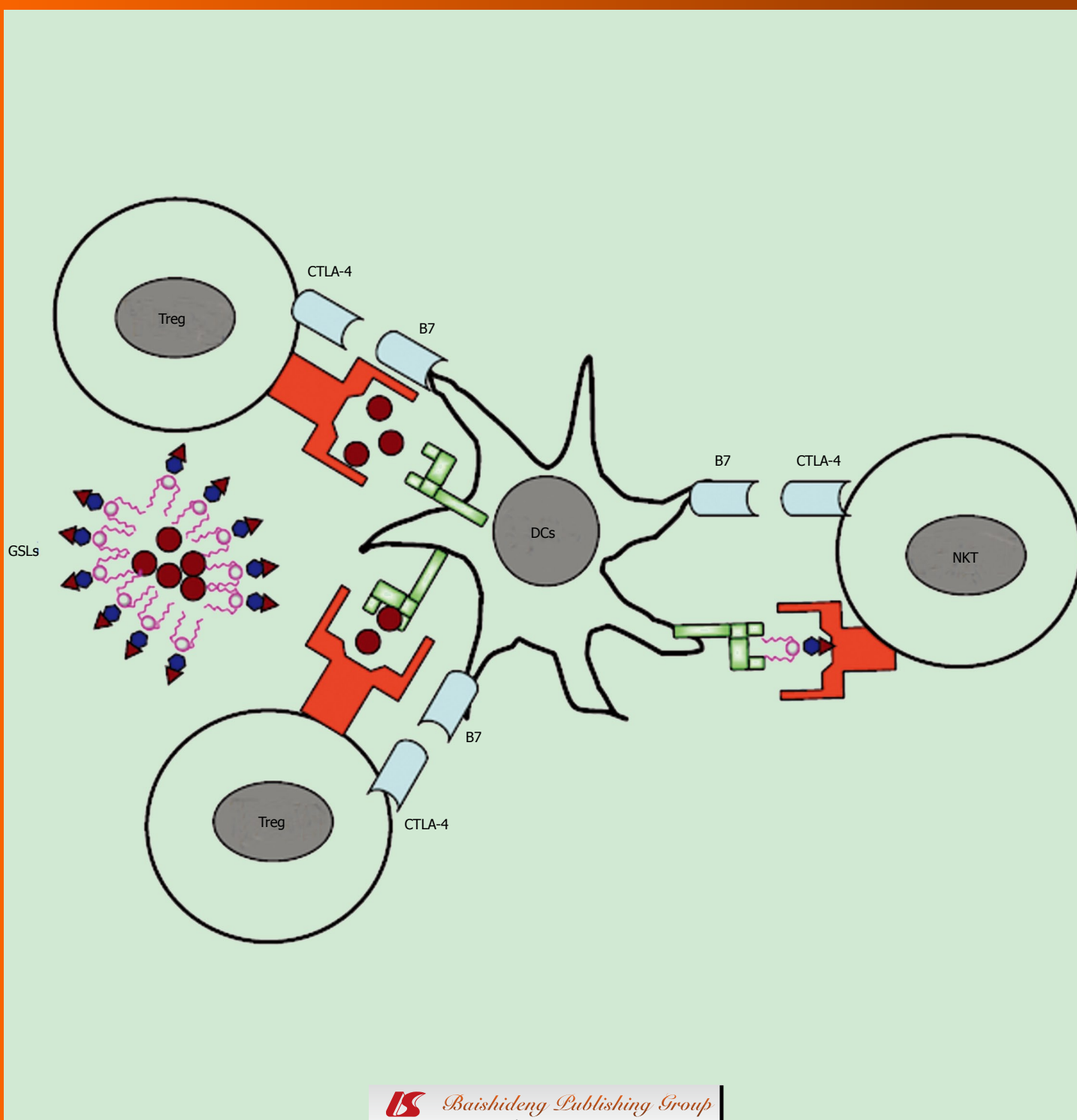


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Genetic susceptibility to autoimmune liver disease

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Abstract

Autoimmune hepatitis (AIH), primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC) are considered as putative autoimmune diseases of the liver. Whereas strong evidence that bacterial infection may trigger PBC exists, the etiologies for PSC and AIH remain unknown. Although there have been significant discoveries of genetic polymorphisms that may underlie the susceptibility to these liver diseases, their associations with environmental triggers and the subsequent implications have been difficult to elucidate. While single nucleotide polymorphisms within the negative costimulatory molecule cytotoxic T lymphocyte antigen 4 (CTLA-4) have been suggested as genetic susceptibility factors for all three disorders, we discuss the implications of CTLA-4 susceptibility alleles mainly in the context of PBC, where *Novosphingobium aromaticivorans*, an ubiquitous alphaproteobacterium, has recently been specifically associated with the pathogenesis of this devastating liver disease. Ultimately, the discovery of infectious triggers of PBC may expand the concept of

genetic susceptibility in immune-mediated liver diseases from the concept of aberrant immune responses against self-antigens to insufficient and/or inappropriate immunological defense mechanisms allowing microbes to cross natural barriers, establish infection and damage respective target organs.

