAM (150 mg)	18 (CPB: 14, CPC: 4)	Mean: 17.9 mo (range: 9-31 mo)	CPB to CPA: 50% (4/14) CBC to CPB: 50% (2/4)	No deaths attributed to liver disease
Yao *et al*[20]	LAM (150 mg)	13 (CPB: 0, CPC: 13)	Mean: 17.5 mo (range: 3-39 mo)	69% (9/13)[2]	Not described
Hann *et al*[22]	LAM (100 mg)	75 (CPA: 4, CPB: 28, CPB: 43)	Mean: 12.7 mo (range: 0.5-33 mo)	31% (23/75)[1]	Not described
Tseng *et al*[23]	LAM (100 mg)	30 (CPB: 16, CPC: 14)	Mean: 39.7 mo (range: 3-128 mo)	CPB to CPA: 62.5% (10/16) CBC to CPB: 35.7% (5/14)	1 yr 70%[4] 2 yr 66%[4] 3 yr 55%[4] 5 yr 55%[4]
Bae *et al*[24]	LAM (100 mg)	17 (CPB: 12, CPC: 5)	Mean: 28 mo (range: 14-42 mo)	CPB to CPA: 83% (10/12) CBC to CPB (1/5) or CPA (3/5): 80% (4/5)	Not described
Shim *et al*[25]	ETV (0.5 mg)	55 (mean CP score 8.1 ± 1.7)	12 mo	49% (27/55)[1]	12 mo: 87.1% 24 mo: 83.0%
Hyun *et al*[26]	LAM (100 mg) ETV (0.5 mg)	86 (CPB: 45, CPC: 41)	Mean: 2 yr	Mean Child-Pugh score Baseline LAM: 9.5, ETV: 9.6 12 mo LAM: 6.7, ETV: 6.6	1 yr LAM: 92.4% ETV: 90.7% 3 yr LAM: 86%[4] ETV: 76%[4]
Liaw *et al*[27]	TDF (300 mg)/FTC (200 mg) + TDV (300 mg)/ETV (0.5 mg or 1 mg)	112 (median CP score: 7, range: 6-9)	48 wk	TDF: 25.9% (7/27)[1] FTC/TDF: 48.0% (12/25)[1] ETV: 41.7% (5/12)[1]	Not described
Chan *et al*[29]	LAM (100 mg), TdT (600 mg)	228 (CPS < 7: 18, CPS 7-9: 155, CPS 9 <: 55)	52-104 wk	52 wk LAM: 38.6% (44/114), TdT: 31.6% (36/114)[1] 104 wk LAM: 40.4% (46/114), TdT: 38.6% (44/114)[1]	52 wk LAM: 88%, TdT: 94% 104 wk LAM: 79%, TdT: 87%

[1]Improvement of Child-Pugh score was defined as a decrease in the CPS greater than or equal to 2-points; [2]Improvement of Child-Pugh score was defined as a decrease in the CPS greater than or equal to 3-points; [3]Cumulative survival rates calculated by Kaplan-Meier method; [4]Approximate value from the published graph. LAM: Lamibudine; ETV: Entecavir; TDF: Tenofovir; FTC: Emtricitabine; TdT: Telbivudine; CPB: Child-Pugh class B; CBC: Child-Pugh class C; CPS: Child Pugh score; CPA: Child-Pugh class A.

Model for End-stage Liver Disease score at 3 mo after beginning a NUC treatment were found to be significant predictors of early mortality.

INCIDENCE OF HCC IN PATIENTS WITH CIRRHOSIS THAT RECEIVED NUC TREATMENT

Worldwide, HBV infection has been identified as an important risk factor for the development of HCC[31]. Longitudinal studies of patients with chronic hepatitis B infection have described the cumulative incidence of HCC[5,31-35]. Incidence of HCC has been found to vary by region and is influenced by the underlying stage or condition of the liver disease present. For patients with compensated cirrhosis that were not treated with NUCs, the annual incidence of HCC has been reported to range from 2.2%-2.8%[5,31,36,37]. In a comparison of cumulative HCC incidence for patients with and without lamivudine treatment, the former had a significantly lower incidence than the latter in a randomized study[38]. Non-randomized studies have also demonstrated that NUCs reduce the incidence of HCC[39-41]. Furthermore, in three meta-analyses[42-44] and a systematic review[45], NUC treatments were found to consistently reduce the risk of HCC compared with an absence of NUC treatment. In addition, two Asian studies reported that entecavir-treated patients had a reduced risk of HCC compared with treatment-naïve patients with cirrhosis[40,46], and Wong *et al*[46] reported that the 5-year cumulative probability of HCC development among cirrhotic patients was 13.8% in an entecavir cohort *vs* 26.4% in a control treatment-naïve cohort (P = 0.036). Hosaka *et al*[40] conducted a propensity score-matched control study and found that the cumulative 5-year incidence of HCC among cirrhotic patients treated with entecavir (7.0%) was lower than that of a control non-treated group (38.9%) (P < 0.001). Furthermore, the entecavir-treated group had a significantly lower incidence of HCC than a lamivudine-treated group of cirrhotic patients (P = 0.043)[40]. Liver cirrhosis has been found to be the strongest risk factor for the occurrence of HCC after NUC treatment[40,47,48], and the long-term cumulative incidence of HCC after NUC treatment remains high in cirrhotic patients[40,45,46].

EFFICACY OF NUC TREATMENTS FOR PATIENTS WITH HBV-RELATED HCC

Several studies have documented that antiviral therapy with NUCs is beneficial after the treatment of HBV-related HCC[49-52]. For example, improved liver function has been observed following curative liver resection and following radiofrequency ablation for the treatment of HCC[48-50]. Moreover, in a longitudinal randomized clinical trial conducted by Yin *et al*[49] to evaluate the effects of NUC treatments following radical hepatectomy, patients who received NUCs exhibited significantly improved liver function and decreased HCC recurrence. NUC treatment following curative therapy for HCC has also improved overall survival[49-52]. In a study by Kuzuya *et al*[50], NUC treatment improved liver function in patients with recurrent HCC, and allowed all of the treated patients to be eligible for curative treatment for recurrent HCC. In contrast, two-thirds of the untreated group were not eligible for curative therapy for recurrent HCC due to deterioration of remnant liver function[50]. Furthermore, an increasing number of treatment options have become available for recurrent tumors[53,54]. We previously reported that a patient with decompensated cirrhosis was able to undergo a right hepatectomy four years after starting a lamivudine treatment regimen[54].

CONCLUSION

Here, the benefits of NUC treatments for patients with HBV-related cirrhosis have been presented. NUC treatments have been found to improve inflammation and fibrosis in the liver of cirrhotic patients. Moreover, even in patients with decompensated cirrhosis, liver function has improved in many cases. Given that hepatitis B can occasionally lead to death, or the need for a liver transplant, in patients with highly deteriorated liver function even after NUC treatment, it is recommended that a NUC treatment be started as early as possible. Furthermore, although NUC treatments can reduce the incidence of HCC, rates of HCC remain high in patients with cirrhosis. However, NUC treatments have been found to improve liver function and the survival of patients with HCC. Improved liver function was also achieved by providing NUC treatments for HBV-related HCC when recurrent tumors developed. Therefore, it is important to select the most appropriate treatment method considering the alterations in liver function that may occur following NUC treatments.

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ORIGINAL ARTICLE

Retrospective Study

Total esophagogastrectomy plus extended lymphadenectomy with transverse colon interposition: A treatment for extensive esophagogastric junction cancer

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Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Sotero del Rio Hospital.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at marco.ceroni@gmail.com. Consent was not obtained but the presented data are anonymized and risk of identification is low. No additional data are available.

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Abstract

AIM: To review the post-operative morbidity and mortality of total esophagogastrectomy (TEG) with second barrier lymphadenectomy (D2) with interposition of a transverse colon and to determine the oncological outcomes of TEG D2 with interposition of a transverse colon.