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Key words: Primary biliary cirrhosis; *Novosphingobium*; Natural killer T cells, Cytotoxic T lymphocyte antigen 4; Diabetes; Susceptibility loci; Non-obese diabetic congenic mice

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INTRODUCTION

Due to its unique and distinct cellular composition with predominant abundance of Kupffer cells (KCs), natural killer (NK) cells and natural killer T (NKT) cells, the liver is considered to be an organ with special innate immune features^[1,2]. While being constantly exposed to microbial products, (toxic) environmental substances and food antigens from the portal stream draining the intestine, the liver plays a pivotal role in the induction and maintenance of immune tolerance^[3,4]. Nonetheless, the liver is involved in many systemic diseases^[5] and can become the target of adverse immune reactions in chronic inflammatory liver diseases^[6] like primary biliary cirrhosis (PBC), autoimmune hepatitis (AIH) and primary sclerosing cholangitis (PSC).

PBC, AIH and PSC are the three major immune-mediated hepatopathies. Variant forms of these diseases are generally called overlap syndromes although no standardized definition exists. Patients with overlap syndromes present with both hepatitic (AIH) and cholestatic (PBC, PSC) serum liver tests and exhibit histological features of AIH and PBC or PSC. A similar genetic predisposition may play a role in the development of these overlap syndromes as all three disorders share some common genetic susceptibility factors^[7-9]. Poor responses to immune suppression suggest that these three diseases are immunopathogenic rather than autoimmune. Association of PSC with inflammatory bowel disease (IBD)^[10] where commensal bacterial flora has been implicated in maintaining/triggering intestinal inflammation^[11] and the specific association of PBC with an ubiquitous alphaproteobacterium^[12-15] suggest that bacterial triggers may be involved in the pathogenesis of the two chronic cholestatic liver diseases.

ASSOCIATION OF PBC WITH *NOVOSPHINGOBIUM* *AROMATICIVORANS*

Chronic cholestasis is the main pathophysiological feature of PBC, one of the two most common chronic cholestatic liver diseases in adults. PBC usually progresses slowly to cirrhosis due to the immune-mediated destruction of small intrahepatic bile ducts, liver failure and death, unless liver transplantation is performed. Signature auto-antibodies to mitochondrial antigens (AMAs) with reactivity to the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2) represent the serological hallmark for the diagnosis of PBC. A curative therapy is not available^[16,17].

Several clinical reports have incriminated *Novosphingobium aromaticivorans*, an ubiquitous alpha proteobacterium^[18,19] and intestinal commensal as a likely etiology for most cases of PBC in humans^[12-14]. Based on these clinical studies, we have established a mouse model of autoimmune liver disease triggered by infection with this bacterium that strikingly resembles PBC in humans^[15]. The onset and severity of liver lesions and anti-PDC-E2 antibody responses in this model were dependent on: (1) mouse genetic background; (2) hepatic persistence of *Novosphingobium* bacteria; and (3) hepatic presence of NKT cells activated by *Novosphingobium* glycosphingolipids (GSLs)^[15,20]. These GSLs replace lipopolysaccharide (LPS) and constitute the unusual gram-negative cell wall of the *Sphingomonadaceae*^[21,22]. NKT cells specifically recognize *Sphingomonadaceae* GSLs and, in the absence of toll-like receptor 4 (TLR4) engagement by LPS, dominate the innate immune response^[20,23]. The preferential activation of NKT cells in the liver where NKT cells are abundant and *Novosphingobium* persists suggest, therefore, the basis for the biased autoreactivity towards autoantigens exposed in the liver environment and, ultimately, the severe, organ-

specific manifestations of *Novosphingobium* infection resembling human PBC. A latent, unrecognized infection with *Novosphingobium* may also account for the striking redistribution of NKT cells from the blood to the livers of PBC patients and the expression of CD1d on biliary epithelial cells^[24-26].

SPONTANEOUS AND INFECTION- INDUCED MOUSE MODELS OF PBC

Infection alone is not likely to be sufficient to confer disease; genetic predisposition plays an important role as well. Based on previous reports describing defined susceptibility loci in distinct autoimmune disorders^[27,28], we evaluated the role of these loci in mediating and/or inhibiting *Novosphingobium*-infection in mice. Commonly used mouse strains, including C57BL/6 (B6), non-obese diabetic (NOD) and SJL, all exhibited chronic anti-mitochondrial autoantibodies including anti-PDC-E2 IgG as well as liver lesions after intravenous inoculation of *Novosphingobium*. However, while some developed severe liver lesions, others exhibited only milder pathology. In order to dissect the genetic susceptibility and resistance alleles within the different genetic backgrounds, we performed infection experiments in NOD congenic mice that were generated by the introgression of diabetes susceptibility loci from chromosome 3 and 4 of B6 and B10 mice onto the NOD background^[29-31]. Utilizing these mouse strains with their concisely defined genetic regions, we aim to uncover the candidate alleles therein predisposing for severe liver disease and to understand the underlying mechanisms of genetic susceptibility to PBC by studying the regulation, expression and function of these candidate alleles. In all experiments, one NOD congenic strain, NOD 1101, consistently developed the most severe biliary pathology^[15]. NOD 1101 is a congenic strain with a restricted B6 chromosomal segment obtained from the NOD.c3c4 mouse^[29-31] that corresponds to the type 1 diabetes susceptibility loci *Idd10* and *Idd18* on chromosome 3. Infected, but not uninfected, NOD 1101 mice progressively develop massive enlargement of the liver in contrast to parental NOD.c3c4 mice that develop spontaneous liver autoimmunity and hepatomegaly^[30]. Currently, we are dissecting these *Idd10* and *Idd18* regions in order to find the candidate gene(s) therein that promote(s) severe PBC.

Although NOD.c3c4 mice do not require an exogenous infection with *Novosphingobium* to exhibit liver lesions and develop autoantibody titers, we reasoned that re-circulation of bacteria or bacterial products from the intestine triggers this process in NOD.c3c4 mice as *Novosphingobium* has been detected in the feces of mice^[32], similar as at mucosal surfaces of humans^[12]. The combination of impaired tolerance and enhanced inflammation promoted by molecules relevant for the immune response encoded in these defined *Idd* regions may render these mice susceptible to lower bacterial inocula. In the

context of genetic predisposition to autoimmunity in combination with bacterial infection, the immune reaction has to be evaluated from two additional perspectives: (1) as an insufficient immune reaction that allows a systemic infection with commensal bacteria; and/or (2) as an overzealous immune reaction that may allow bacterial eradication but only for the cost of collateral tissue damage; and, next to the traditional ones: (3) as an aberrant immune reaction towards self; and/or (4) as a failure to eliminate autoreactive lymphocytes before their migration into the periphery.

Here, we discuss these possibilities with respect to one candidate gene that has been associated with PBC and also with AIH and PSC in humans. Having unique tools for the dissection of these genetic loci with NOD congenic mice at hand, our studies in the mouse model allow not only the identification of susceptibility genes but also open the unique tool for directly testing them in translational studies for human disease.

GENETIC PREDISPOSITION FOR IMMUNE-MEDIATED LIVER PATHOLOGIES

Naturally occurring genetic polymorphisms determine the susceptibility of an individual to autoimmune diseases. Single nucleotide polymorphisms (SNPs) most likely evolved due to microbial pressure and reveal a consequence of natural selection for enhanced resistance/susceptibility to certain pathogens. Association studies mainly focused on immune genes that affect the immune system belonging to both the human leukocyte antigen (HLA) family and non-HLA immune modulator genes. Allelic variations in MHC class II (DQ, DR) and in components of the innate (C4*Q0, C4B*2, MBL, NRAMP1/SLC11A1, VDR) and of the adaptive (Cytotoxic T-Lymphocyte Antigen 4, [CTLA4, interleukin-1beta (IL-1beta), IL-12A, IL-12RB2, Tumor necrosis factor (TNF) alpha] immune system have been associated with susceptibility to PBC^[33-37]. Next, with several associations with HLA genes in PSC^[7,38,39] and AIH^[40,41], SNPs within the CTLA-4 gene have been implicated with PSC^[7] and AIH^[8]. The potential role of allelic variations within several genes encoding components of the innate and adaptive immune system suggests some disturbances of host resistance to microbial infection and their implication in the initiation and/or perpetuation of inflammatory processes. This may apply in particular for genetic variations of the IL-12 pathway since several data link inherited deficiencies of IL-12, IL-12R and Interferon-gamma to increased susceptibility to microbial infection and severity of infectious diseases, in particular mycobacterial diseases^[42]. As SNPs within CTLA-4 have been also associated with PSC and AIH^[7-9], we will focus our discussion of genetic susceptibility to autoimmunity in the context of bacterial infection on this negative costimulatory molecule.