METHODS: This study consisted of a retrospective review of patients with a cancer diagnosis who underwent TEG between 1997 and 2013. Demographic data, surgery

protocols, complications according to Clavien-Dindo classifications, final pathological reports, oncological follow-ups and causes of death were recorded. We used the TNM 2010 and Japanese classifications for nodal dissection of gastric cancer. We used descriptive statistical analysis and Kaplan-Meier survival curves. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS: The series consisted of 21 patients (80.9% men). The median age was 60 years. The 2 main surgical indications were extensive esophagogastric junction cancers (85.7%) and double cancers (14.2%). The mean total surgery time was 405 min (352-465 min). Interposition of a transverse colon through the posterior mediastinum was used for replacement in all cases. Splenectomy was required in 13 patients (61.9%), distal pancreatectomy was required in 2 patients (9.5%) and resection of the left adrenal gland was required in 1 patient (4.7%). No residual cancer surgery was achieved in 75.1% of patients. A total of 71.4% of patients had a postoperative complication. Respiratory complications were the most frequently observed complication. Postoperative mortality was 5.8%. Median follow-up was 13.4 mo. Surgery specific survival at 5 years of follow-up was 32.8%; for patients with curative surgery, it was 39.5% at 5 years.

CONCLUSION: TEG for cancer with interposition of a transverse colon is a very complex surgery, and it presents high post-operative morbidity and adequate oncological outcomes.

Key words: Esophagogastric junction cancer; Total esophagogastrectomy; Transverse colon interposition; Total gastrectomy; Total esophagectomy

Core tip: Esophagogastric junction cancers are rare tumors that have increased in prevalence in recent decades due to lifestyle changes. Key results of this study are as follows: (1) We describe a treatment for patients with esophago-gastric junction cancer; (2) We show the surgical details in high quality artwork to better represent the surgery; and (3) We show the postoperative and oncological outcomes of this technique.

Ceroni M, Norero E, Henríquez JP, Viñuela E, Briceño E, Martínez C, Aguayo G, Araos F, González P, Díaz A, Caracci M. Total esophagogastrectomy plus extended lymphadenectomy with transverse colon interposition: A treatment for extensive esophagogastric junction cancer. *World J Hepatol* 2015; 7(22): 2411-2417 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i22/2411.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i22.2411

INTRODUCTION

The incidence of esophagogastric junction (EGJ) cancer has increased steadily in the West for the last 4 decades, with a currently reported prevalence of 5 cases per 100000 people. EGJ cancer corresponds to the increment of adenocarcinoma of the distal esophagus and cardia, which are related to Barrett's esophagus, obesity, pathological gastro-esophageal reflux and smoking[1-4].

Globally, these types of cancers are considered to have a poor prognosis, with a survival rate of 20% at 5 years follow-up in patients who underwent surgery[5]. Another study that included more patients was conducted by Rüdiger Siewert *et al*[6], and a 5-year survival of 32.5% was reported when surgery was performed.

The management of EGJ cancer is controversial. There are several surgical treatment alternatives that differ in morbidity, chance of no residual tumor (R0), and type of lymph node dissection, and there is no consensus on which method is considered to be the best alternative.

Total esophagogastrectomy (TEG) with the interposition of a transverse colon is recommended for patients with extensive EGJ cancers (cancer that has a significant invasion of the esophagus and stomach) or for patients with a concomitant esophageal and gastric cancer (also called double cancer).

TEG with second barrier lymph node dissection (D2) is an infrequently performed surgery because it has been associated with a high postoperative morbidity. Considering national series, only series of less than 20 patients with EGJ cancer have been published to date[7-9].

The main objectives of this study are: (1) to review the post-operative morbidity and mortality of TEG D2 with interposition of a transverse colon; and (2) to determine the oncological outcomes of TEG D2 with interposition of a transverse colon.

MATERIALS AND METHODS

This study consisted of a retrospective review of patients with cancer who underwent TEG between 1997 and 2013. Demographic data, surgery details, postoperative complications, pathological reports of the surgical specimen and oncologic follow-up were recorded.

Information was obtained from medical records, surgery protocols, oncological controls and the national registry of civil information. We used the TNM 2010 and the Japanese classifications for nodal dissection of gastric cancer[10,11]. This study was approved by the ethics committee of our center.

Preoperative study and treatment

Preoperative evaluation of patients included upper digestive endoscopy; computed tomography of the

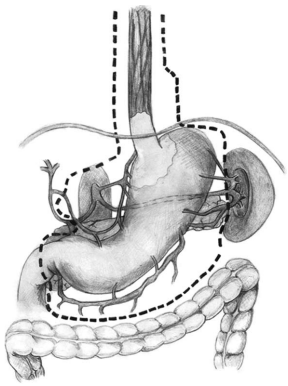

Figure 1 Lymph node dissection when performing total esophagogastrectomy.

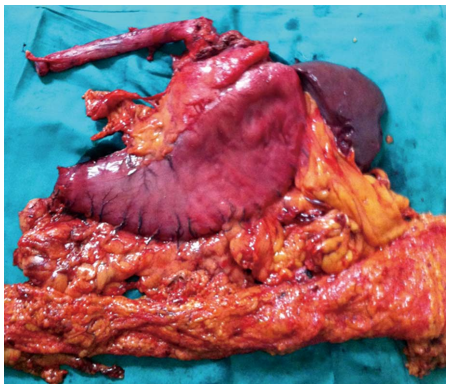

Figure 2 Surgery specimen, which was dissected in the operating room after surgery.

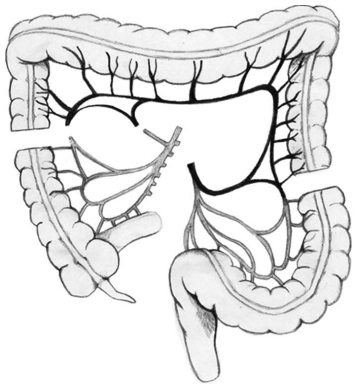

Figure 3 Colon section at the origin of middle colic artery, preserving the marginal arcade.

chest, abdomen and pelvis with use of intravenous contrast; spirometry; colonoscopy; arterial gasometry; serum albumin; general laboratory tests and specific evaluations for each patient's comorbidities. Once the evaluations were complete, the patient was presented in a clinical-radiological meeting of the center, where the best treatment alternative was discussed.

Preoperative treatment of patients included motor and respiratory physiotherapy and colon preparation. A nutritionist assessed all patients. In the cases of patients who presented with significant dysphagia or did not improve their preoperative nutritional status, parenteral nutrition was recommended.

Surgical technique steps

The patient is placed in a supine position over the operating table with the neck rotated to the right side. An upper midline laparotomy is performed, extending it by 2 cm below the umbilicus, and a left cervical approach is also performed.

Resection steps

(1) Exploration of the abdominal cavity searching for the presence of metastasis; (2) Resection of the round ligament of the liver; (3) Dissection of the left triangular ligament and enlargement of the hiatus by a vertical incision on the diaphragm (Pinotti maneuver); (4) Determination of concomitant extension of EGJ cancer into the esophagus and stomach and, if it is significant, performance of the TEG; (5) Performance of an esophagectomy using a transhiatal plus left cervical approach. Dissection of the lower mediastinum and EGJ lymph nodes; (6) Sectioning of the cervical region of the esophagus and near total displacement of the mobilized esophagus into the abdominal cavity through the hiatus. A suture or nasogastric tube is used to guide the colon into the neck through the posterior mediastinum; (7) Performance of a total D2 gastrectomy with omentectomy and bursectomy (Figure 1); (8) Performance of a splenectomy in patients with macroscopic group 11 lymph node metastasis (LNM), or if spleen cancer invasion is observed; and (9) Placement of the surgical specimen on a secondary surgical table for dissection by the surgeon at the end of the surgery (Figure 2)[12].