CTLA-4 is one of the leading examples of genetic

variants that confer risk for developing diverse human autoimmune diseases as underscored by genome-wide association studies^[43] on the one hand. On the other hand, it has been associated with an increased risk of infections with parasites^[44-46], viruses^[47-50] and invasive bacterial infections^[51]. CTLA-4 and CD28 belong to the best-characterized co-receptors for T cells. Whereas CD28 activation propagates T cell activation, engagement of CTLA-4 can protect against the development of autoproductive and/or autoimmune disease due to the inhibition of T cell responses. Indeed, CTLA-4 is an indispensable negative regulator of peripheral T cell function^[52]. CTLA-4^{-/-} mice display a lymphoproliferative disorder and a severe autoimmune phenotype with the development of myocarditis and pancreatitis and die within the first month of life^[53,54]. No CTLA-4 deficient human has been reported so far. Two of the single nucleotide polymorphisms (SNPs) found within the CTLA4 gene, 49AG (rs231775) and CT60 (rs3087243), have been associated with susceptibility to PBC and/or correlated with autoantibody titers^[9,55], although some controversy exists^[56]. The 49AG SNP lies within the coding region of the CTLA4 signal peptide and is characterized by a threonine (A allele) to alanine (G allele) substitution, which has been shown to reduce the cell surface expression of CTLA4^[57,58]. Thus, the G allele is proposed to contribute to autoimmune risk, resulting in increased T-cell proliferation in response to immune activation. The CT60 SNP of CTLA4 is located in the 3' untranslated region of the gene and the slightly less common A allele is suggested to be protective for autoimmunity^[43]. Conversely, the G allele is thought to impart autoimmune risk by interfering with splicing processes, resulting in reduced production of a soluble form of CTLA4^[43] that has been shown to inhibit T-cell activation *in vitro*^[59].

Whereas CD28 is constitutively expressed at moderate levels, CTLA-4 expression on most T cell populations is dependent on T cell activation although some subsets of regulatory T cells (Tregs) have been reported to constitutively express CTLA-4^[60,61]. Whereas it is well-established that CTLA-4 is required for the optimal function of Tregs^[62], its role on natural killer T (NKT) cells, conventional (T conv cells) or effector T (Teff) cells is less clear (Figure 1). A recent study showed that self-reactive T cells which escape negative selection must either express CTLA-4 by themselves or become subject to peripheral control by regulatory T cells that also depend on CTLA-4 for their function^[63]. This may at least partially explain the differential expression of CTLA-4 among T cell subsets^[64], not considering differences in the kinetics of its induction on different T cell subsets. Nonetheless, there is an important role for CTLA-4 expression by effector T cells in restraining tissue-specific CD4⁺ T cells from infiltrating, expanding their populations and/or surviving in target organs and provides evidence that CTLA-4 can control the pathogenicity of self-reactive T cell at multiple levels. It is not yet clear whether CTLA-4 acts directly on the T cell that expresses it or acts on the antigen-presenting cell,