Reconstruction steps

(1) Dissection of the colon from the cecum to the sigmoid; (2) Selective clamping of the middle and left colic arteries to determine the quality of transverse colon irrigation (colon coloration is observed and the pulse of the marginal artery is observed and manually estimated); (3) Sectioning of the middle colic artery at its origin, preserving Drummond's vascular arcade (Figure 3); (4) Measurement of the transverse colon; *i.e.*, distance from the patient's neck to the root of the mesentery. This measure is applied on the surface of the colon following irrigation from the root of the mesentery to the proximal end; (5) Sectioning of the proximal transverse colon with a blue load of a linear cutting stapler (in the place previously measured; Figure 3); (6) Sectioning of the distal transverse colon where the left colic artery arrives with a blue load of a linear cutting stapler, preserving the continuity of the marginal artery (Figure 3); (7) Ascension of the isoperistaltic transverse colon through the posterior mediastinum to the neck (Figure 4); (8) Manual performance of a coloesophageal anastomosis, colojejunal anastomosis and colo-colonic

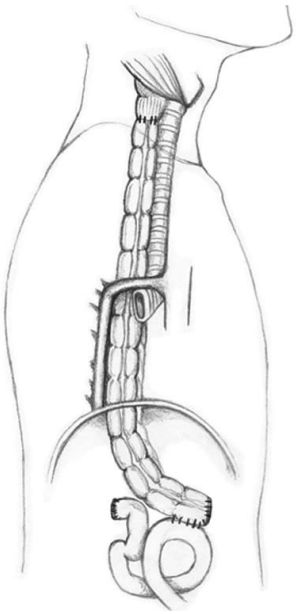

Figure 4 Transverse colon interposition through the posterior mediastinum. Coloesophageal anastomosis and colojejunal anastomosis are shown lateral view.

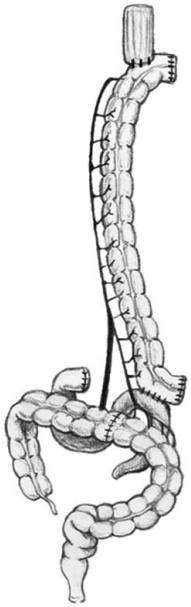

Figure 5 Transverse colon interposition through the posterior mediastinum. Coloesophageal anastomosis and colojejunal anastomosis are shown front view.

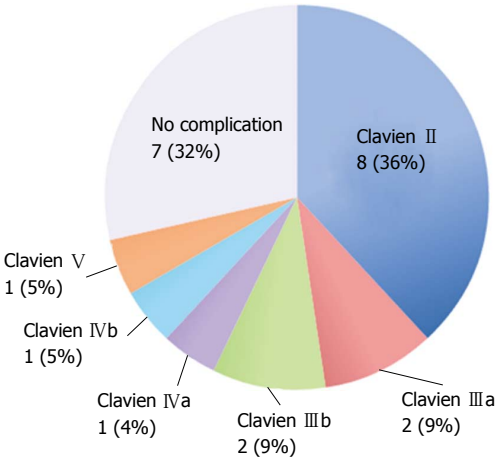

Figure 6 Complications observed according to Clavien Dindo classifications.

anastomosis (Figure 5); (9) Performance of a Witzel type jejunostomy; (10) Installation of one drainage tube to each pleura; and (11) Installation of a Jackson-Pratt type drainage tube to the neck.

Statistical analysis

A statistical analysis was performed using GraphPad Prism software, version 5.0. A Kaplan-Meier method was used to estimate the years of post-operative survival. A *P*-value of less than 0.05 was considered as statistically significant.

RESULTS

TEG and D2 lymph node dissection with transverse colonic interposition was performed between 1997 and 2013 in 21 patients who had extensive EGJ cancer or double cancer. There were 17 (80.9%) men and 4 women (19.1%), with a median age of 60 years (46.7 to 64.2 years). Twelve patients were classified according to the American Society of Anesthesiologists (ASA-1), 8 patients were classified as ASA-2 and 1 patient was classified as ASA-3. The pre-surgery median serum albumin value was 4 g/dL (3.5-4.2 g/dL).

The mean total surgery time was 405 min (352-465 min). Interposition of the transverse colon through the posterior mediastinum was used for replacement in all cases. Splenectomy was required in 13 patients (61.9%), distal pancreatectomy was required in 2 patients (9.5%) and resection of the left adrenal gland was required in 1 patient (4.7%).

The indications of the TEG D2 were: (1) extensive EGJ cancer in 18 patients (85.7%); and (2) two concomitant cancer (esophageal and gastric) in 3 patients (14.2%).

The median hospital stay was 17 d (7 to 26 d), and the median re-feeding time was 11.5 d (8 to 19 d). A total of 15 (71.4%) patients presented with the following complications (detailed according to the Clavien-Dindo classifications in Figure 6): 7 patients (33.3%) presented with a cervical esophagus-colonic anastomosis leak that was managed with medical treatment; 2 patients (9.5%) presented with a colo-colonic anastomosis leak, and both of these patients underwent reoperation. One of them received a new anastomosis and died afterwards, and the other patient underwent a colostomy with a mucous fistula; therefore, the intestinal transit was successfully reconstituted. One patient (4.7%) presented with a colo-duodenal anastomosis leak that was treated medically; 2 patients (9.5%) presented with obstruction of the jejunostomy. Of those patients, 1 patient needed to

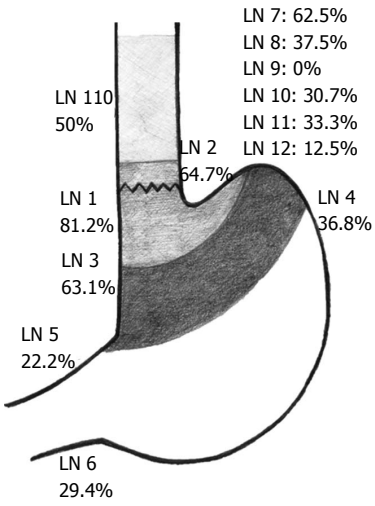

Figure 7 Lymph node metastasis distribution. Only patients with esophagogastric junction cancer are considered. LN: Lymph node according to the Japanese classifications.

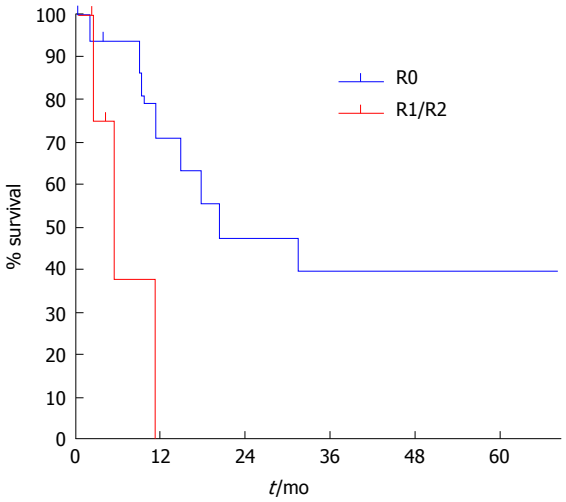

Figure 8 Cancer specific survival at 5 years, according to residual tumors.

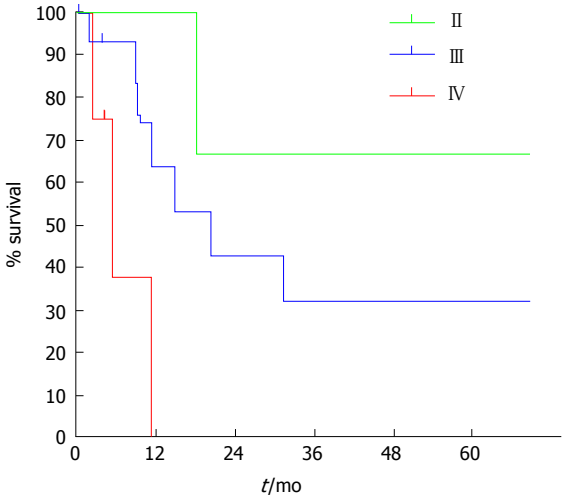

Figure 9 Five year survival follow-up by tumor stage according to the TNM classifications.

Table 1 Cumulative complications (patients may have presented with more than one complication)

Surgical
Esophagocolonic anastomosis leak: 7 (33.3%)
Colocolonic anastomosis leak: 2 (9.5%)
Colojejunal leak: 1 (4.7%)
Jejunostomy obstruction: 2 (9.5%)
Intraabdominal collection: 4 (19%)
Infection of surgery wound: 4 (19%)
Medical
Respiratory: 9 (42.8%)
Sepsis by central venous catheter: 2 (9.5%)
Atrial fibrillation: 1 (4.7%)
Clostridium difficile diarrhea: 1 (4.7%)

be reoperated due to a twist of the jejunostomy, and the second patient, who presented with only a partial obstruction, was resolved by removing the jejunostomy tube. The main medical complications were of the respiratory type, occurring in 9 patients (42.8%). The details of the complications are presented in Table 1.

When observing patients with EGJ cancer, they all presented with pathological evidence of adenocarcinoma. Three patients presented with more than one tumor: 2 of these patients had concomitant esophageal squamous cancer and gastric adenocarcinoma, and the other patient had 3 tumors, including two gastric adenocarcinomas and one squamous esophageal cancer. The median tumor size was 8 cm (6.3 to 12 cm), and the median count of lymph nodes removed was 37 (27 to 49 lymph nodes).

R0 surgery was achieved in 16 patients (76.1%), 1 patient (4.7%) underwent R1 surgery (presenting with positive radial edges) and 4 patients (19%) underwent R2 surgery (3 of those patients presented with isolated peritoneal metastasis and 1 patient presented with incidental finding of pleural metastasis).

The distribution of LNM is shown in Figure 7. The lymph node groups most associated with metastasis were group 1 (81.2%); group 2 (64.7%); group 3 (63.1%); group 7 (62.5%) and group 110 (50%).

Three (14.2%) patients were staged as ⅡB, 1 (4.7%) was staged as ⅢA, 1 (4.7%) was staged as ⅢB, 12 (57.1%) were staged as ⅢC and 4 (19%) were staged as Ⅳ.

To date, a total of 17 patients (80.9%) died: 11 died of cancer (64.7%), 1 died in the post-operative period (5.8%) and 5 died because of non-cancer related causes (29.4%).

The estimated specific survival rate for patients at 5 years follow-up was 32.8% with a median of 18.1 mo. The estimated specific survival rate for patients with R0 surgery at 5 years follow-up was 39.6% with a median of 20.5 mo (Figures 8 and 9).

DISCUSSION

EGJ cancer is an epidemiologically relevant pathology.

There has been a significant increase in its incidence in recent decades, and there will be a greater number of patients with this disease in the future[2-4,13].

The increasing incidence of EGJ cancer is related to changes in lifestyle in developed countries, such as changes in eating habits. In those countries, there are also higher prevalences of gastro-esophageal reflux, Barrett's esophagus and obesity[14]. However, the exact pathophysiological mechanisms linking those changes to EGJ cancer are still not clear.

Whether EGJ cancer should be considered in the same group as esophageal and gastric cancer or if it corresponds to a different type of cancer because of its different oncological prognosis and lymph nodal spreading is controversial. Therefore, various surgical treatment alternatives have been described, such as total esophagectomy, partial esophago-gastrectomy (Ivor-Lewis and Merendino) and TEG. No consensus has been established when deciding which method is the most appropriate. In the last decade, this cancer was imprecisely classified in the 2006 TNM, and subsequently, it was grouped together with esophageal cancer in the 2010 TNM classifications[10]. This difficulty in grouping corresponds to the several different types of tumors than can compromise the EGJ, with some of the tumors behaving as an esophageal cancer and others having a gastric cancer behavior.

In 1996, Siewert *et al*[15,16] made a topographic classification, which allows planning and standardization of the surgical treatment and comparing outcomes. EGJ cancer type Ⅰ is treated similarly to esophageal cancer, with esophagectomy plus proximal gastrectomy[17], and EGJ cancer type Ⅲ is treated with a total gastrectomy[18]. EGJ type Ⅱ tumors are usually treated with methods similar to those used for type Ⅲ tumors because of the frequent involvement of the intra-abdominal lymph nodes, unless they present a greater esophageal involvement in the preoperative study, in which a proximal esophagogastrectomy plus abdominal and mediastinal lymphadenectomy is preferred. The type of approach and reconstruction in these cases can vary according to the center; some centers prefer a combined abdominal and thoracic approach, and other centers prefer exclusively a transhiatal approach. Regarding the type of reconstruction, it is possible to perform reconstruction with interposition of the stomach into the thorax (Ivor Lewis) or with interposition of a distal organ (colon or bowel).

One of the most difficult aspects to determine in patients with Siewert's type Ⅱ or type Ⅲ large EGJ tumors, also called EGJ cancer type Ⅳ by Burmeister *et al*[8], is the length of the extension of the esophageal tumor involvement when performing preoperative studies. Therefore, in some cases, the choice of the treatment alternative for surgery is made during the intra-operative period. It has been established that in order for the treatment to meet oncological criteria, it has to ensure a macroscopic tumor-free margin of at least 5-10 cm in the esophagus[19-22]. At the other extreme, 2 cm is considered as a macroscopic tumor-free margin of poor prognosis or insufficient in a Japanese series. Therefore, we performed a total esophagectomy with total gastrectomy if an esophageal macroscopic tumor-free margin of less than 10 cm was determined while in surgery[19-22].

The aforementioned situation is why patients with EGJ cancer should be prepared for an eventual TEG and colon interposition plus an extended lymph node dissection (D2), due to the frequent lymph node metastasis of groups 1, 2, 3 and 7 of the Japanese classifications[6,7,23].

A second group of patients in whom the indication of TEG is clearer, are those who have a concomitant esophageal and gastric cancer (double cancer).

In this study, a splenectomy was performed only when evidence of invasion of the splenic hilum was found or in cases when significant LNM of groups 10 and 11 of the Japanese classifications were found. Resection of other organs such as the pancreas or adrenal gland is recommended only in cases with evidence of direct invasion by the tumor.

While reviewing this series, we observed a high morbidity (68%) and respiratory complications were the most frequently observed (40%), followed by leakage of the esophageal-colon anastomosis (30%); however, only 1 postoperative death (5%) was observed. These results were similar to results reported in other series of TEG and can even be compared to a series of patients who underwent proximal esophagogastrectomy[8], suggesting that multidisciplinary management is crucial for the management of postoperative complications.

Oncological results observed in this study were very satisfying when compared to large series, resulting in a 39.6% survival rate at 5 years follow-up and a median of 20.5 mo of survival when curative surgery was achieved. The surgical results obtained in this study showed that it is still controversial to perform this procedure with palliative intention; however, it showed better outcomes than other palliative procedures such as esophageal prosthesis, a gastrostomy or palliative chemotherapy. Similar to our series, Siewert's study even had patients who underwent palliative TEG who were reported to have survival after 1 year of follow-up[6].

TEG D2 has a high postoperative morbidity rate, which suggests that it should be performed only in specialized centers that are dedicated to high complexity oncological surgeries.

COMMENTS

Background

Total esophagectomy associated with total gastrectomy for cancer is a highly complex surgery, associated high postoperative morbidity and infrequently indicated. The literature reported about 20% overall survival at 5 years.

Research frontiers

Describe surgery in patients with cancers of the gastroesophageal junction, which is feasible, the main complication is respiratory cancer and has good

oncological results.

Innovations and breakthroughs

The difference with other studies, is that described in detail the surgical steps, also includes high quality diagrams to understand step by step surgery.

Applications

This study provides interesting information to indicate total esophagogastrectomy, in selected patients, which is useful even in patients in advanced stages, with more than 12 mo survival.

Terminology

TEG: Total esophagogastrectomy; EGJ: Esophagogastric junction.

Peer-review

This is a interesting article for surgeon. The authors' work is very meaningful.

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ORIGINAL ARTICLE

Observational Study

Effectiveness of hepatitis B virus vaccination program in Egypt: Multicenter national project

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Author contributions: All the authors contributed to this work.

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Institutional review board statement: The study was reviewed and approved by the ethical committees of Ministry of Health (MOH), National Research Center and Ministry of Education-Egypt in accordance with the Helsinki Declaration.

Informed consent statement: All the legal guardians of the study participants were provided informed written consent prior to study enrollment. In addition, children aged above 10 years were enrolled after getting their verbal assent.

Conflict-of-interest statement: The authors declare no conflict of interest.

Data sharing statement: Statistical code and dataset are available from the first author at salamaiman@yahoo.com. Consent for data sharing was not obtained from participants but the presented data are anonymized and risk of identification is nil. No additional data are available.

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Abstract

AIM: To assess the effectiveness of hepatitis B virus (HBV) vaccination program among fully vaccinated children.