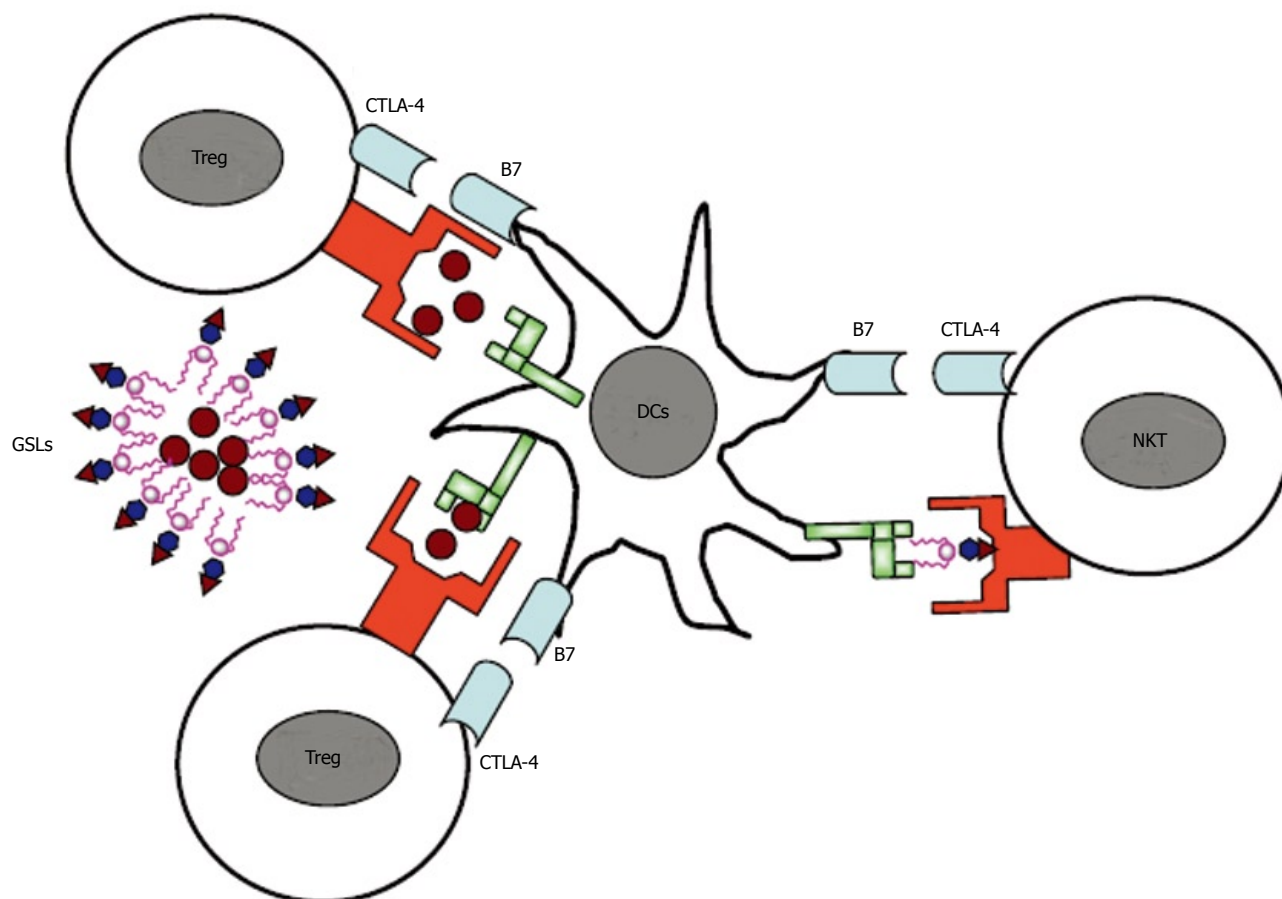


Figure 1 Regulation of T cell activation by cytotoxic T lymphocyte antigen 4. While cytotoxic T lymphocyte antigen 4 (CTLA-4) is constitutively expressed on regulatory T cells (Tregs), its expression is induced on effector T cells (Teff) upon activation. While it is well-established that CTLA-4 is required for the optimal function of Tregs, its role on natural killer T cells or effector T cells is less clear. DCs: dendritic cells; NKT: natural killer T; GSLs: glycosphingolipids.

either by binding of the ligand B7 by CTLA-4 which leads to back-signaling into antigen-presenting cells^[65,66] or by down-modulating B7 expression^[62].

Considering the fact that (1) PBC can be induced in our model due to the infection with *Novosphingobium*, (2) Tregs promote infection; and (3) CTLA-4 is indispensable for Treg activity, expression of CTLA-4 variants may also influence the susceptibility of an individual towards infection. SNPs within CTLA-4 that may dampen the expression and/or function of the CTLA-4 protein may enhance autoreactive T cell responses due to the lack of intrinsic T cell or Treg control while decreasing bacterial infection at the same time. On the other hand, gain of function mutations may create a tolerogenic environment that allows the outgrowth of bacteria and, subsequently, tissue damage due to recruited macrophages that are independently of IFN-gamma activated. Therefore, it has to be determined if CTLA-4 activation affects T cell activation in general or affects the activation of defined T cell populations. Accordingly, (1) enhanced CTLA-4 activation decreases T cell activation in general, promoting bacterial infection and subsequently tissue damage due to impaired bacterial clearance and/or prolonged bacterial persistence; and/or (2) specific activation in T cell sub-

populations, for example, regulatory T cells, allows tissue damage due to autoreactive T cells (that may be even less controlled by the respective CTLA-4 allele). This needs to be addressed in further studies. In addition, infection may modulate the surface expression of the CTLA-4 ligands, CD80 and CD86 on APCs.

CONCLUSION

Although genetic susceptibility factors have been often associated with aberrant immune responses to self-antigens and/or the inability of the immune system to eliminate autoreactive lymphocytes before emigration from the thymus, genetic susceptibility in light of bacterial infection may also refer to the susceptibility of an individual to develop an infection and/or to exhibit an inappropriate immune response causing collateral tissue damage and/or to prevent the development of an appropriate immune response to clear bacterial infection. In order to understand these mechanisms, animal models need to be developed which complement clinical and epidemiological studies. Uncovering the etiologies for these devastating liver diseases in the context of genetic susceptibility requires therefore further attention and

research efforts. NOD congenic mice not only provide a unique tool for the identification of genetic susceptibility region, but also allow the translation into human disease as the regulation of candidate molecules in humans and mice can be directly compared.

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Radiofrequency ablation of liver tumors: Actual limitations and potential solutions in the future

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Abstract

Over the past decade, radiofrequency ablation (RFA) has evolved into an important therapeutical tool for the treatment of non resectable primary and secondary liver tumors. The clinical benefit of RFA is represented in several clinical studies. They underline the safety and feasibility of this new and modern concept in treating liver tumors. RFA has proven its clinical impact not only in hepatocellular carcinoma (HCC) but also in metastatic disease such as colorectal cancer (CRC). Due to the increasing number of HCC and CRC, RFA might play an even more important role in the future. Therefore, the refinement of RFA technology is as important as the evaluation of data of prospective randomized trials that will help define guidelines for good clinical practice in RFA application in the future. The combination of hepatic resection and RFA extends the feasibility of open surgical procedures in patients with extensive tumors. Adverse effects of RFA such as biliary tract damage, liver failure and local recurrence remain an important task today but overall the long term results of RFA application in treating liver tumors are promising. Incomplete ablation of liver tumors due to insufficient

technology of ablation needles, tissue cooling by the neighbouring blood vessels, large tumor masses and ablation of tumors in close vicinity to heat sensitive organs remain difficult tasks for RFA. Future solutions to overcome these limitations of RFA will include refinement of ultrasonographic guidance (accuracy of probe placement), improvements in needle technology (e.g. needles preventing charring) and intraductal cooling techniques.