METHODS: A national community based cross-sectional study was carried out in 6 governorates representing Egypt. A total of 3600 children aged from 9 mo to 16 years who were fully vaccinated with HBV vaccine during infancy were recruited. Face to face interviews were carried out and sera were evaluated for hepatitis B surface antigen (HBsAg), anti-HBV core antibodies (total) and quantitative detection of hepatitis B surface antibody using enzyme linked immunoassays techniques. Samples positive to HBsAg/anti-HBV core antibodies were subjected to quantitative HBV-DNA detection by real time polymerase chain reaction with 3.8 IU/L detection limit.

RESULTS: Sero-protection was detected among 2059 children (57.2%) with geometric mean titers 75.4 ± 3.6 IU/L compared to 3.1 ± 2.1 IU/L among non-seroprotected children. Multivariate logistic analysis revealed that older age and female gender were the significant predicting variables for having non sero-protective level, with adjusted odds ratio 3.3, 9.1 and 14.2 among children aged 5 to < 10, 10 to < 15 and ⩾ 15 years respectively compared to those < 5 years and 1.1 among girls compared to boys with P < 0.01. HBsAg was positive in 0.11% and breakthrough infection was 0.36% and 0.39% depending on positivity of anti-HBc and DNA detection respectively. The prevalence of HBV infection was significantly higher among children aged ⩾ 7 years (0.59%) compared to 0.07% among younger children with odds ratio equal to 8.4 (95%CI: 1.1-64.2) and P < 0.01.The prevalence was higher among girls (0.48%) than boys (0.29%) with P > 0.05.

CONCLUSION: The Egyptian compulsory HBV vaccination program provides adequate protection. Occult HBV infection exists among apparently healthy vaccinated children. Adherence to infection control measures is mandatory.

Key words: Hepatitis B virus; Immunization; Sero-protection; Breakthrough infection; Children

Core tip: To assess the effectiveness of hepatitis B virus (HBV) vaccination program, a national community based survey was carried out in six governorates representing Egypt on 3600 children aged 9 mo to 16 years (received 3 doses HBV vaccine during infancy). Anti-hepatitis B surface (anti-HBs) titer, anti-HBc and HBs antigen were assessed. HBV DNA detection was done for suspected cases. Prevalence of HBV sero-protection, breakthrough HBV infection, and chronic carrier were 57.2% 0.39% and 0.11% respectively. Multivariate analysis revealed that older age and girls were the significant predictor variables for non sero-protection. Despite waning of anti-HBs over time, HBV vaccination program is effective in Egypt.

Salama II, Sami SM, Said ZNA, El-Sayed MH, El Etreby LA, Rabah TM, Elmosalami DM, Abdel Hamid AT, Salama SI, Abdel Mohsen AM, Emam HM, Elserougy SM, Hassanain AI, Abd Alhalim NF, Shaaban FA, Hemeda SA, Ibrahim NA, Metwally AM. Effectiveness of hepatitis B virus vaccination program in Egypt: Multicenter national project. *World J Hepatol* 2015; 7(22): 2418-2426 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i22/2418.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i22.2418

INTRODUCTION

The hepatitis B vaccine is the mainstay of hepatitis B prevention. In 1992, the World Health Organization (WHO) recommended the implementation of universal childhood vaccination worldwide and by the end of 2012, 181 countries had adopted this measure[1]. The complete vaccination series induces protective antibody levels in more than 95% of infants, children and young adults[2]. Persistence of hepatitis B surface antibody (anti-HBs) and thus the protection against infection and carrier state depends on the peak anti-HBs concentration achieved after primary vaccination. However, anti-HBs decay exponentially with length of time since vaccination[3].

Hepatitis B virus (HBV) is considered moderately endemic in Egypt with 4% of the population having evidence of chronic HBV infection[4]. A key goal of HBV immunization program is to reduce the prevalence of hepatitis B surface antigen (HBsAg) among cohorts born since the program implementation. A practical means to determine the long term protection provided by HB vaccine is to estimate the incidence of break-through infection (positive anti-HBc) as well as chronic carrier state (positive HBsAg) among previously vaccinated individuals[5]. In Egypt, the HBV vaccination program was applied in 1992 with a schedule of 2, 4 and 6 mo of age, while routine screening of pregnant women for HBsAg was not applied[6]. There have been no sero-surveys among children born since the introduction of the vaccine in Egypt; however, the finding of acute disease transmission in these cohorts indicates there is ongoing HBV transmission and more in-depth evaluation of the immunization program is needed[7]. Although several studies have been done in Egypt to measure the effectiveness of HBV vaccination, yet these studies were done on a relatively small scale and in certain areas of Egypt, from which arose the need for a large national study including numerous areas to be representative of all Egypt to give a clear picture of the situation. The present study aimed to assess the effectiveness of compulsory HBV vaccination on national basis and to determine health disparities and risk factors associated with non-seroprotective levels among Egyptian children aged from 9 mo to 16 years. It also aimed to assess the prevalence of breakthrough HBV infection and carrier state among the studied children.

MATERIALS AND METHODS

This is a community based national project using a multi-stage cluster sampling technique. It was carried out in 6 representative governorates in Egypt from July 2010 to June 2013. These governorates included the Capital (Cairo), two in Lower Egypt (Gharbeya and Dakahleya), two in Upper Egypt (Assiut and Beni-Suef), and one Frontier (Red Sea). The age of the participating children ranged from 9 mo to 16 years. They were fully vaccinated by the 3 compulsory HBV vaccine doses during infancy. Probability proportional to size sampling was used for the sampling process and selection of clusters. The design effect used was considered equal to 2. From previous Egyptian studies carried out on small scales, the prevalence of sero-protection level among fully vaccinated children ranged from 95% among infants aged 9 mo to 35% among children aged 11 years[8,9]. We assumed that it might be about 25% among older children. The sample size allowed an estimated precision (margin of error) of 5% and 95% confidence level of being within 5% of the true value, with response rate estimated to be 90%. The sample frame for the survey was based on the most recent population census of 2006. List of cities and villages were arranged in serpentine order after the implicit stratification by geographic location independently for urban and rural areas of each governorate. After calculating a sampling interval, a random number was selected from the table of random numbers. Out of this list, the number of participating areas in each governorate was identified according to its population size. So, we identified 5 urban areas from Cairo governorate, 4 areas from Gharbeya governorate (1 city and 3 villages), 5 areas from Dakahleya governorate (2 cities and 3 villages), 3 areas from Assuit governorate (1 city and 2 villages), 2 areas from Beni-Suef governorate (1 city and 1 village), and one city area from Red Sea governorate. In each selected area, one maternal and child health center in urban areas or health unit (in rural areas) was randomly selected. Then according to the age of the targeted children within the catchment areas, one kindergarten and 3 schools (primary, preparatory and secondary) were randomly selected.

The study was reviewed and approved by the ethical committees of Ministry of Health, National Research Center and Ministry of Education. All the legal guardians of the study participants were provided informed written consent prior to study enrollment. In addition, children aged above 10 years were enrolled after getting their verbal assent. Through face to face interview, children's personal data, demographic and socioeconomic variables and current and past medical history were collected through a designed pre-tested close-ended questionnaire. Children's HBV vaccination was confirmed by taking a full detailed vaccination history from parents as well as revising the vaccination cards available with their parents or in the child's school file. For quality assurance, Ministry of Health staff, supervisors and interviewers attended several training sessions before the study implementation in each governorate. To ensure tracing blood samples and linking laboratory results with other survey, data peel-off barcode sheets were used. To assess the nutritional status, anthropometric measurements including height and weight were also taken.

Laboratory analysis

A blood sample (3-5 mL) was withdrawn from each child aseptically and serum was aliquoted into two labeled sterile cryotubes and stored at -20 ℃. Detection of HBV markers was performed in the Virology lab - Microbiology and Immunology Department-Faculty of Medicine (for girls), Al-Azhar University, Cairo. Serum total anti-HBc, HBsAg and anti-HBs were assessed using commercially available enzyme linked immunoassays (Dia Sorin-Italy) according to the manufacturer instructions. Anti-HBs $\geqslant$ 10 IU/L, was considered to be protective against HBV infection[10].

Repeatedly positive samples for either anti-HBc or HBsAg were subjected to quantification of HBV DNA by Real-time PCR using automated system. Viral DNA was extracted from serum samples using QIAxtractor®, and VX kit as recommended by the manufacturer (QIAGEN, Germany). PCR setup was automated *via* QIAgility (QIAGEN, Germany). HBV real-time assays were performed in combination of Artus HBV RG PCR Kit (Artus™ GmbH, Hamburg, Germany) and the Real time PCR instrument, Rotor-Gene Q (QIAGEN, Germany). Thermal profile was set according to manufacturer's guideline. Detection limit of HBV DNA in the current study assay was 3.8 IU/L assessed by the WHO international standard (97/750)[11]. At least two negative controls, one non template control and four standards (provided by the manufacturer) were added per run. Strict precautions were taken to avoid possible contamination. Only reproducible data that revealed no false positive results in the negative controls was used.

Statistical analysis

Data entry and statistical analysis were done using SPSS software program version 18.0. Anti-HBs geometric mean titer (GMT) was calculated to estimate the central tendency of anti-HBs level in consideration to its skewed distribution. Children who had an undetectable anti-HBs titer were assigned a titer value of 0.05 IU/L[12]. For qualitative data that presented by numbers and percentages, χ^2 was done. For comparison between two means, *t*-test was done while one way ANOVA was used for more than two means. Multivariate logistic analysis was carried out to predict risk factors significantly associated with non-serprotection. $P < 0.05$ was considered statistically significant and $P < 0.01$ was considered statistically highly significant.

The statistical methods of this study were performed by first author Iman I Salama, professor of public Health and Preventive Medicine at National Research Center and she is a Bio-statistical reviewer in Medical

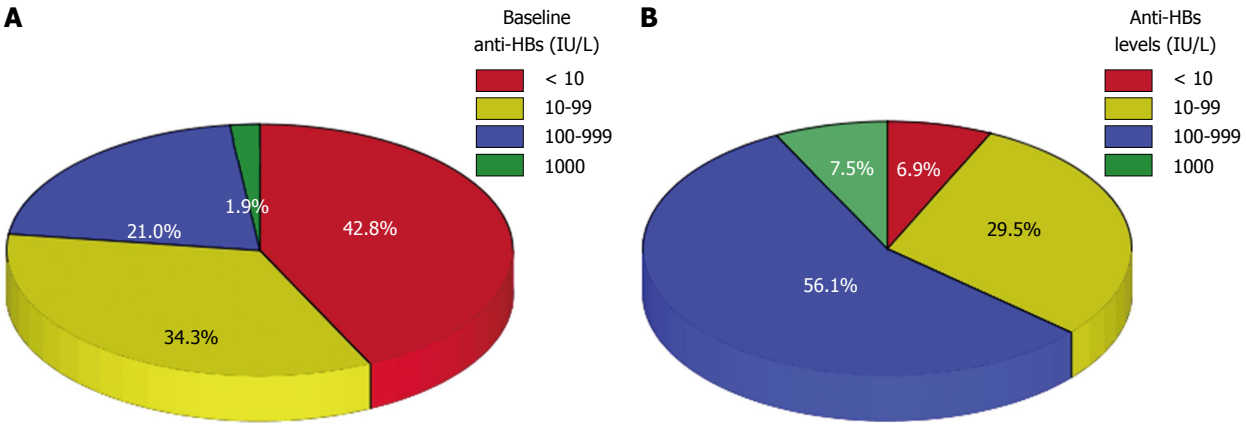

Figure 1 Prevalence of hepatitis B surface antibody levels among all the studied children (A) and among children aged ⩽ 1 year (B). Anti-HBs: Hepatitis B surface antibody.

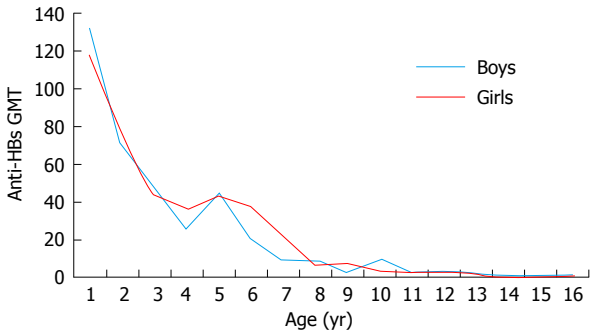

Figure 2 Hepatitis B surface antibody geometric mean titer among the studied children according to age and gender. Anti-HBs: Hepatitis B surface antibody; GMT: Geometric mean titer.

Research J.

RESULTS

Short and long term HBV sero-protection among the fully vaccinated children

The present study was carried out on 3600 children and adolescents from six Egyptian governorates, 1909 (53%) from urban and 1691 (47%) from rural areas. There were 1743 (48.4%) boys and 1857 (51.6%) girls with a mean age of 9.1 ± 5.5 years. Overall sero-protection rate among the studied children was 57.2% (95%CI: 55.6% to 58.8%). Figure 1A shows anti-HBs level among all the studied children, while Figure 1B shows anti-HBs level among children ⩽ 1 year representing the primary response to HBV vaccine 3-6 mo after receiving the 3 compulsory doses. The GMT of the anti-HBs was significantly lower among children ⩾ 5 years compared to younger, among girls than boys, in lower socioeconomic status, in Assuit and Red Sea compared to other governorates and in rural compared to urban areas ($P < 0.05$), Table 1. The table also showed that the distribution of anti-HBs levels was significantly different as regards all studied socio-demographic characteristics, $P < 0.05$. Antibody concentrations decline more quickly during the first 4 years after vaccination than they do later on (Figure 2). There was no significant difference between boys and girls in the different governorates except in Gharbeya and Assuit, where males had significantly higher sero-protection rate (66.4% and 59.3%) compared to females (49.9% and 48.4%) respectively, $P < 0.01$.

Children with history of hospital admission, abscess incision, surgical operation, regular medical injection, blood transfusion and rheumatic fever had significantly higher non sero-protective rates compared to children with no such history ($P < 0.001$). Children with Height for age percentile (HAP) and Weight for age percentile (WAP) < 5th percentiles had significantly lower non sero-protection rate compared to normal children with odds ratio 1.3 for both, $P < 0.05$ (Table 2). Multivariate logistic analysis revealed that older age and female gender were the significant predicting variables for having non sero-protective level, with adjusted odds ratio 3.3, 9.1 and 14.2 among children aged 5 to < 10, 10 to < 15 and ⩾ 15 years respectively compared to those < 5 years and 1.1 among girls compared to boys, $P < 0.01$ (Table 3).

HBV breakthrough infection among the fully vaccinated children

Fourteen children (9 females and 5 males) showed HBV breakthrough infection; 14/3600 (0.39%) were positive for HBV-DNA, 13/3600 (0.36%) had positive anti-HBc and 4/3600 (0.11%) had positive HBsAg. HBV infection was not found among children aged < 3 years (Table 4). The prevalence of HBV infection was significantly higher among children aged ⩾ 7 years (0.59%) compared to 0.07% among younger children, odds ratio 8.4 (95%CI: 1.1-64.2), $P < 0.01$. The prevalence was higher among girls (9/1857; 0.48%) than boys (5/1743; 0.29%), $P > 0.05$. Table 5 shows the demographic characteristics and HBV markers of breakthrough infection among the studied children. Five out of thirteen children with positive anti-HBc had anit-HBs above 100 IU/L and 11 out of 14 children presented HBV DNA ⩾ 200 IU/L. Three children, positive for HBsAg, had a family history

Table 1 Prevalence of hepatitis B virus anti-hepatitis B surface in relation to some socio-demographic characteristics among 3586[1] studied children *n* (%)