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Key words: Radiofrequency ablation; Hepatocellular carcinoma; Thermoablation; Colorectal cancer; Liver metastases

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INTRODUCTION

Although surgical resection still remains the treatment of choice for primary and secondary hepatic tumors^[1], several local ablative therapeutic modalities have emerged as reliable alternatives to resection^[2-5] or as adjuncts in oncological treatment. Throughout the past two decades, the importance of radiofrequency ablation (RFA) has continuously increased in the treatment of localized primary or secondary cancers in the liver^[6-10]. Whereas the initial indication and clinical application of RFA in visceral surgery

included the treatment of small circumscribed liver lesions, the indication has gradually expanded to more complex disease^[8,11] and combination of other techniques such as transarterial chemoembolization (TACE) or microwave ablation (MWA)^[12]. RFA represents an effective therapeutic tool for destruction of non-resectable primary and metastatic liver tumors of variable size and location and has proven to be successfully performed transcatheterally or by laparotomy or laparoscopy using sonographic or computer tomographic guidance^[13]. Different approaches have been continuously evaluated and RFA has been combined with other invasive techniques, e.g. thermal ablation and/or MWA, to engage liver tumors with different therapeutic methods^[14]. RFA has become widely accepted as an approach against primary liver tumors because of its ease and safety of use, lower level of invasiveness and high level of effectiveness. RFA has been repeatedly compared to open surgical procedures in different stages of liver tumor disease and the rate of complications within the RFA treated patients has displayed a lower frequency of severe complications; thus the efficacy of RFA is comparable to open surgery^[4].

ADVERSE EFFECTS OF RFA

Severe adverse effects of RFA of liver tumors have been critically investigated and the mortality in a comprehensive monocentric series of 143 RFA procedures in 122 patients resulted in a 1.4% fatal outcome^[15]. Furthermore, major complications including biliary tract damage (4.9%), liver failure (2.8%), hepatic abscess formation (2.1%), peritoneal infection (1.4%), intrahepatic haematoma and pulmonary embolism occurred in less than 1% of cases^[15]. The procedure specific complication rate was about 10% and the overall complication rate around 20%. In long term analysis, biliary stricture, hepatic failure, vascular damage and hepatic abscess formation were the most common major complications of RFA, reported in several studies^[15,16]. Another prospective randomized trial indicated the long term survival after RFA of colorectal liver metastases in a 10 year experience^[17]. They described an actuarial survival of 24 mo, with an actual 3 and 5 year survival of 20.2% and 18.4% respectively^[17].

NUMBER OF LIVER LESIONS ARE LIMITING RFA OUTCOME

The number of liver lesions was critical and median survival of patients with less or equal three hepatic lesions was significantly better than that of patients with more than three metastatic hepatic lesions^[17]. Furthermore, it has been concluded that not only the number and dominant size of hepatic lesions but also the preoperative serum level of carcinoembryonic antigen (CEA) value were strong predictors of survival^[17]. Interestingly, extrahepatic disease did not adversely affect survival in this large study, an overview of a total of 292 RFA procedures^[17].

EFFECTS OF RFA IN BRIDGING FOR LIVER TRANSPLANTATION

RFA has also been propagated as a loco regional therapy for bridging HCC prior to liver transplantation. In a retrospective analysis of 123 patients, the impact of transcatheter arterial chemoembolization (TACE), yttrium-90 (⁹⁰Y) and RFA prior to OLT was investigated^[18]. Interestingly, survival did not statistically change in the treatment groups compared to the non-treatment groups. Twelve patients were successfully down-staged but did not have a significant advantage in survival compared to patients that were transplanted without therapy. The authors concluded that loco-regional therapy is a safe method for patients on the transplant list that can downstage selected patients awaiting liver transplantation (OLT) but does not have an impact on survival of HCC^[18].

RFA AND THE OUTCOME IN PATIENTS WITH LIVER CIRRHOSIS

Another important topic of RFA is addressed in a study investigating the ablation of small hepatocellular carcinomas (HCC) in patients with liver cirrhosis. Mazzaferro *et al.*^[19] investigated RFA in 60 HCC in patients who underwent (OLT) (according to the Milan criteria). Single session RFA was performed and histological response was determined on the explanted livers. The post-RFA complete response rate was 55% rising to 63% for HCC with a diameter ≤ 3 cm^[19]. Tumor satellites or new formations of HCC lesions were unaffected by RFA and significantly correlated with HCC > 3 cm in diameter. Radiological response rates were around 70% and not significantly different from histology. Major post-RFA morbidity was described with 8% and no mortality occurred^[19]. Nevertheless, hepatic failure occurred in 2% and deterioration of Child-Pugh status was described^[19].