	Total	Anti-HBs IU/L			
	n = 3586	Mean GMT	< 10	10-99	⩾ 100
			n = 1535	*n* = 1229	*n* = 822
Gender					
Boys	1738	220.4 ± 13.4	681 (39.2)	643 (37.0)	414 (23.8)
Girls	1848	161.7 ± 11.2	854 (46.2)	586 (31.7)	408 (22.1)
P value		< 0.05	< 0.001		
Age in years					
< 5 yr	1114	64.0 ± 8.0	152 (13.6)	435 (39.0)	527 (47.3)
5 to < 10 yr	625	18.7 ± 13.8	206 (33.0)	240 (38.4)	179 (28.6)
10 to < 15 yr	1026	3.0 ± 15.0	606 (59.1)	346 (33.7)	74 (7.2)
⩾ 15 yr	821	1.5 ± 17.0	571 (69.5)	208 (25.3)	42 (5.1)
P value		< 0.001	< 0.001		
Socio-economic status[2]					
Very low	992	22.3 ± 6.3	465 (46.9)	345 (34.8)	182 (18.3)
Low	650	24.4 ± 6.2	301 (46.3)	220 (33.8)	129 (19.8)
Middle	934	37.2 ± 6.4	349 (37.4)	324 (34.7)	261 (27.9)
High	916	33.3 ± 6.5	376 (41.0)	308 (33.6)	232 (25.3)
P value		< 0.001	< 0.001		
Governorate					
Cairo	815	32.3 ± 6.8	320 (39.3)	271 (33.3)	224 (27.5)
Dakahleya	898	29.6 ± 5.8	380 (42.3)	328(36.5)	190 (21.2)
Gharbeya	762	29.8 ± 6.5	328 (43.0)	256 (33.6)	178 (23.4)
Beni-Suef	358	35.3 ± 6.2	155 (43.3)	126 (35.2)	77 (21.5)
Assuit	564	22.3 ± 6.7	264 (46.8)	191 (33.9)	109 (19.3)
Red Sea	189	21.2 ± 7.0	88 (46.6)	57 (30.2)	44 (23.3)
P value		< 0.01	< 0.05		
Residence					
Urban	1902	11.0 ± 18.4	784 (41.2)	649 (34.1)	469 (24.7)
Rural	1684	7.3 ± 20.8	751 (44.6)	580 (34.4)	353 (21.0)
P value		< 0.001	0.021		

[1]Breakthrough infected children were excluded; [2]Data for Socio-economic status was fulfilled for only 3492 children. Anti-HBs: Hepatitis B surface antibodies; GMT: Geometric mean titer.

Table 2 Hepatitis B virus immunity in relation to the child's medical history *n* (%)

Risk factors	Total	Level of anti-HBs		Odds ratio (95%CI)
		< 10 IU/L	⩾ 10 IU/L	
		n = 1535	*n* = 2051	
Hospital admission				
Yes	1196 (33.4)	558 (46.4)	638 (53.6)	1.3 (1.0-1.5)[b]
No	2390 (66.6)	977 (40.9)	1413 (59.1)	®
Open abscess				
Yes	280 (7.8)	150 (53.5)	130 (46.5)	1.6 (1.2-2.0)[b]
No	3306 (92.2)	1385 (41.6)	1921 (58.4)	®
Surgical operation				
Yes	683 (19.0)	339 (49.6)	344 (50.4)	1.4 (1.2-1.7)[b]
No	2903 (81.0)	1196 (41.2)	1707 (58.8)	®
Regular medical injection				
Yes	112 (3.1)	59 (52.7)	53 (47.3)	1.5 (1.0-2.2)[a]
No	3474 (96.9)	1476 (42.5)	1998 (57.5)	®
Blood transfusion				
Yes	90 (2.5)	48 (53.3)	42 (46.7)	1.5 (1.0-2.4)[a]
No	3496 (97.5)	1487 (42.5)	2009 (57.5)	®
Rheumatic fever				
Yes	140 (3.9)	74 (52.9)	66 (47.1)	1.5 (1.1-2.1)[a]
No	3446 (96.1)	1461 (42.3)	1985 (57.6)	®
HAP (total = 3256)				
< 5th percentile	569 (17.5)	218 (38.3)	351 (61.7)	1.3 (1.1-1.6)[b]
⩾ 5th percentile	2687 (82.5)	1208 (45.0)	1479 (55.0)	®
WAP (total = 3317)				
< 5th percentile	240 (7.2)	89 (37.1)	151 (62.9)	1.3 (1.0-1.7)[a]
⩾ 5th percentile	3077 (92.8)	1341 (43.6)	1736 (56.4)	®

[a]$P < 0.05$, [b]$P < 0.01$. ®: Reference group; Anti-HBs: Hepatitis B surface antibodies.

Table 3 Univariate and multivariate logistic analysis to determine predictors for risk of non sero-protection *n* (%)

Variable	Non-seroprotection rate	Crude odds ratio (95%CI)	AOR (95%CI)
Age in years			
< 5	152 (13.6)	®	®
5 to < 10	256 (34.7)	3.4 (2.7-4.2)[b]	3.3 (2.5-4.2)[b]
10 to < 15	606 (59.1)	9.1 (7.4-11.3)[b]	9.1 (7.3-11.2)[b]
⩾ 15	571 (69.5)	14.5 (11.5-18.1)[b]	14.2 (11.3-17.9)[b]
Gender			
Boys	681 (39.2)	®	®
Girls	854 (46.2)	1.3 (1.2-1.5)[b]	1.1 (1.0-1.4)[b]

Variables entered in model: Age group, gender, socio-economic levels and history of rheumatic fever, diabetes mellitus, surgical operation, regular medical injection, blood transfusion, hospital admission, open abscess. [b]$P < 0.01$. AOR: Adjusted odds ratio; ®: Reference group.

Table 4 Hepatitis B virus breakthrough infection among the studied children in different age groups

Age group (yr)	Total	Sero-protection rate Anti-HBs ⩾ 10 IU/L		HBV infection markers		
		n (%)	95%CI	Anti-HBc %	HBsAg %	HBV-DNA %
< 3	702	633 (90.2)	88.0-92.4	0 (0.0)	0 (0.0)	0 (0.0)
3-	705	557 (79.0)	76.0-82.0	1 (0.14)	0 (0.0)	1 (0.14)
7-	493	282 (57.2)	52.9-61.6	4 (0.81)	2 (0.41)	5 (1.00)
11-	875	335 (38.3)	35.1-41.5	4 (0.46)	1 (0.11)	4 (0.46)
⩾ 15	825	252 (30.5)	27.4-33.6	4 (0.48)	1 (0.12)	4 (0.48)
Total	3600	2059 (57.2)	55.6-58.8	13 (0.36)	4 (0.11)	14 (0.39)

HBV: Hepatitis B virus; Anti-HBs: Hepatitis B surface antibodies; Anti-HBc: Hepatitis B core antibody; HBsAg: Hepatitis B surface antigen.

Table 5 Demographic characteristics and hepatitis B virus markers of breakthrough infection among the studied children

N	Age (yr)	Gender	Residence	Governorate	Base line HBV markers			
					Anti-HBs (IU/mL)	HBsAg	Anti-HBc	HBV DNA
1	10	Boy	Urban	Beni-Suef	0	+	+	10000
2	15.8	Girl	Urban		37	-	+	1280
3	16.8	Girl	Urban		3	+	+	866
4	11	Girl	Rural		0	-	+	24100
5	11	Girl	Urban	Assuit	992	-	+	953
6	10	Boy	Urban		404	-	+	2850
7	11.8	Girl	Rural	Dakahleya	4	-	+	4170
8	15.8	Girl	Rural		3	-	+	455
9	16	Boy	Rural		559	-	+	781
10	12	Boy	Rural		0	+	+	26
11	9.3	Girl	Urban	Cairo	439	-	+	48
12	9	Girl	Urban		210	-	+	3920
13	9.8	Girl	Urban		24	+	-	2440
14	3.3	Boy	Urban		15	-	+	209

HBV: Hepatitis B virus; Anti-HBs: Hepatitis B surface antibodies; Anti-HBc: Hepatitis B core antibody; HBsAg: Hepatitis B surface antigen.

of positive HBV infection where two mothers and one father were positive. None of the studied children had elevated liver enzymes or was hepatitis B e antigen (HBeAg) positive. Follow up after one year showed that six children only retained anti-HBc positivity, three of them were also positive for HBsAg while the other three children had isolated anti-HBc indicating occult HBV infection.