INDICATIONS AND LIMITATIONS IN THE USE OF RFA

Even though RFA has become a standard technique of ablation of liver tumors, it is of great importance to evaluate the right indications for the best possible benefit for patients. Current indications for RFA consist of limited but inoperable liver tumors, extent of distribution that permits ablation but not resection, non-operable liver tumors due to co-morbidity and non-operable due to inadequate residual functionality of liver tissue^[20,21]. To further dissect the question 'who benefits most of RFA', a study by McGrane *et al.*^[20] approached the question while highlighting the efficacy, the local recurrence rate and the safety of RFA application in the treatment of colorectal liver metastases. They found that RFA is not currently considered the method of choice for resectable colorectal metastases because surgical resection is, according to the reviewed literature, still superior to RFA^[22]. Nevertheless,

RFA appeared safe and highly effective in tumor destruction depending on tumor size^[23]. Importantly, the recurrence rate of 21.6% for tumors ≤ 2.5 cm, 52.8% for tumors 2.6 - 4.0 cm in size and 68.8% for tumors larger than 4.1 cm have been reported^[24], indicating a strong argument for RFA application in smaller tumors. The reviewed data today are not sufficient to judge whether RFA prolongs survival in patients with advanced colorectal liver metastases^[23]. A prospective trial by our group^[25] has shown an overall survival rate at 1, 2 and 3 years of 88%, 80% and 57% respectively. An overall recurrence rate occurred in 8.8% and in lesions smaller than 3 cm in diameter was 1.6%^[25]. In the conclusion of this study, the minimal local recurrence rate of colorectal liver metastases of less than 3 cm may competitively challenge the results of open surgery^[25]. Despite promising single centre results, there is a strong body of opinion that does not recommend extensive RFA application today^[21]. Future multi-centric studies will hopefully address this important question. In conclusion, there is an existing consensus that RFA indications can be proposed for colorectal liver lesions from 1 to 4, maximum 5 cm in diameter and that diameter and location of colorectal metastases are still the most limiting factors that challenge the use of RFA.

MODERN ULTRASOUND POTENTIATES THE OUTCOME OF RFA

The role of ultrasound guidance is increasing in the application of RFA and became an optimal method for accurate targeting of liver tumors. Ultrasound guidance for RFA is advantageous to the CT or MRT guidance because it is more mobile, practical, readily available as well as rapid and cost-effective^[26]. Advances in non-linear imaging modes and the development of 3-dimensional (3D) ultrasound probes have led to a significant improvement in the real-time contrast enhanced volumetric imaging. This progress impacts the detection, the planning and the targeting strategy of RFA needles. Although general limitations of RFA such as treatment of perihilar hepatic lesions due to the risk of biliary damage and consecutive fistula still are an issue, the technical progress is not to stop. Intraoperative ultrasound (IOUS) has been proven to gain significant new information not identified in preoperative radiological imaging. Therefore, IOUS has become the gold standard for the final evaluation of respectability of liver tumors^[27]. Contrast-enhanced ultrasound (CE-US) has been shown to be highly accurate in detecting the extent and distribution of liver tumors^[27]. Some recent studies have shown that new CE-US applications are of comparable sensitivity and accuracy as CT and MR scans^[28,29]. A further advantage of CE-US is the possibility to restage the actual situation directly before the therapeutic intervention. The application of advanced 3 dimensional CE-US (CE-3DUS) offers new possibilities to detect liver tumors more comprehensively. The CE-3DUS data set may be transferred and computed online by a sonolo-

gist. Furthermore, dealing with larger liver tumors and recurrent liver disease, the CE-3DUS can define active tumor tissue more clearly^[26]. Another important topic in targeting tumors is the selection of RFA needle electrodes. That highly depends on the tumor size to be ablated. For smaller tumors (2 to 3 cm in diameter) the geometry of tumors is usually spherical. However, larger tumors (> 3 cm) may become more ellipsoid. Local advanced disease often manifests with satellite metastases, indicating a more lobular growing tumor mass^[26]. Therefore, the 3D-ultrasonographic assessment of the tumor geometry and determination of the lesion's long axis is required to plan the RFA intervention. Accurate delineation of the active tumor margins in 3 dimensions is critical to determine the actual size of the liver lesion. In an analogy to surgical liver resection, a safety margin of 0.5 to 1.0 cm should be reached in any circumstance. Especially during the ablation process, the 3D ultrasonographic technique is vastly superior to standard ultrasound^[26]. The importance of the true delineation and geometry of liver tumors is also largely related to the limited ablative capability of RFA needle electrodes available today. In CE-3DUS the exact volume and rim of the liver mass can be calculated automatically, depending on the software, giving another important tool for planning RFA. The placement of RFA needle electrodes can be performed with reference to the probe position with the aid of a needle-guide or free-hand control^[26]. The more specified and computed information provided by CE-3DUS enables a more aggressive approach to the ablation of large liver lesions. The combination of several RFA needle electrodes and/or electrodes with multiple antennas create coagulation necrosis beyond 7 cm^[26]. Importantly, the number of electrodes should be minimized to prevent tumor cell spill and further complications.