DISCUSSION

HBV vaccine is the first vaccine against a major human cancer[13]. The present study has the greatest sample size to study the effectiveness of HBV vaccine among Egyptian children (n = 3600). Subjects were relatively homogenous: All had received the same recombinant HBV vaccine with the same schedule during infancy. The

overall sero-protection rate among the studied children was 57.2%, which decreased significantly from 90.2% among children < 3 years to 30.5% among children ⩾ 15 years. Similarly, other Egyptian studies carried out on smaller sample sizes[8,14], reported 54% and 39.7% sero-protection rate among vaccinated children aged 6-12 years respectively. In Slovakia, 10-11 years after primary vaccination, 48.4% children had persisting sero-protection anti-HBs[15]. Similar to the present study, Afifi *et al*[9], found that the mean anti-HBs level decreased significantly with increasing age, being 426.8, 79.2 and 32.1 IU/L at 9 mo, 6 years and 11 years post vaccination respectively. On the other hand, in Italy higher sero-protection rate (64% of children aged over 10 years) was reported by Zanetti *et al*[16]. Similar to our results, anti-HBs concentrations decline more quickly during the first few years after vaccination than they do later on which was mentioned in a Turkish study on children aged 2 to 12-years[17]. Using multivariate logistic analysis, the current study showed that age and gender were the only two risk factors for non-seroprotection among the studied children. The risk of non-sero-protection was significantly slightly higher among girls than boys with odds ratio of 1.1. However, other studies in the United States and Iran found no gender difference[18,19].

Some investigators correlate the socioeconomic status (SES) with vaccine response[20]. In this study, the percentage of non sero-protection was significantly high among very low and low SES when compared to non sero-protection among middle SES, with odds ratio 1.5 and 1.4 respectively. Wang *et al*[21] reported that HBV vaccination program was less effective in socio-economically disadvantaged area and was affected by factors associated with urbanization[22]. However, Zanetti *et al*[16] showed that socioeconomic factors such as residential location, family size, fathers' level of education did not affect the level of protective antibody concentrations.

In the current study, it was found that children with HAP and WAP < 5th percentiles had significantly lower non sero-protection rate compared to normal children with odds ratio 1.3 for both. These results are in accordance to Karimi *et al*[23]. However, another Egyptian study done on 200 children showed no difference in sero-protection rates as regards children's growth and nutritional status[22].

The fall in the antibody titer does not necessary indicate loss of immunity. Protection against clinically important disease outlasts the presence of detectable antibodies[24]. No HBV infection was detected among children aged < 3 years, indicating absence of perinatal infection. However, three older children found positive for HBsAg had a family history of positive HBV infection (2 mothers and 1 father were positive). Transmission from chronically infected women to their infants during delivery is one of the most common routes of HBV infection worldwide. The risk of perinatal infection is 5%-20% in infants born to HBsAg-positive mothers and 70%-90% if the mother is HBeAg positive[25,26]. However, it was previously shown that combined active and passive immunization of newborns of HBsAg$^+$ mothers against HBV demonstrates persistent protection up to adolescence despite a frequent waning of anti-HBs antibodies[27]. A recent meta analysis showed that HBV vaccine alone seems to be equally effective to a combination of HBIG and hepatitis B vaccine for neonates of HBsAg$^+$/HBeAg$^-$ mothers in preventing infection[28]. Currently, WHO recommends that all infants receive the hepatitis B vaccine as soon as possible after birth, preferably within 24 h. The birth dose should be followed by 2 or 3 doses to complete the primary series. The complete vaccination series induces protective antibody levels in more than 95% of infants, children and young adults. Protection lasts at least 20 years and is possibly lifelong[29].

None of the studied children aged < 7 years was a carrier for HBsAg. This was in accordance with a recent Egyptian study which did not find HBsAg positive sera among 180 children < 5 years[30]. Whereas another Egyptian study in 2003 reported higher prevalence (0.8%) of 6-year-old children having positive HBsAg[31]. In Taiwan, anti-HBc was detectable among 33% of vaccinated children aged 15 years *vs* 0.48% of children of the same age in the present study, and only one child had detectable HBsAg in both the Taiwanese and the present study[32]. In Italy, 3 out of 1543 vaccinated children aged 5 years were found to be anti-HBc positive[10].

However in long-term follow-up studies, breakthrough infections do occur, illustrated by the sero-conversion of anti-HBc, but few clinically significant infections are diagnosed and few new carriers are reported[33,34].

A meta-analysis revealed that the overall cumulative incidence of HBV breakthrough infection 5-20 years post-primary vaccination was 0.007 with a variation among studies ranging from 0 to 0.094[5]. In the present study, the highest prevalence of HBV infection detected by DNA (0.59%) was found in the age group ⩾ 7 years compared to 0.07% in the age group < 7 years and it was higher among girls (9/1857; 0.48%) than boys (5/1743; 0.29%). Results also suggested that monitoring the presence of HBV DNA (by using qPCR) is a better diagnostic parameter than anti-HBc for detecting viral infection. The effect of increasing age on the prevalence of breakthrough infection was also detected by other studies[35-37]. On the contrary as regards to gender, infection was higher among males than females in both Gambia[37] and in Iran[38].

In the current study, five out of thirteen children with positive anti-HBc had anti-HBs above 100 IU/L. It was reported that immunological responses to exposure to HBV, so-called breakthrough infections, have been observed in successfully vaccinated individuals who were later exposed to HBV. Such an exposure may simply boost the titer of anti-HBs[39]. The results also showed that one year later, six children retained anti-HBc positivity, three of them were also positive for

HBsAg while the other three children had isolated anti-HBc indicating occult HBV infection. Similar results were obtained by Su *et al*[40], who recommended a single HBV booster dose of vaccine for those with isolated anti-HBc who were fully vaccinated with HBV vaccine as infants.

From this study it can be concluded that the Egyptian national HBV vaccination in infancy produces adequate protection 1 to 16 years post vaccination. Successful implementation of universal vaccination policies in Egypt with a good coverage rate, together with the general improvement in infection control measures and safety blood donation can minimize the hepatitis B disease burden. Strict adherence to infection control measures and safe blood transfusion are needed especially for high-risk infants to augment the effectiveness of the vaccine.

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COMMENTS

Background

Hepatitis B virus (HBV) is considered moderately endemic in Egypt with 4% of the population having evidence of chronic HBV infection. World Health Organization recommended in 1992 to implement universal childhood vaccination worldwide. The complete vaccination series induces protective antibody levels in more than 95% of infants, children and young adults. A key goal of HBV immunization program is to reduce the prevalence of hepatitis B surface antigen (HBsAg) among cohorts born since program implementation.

Research frontiers

Although several studies have been done in Egypt to measure the effectiveness of HBV vaccination, yet these studies were done on a relatively small scale and in certain areas of Egypt from which arose the need for a large national study including numerous areas to be representative and give a clear picture of the situation in Egypt.

Innovations and breakthroughs

There have been no sero-surveys among children born since the introduction of the vaccine in Egypt. However, the finding of acute disease transmission in these cohorts indicates ongoing HBV transmission, thus the need for more in-depth evaluation of the immunization program. The present study had the greatest sample size (n = 3600) and aimed to assess the prevalence of breakthrough HBV infection (positive anti-HBc) as well as chronic carrier state (positive HBsAg) among the previously vaccinated studied children as a practical means to determine the long term protection provided by hepatitis B vaccine. It also aimed to determine health disparities and risk factors associated with non-seroprotective levels among Egyptian children aged from 9 mo to 16 years.

Applications

Successful implementation of universal vaccination policies in Egypt with a good coverage rate, together with the general improvement in infection control measures especially for high risk infants and safe blood donation can minimize the hepatitis B disease burden.

Terminology

Hepatitis B is a serious disease caused by a virus that attacks the liver. The virus, which is called HBV, can cause lifelong infection, cirrhosis (scarring) of the liver, liver cancer, liver failure, and death; Sero-protection: Following a standard hepatitis B vaccination course, antibody to HBsAg is established in the bloodstream, the antibody is known as antibodies to hepatitis B surface (anti-HBs). About 90%-99% of healthy neonates, children, adolescents and adults develop protective levels of anti-HBs; Breakthrough infection: (Positive anti-HBc): HBV infection in previously vaccinated subjects. Vaccinated subjects with anti-HBs antibody titers below the protective level are still susceptible to HBV infection, especially if they are exposed to a high viral load; HBV infections positive HBsAg: Chronic carrier state among previously vaccinated individual.

Peer-review

The manuscript is well written and is based in a large and well selected cohort that represents the Egypt young population. The conclusions and statements are well made in face of the obtained results. HBV vaccine is worldwide used and other studies have demonstrated its effectiveness in other populations. This study was focused in Egypt population and evaluated the behavior of anti-HBV response build after HBV vaccination. The expected result "The Egyptian compulsory HBV vaccination program has produced adequate protection" was correctly placed and support by the data collect.

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