Another important part of the RFA treatment is to monitor the response. To assess the initial response to RFA treatment, CE-3DUS can be performed around 10 min after the RFA treatment. The absence of any intralesional enhancement or moving microbubbles is consistent with the complete coagulation necrosis of affected liver tissues. The security of total coagulation necrosis is detectable in highly vascularized tumors^[26].

WHAT ARE THE LIMITATIONS OF ULTRA-SONOGRAPHIC TECHNIQUES?

The limitations of this new ultra-sonographic technique consist of limited spatial resolution of the current 3D probes. The volumetric measurement may be distorted as a result of motion when using the mechanical probe. Furthermore, the presence of gas production during the ablative process may cause a shadowing artefact that may lead to inaccurate assessment of treatment response and calculation of ablation zone dimensions. A significant learning curve in the adoption of the new technique is further discussed^[26]. This aspect of highly advanced 3D

ultra-sonographic application in combination with RFA shows how future therapeutical tools may have significant impact in treatment of metastatic liver disease and how a combination of two or more medical modalities can enlarge our therapeutical capacity to defeat malignant disease of the liver.

WHAT ARE THE PROGNOSTIC PREDICTORS AFTER RFA?

In general, authors reported that the prognosis of patients with hepatocellular carcinoma (HCC) treated by RFA is highly dependent on tumor characteristics and liver function^[30,31]. The usefulness of α -fetoprotein (AFP), *Lens culinaris agglutinin A-reactive fraction of AFP* (AFP-L3) and prothrombin induced by vitamin K absence of antagonist II (PIVKA-II) has been detailed in previous studies^[32]. Depending on the Child-Pugh stage, PIVKA-II was found to be the best prognostic predictor after curative RFA in Child-Pugh stage A liver disease^[33]. PIVKA-II levels above 100 mAU/ml prior to RFA therapy significantly predicted the recurrence and shortening of the period within the Milan criteria^[33]. In the ablation of HCC, des-gamma-carboxy prothrombin (DCP) not only reflected the biological aggressiveness and progression of HCC tumors but DCP levels were significant predictors of survival^[34]. Likewise, DCP levels were also significant predictors in recurrence free survival^[34]. Another study investigated predictors of survival after RFA of colorectal cancer metastases in the liver. They concluded, after analyzing patients demographics and tumor characteristics, that the number and size of liver metastases and serum CEA are among the most significant factors to predict patient outcome after RFA treatment for colorectal cancer metastases^[35].

COST EFFECTIVENESS OF RFA

In times of financial restrictions where health insurance rates are increasing every year, the cost effectiveness of a new medical procedure needs to be addressed carefully. Today there is no doubt that RFA is a standardized and safe tool in experienced hands to treat patients with malignant liver disease where options of curative resection and cure are limited. Nevertheless, the cost analyzes has to be made critically. A prospective randomized trial has reported average costs of hospital stays with RFA at 1584 € for percutaneous RFA in an outpatient setting, 3824 € for percutaneous RFA in an inpatient setting, 8194 € for laparoscopic RFA and 12967 € for intraoperative RFA combined with surgical resection^[36]. The economical impact of RFA complications was a longer duration of hospital stay (2-6 d on average and an added cost of 1660 €)^[36]. These sober numbers only display the treatment budget without any emotional background, mandatory in an ethical concept to globally address the question of cost effectiveness between economical and social resources.

Cost benefits of RFA is an area that is difficult to asse-

ss simply because, although total costs of a medical procedure can be relatively easy assessed, the total economic costs are very difficult to measure as the earlier start of reintegration in the patient's normal life cannot be "financially" measured by simple figures.

FUTURE CONCEPTS IN RFA

According to the American Association for the Study of Liver Diseases (AASLD) guidelines, RFA is a safe and effective method to treat patients with advanced liver tumors^[37]. For hepatic tumors less than 3 cm in diameter, initial complete tumor response rates of $\geq 90\%$ and local tumor progression rates between 10% to 20% have been reported^[37]. A three year local recurrence free interval has been reported in more than 95%. Although these are promising clinical outcomes, the complete tumor response rates for HCC following RFA in histopathological diagnostic criteria has reported to be less than 50% in certain reports^[38,39]. Regarding conventional histopathological diagnostic criteria as the gold standard, RFA must be considered as only a palliative treatment modality due to the apparently poor histopathological tumor response rates^[37]. This discrepancy between radiological and histopathological findings is a result of at least two main effects: conventional histological assessment based on H&E stained tissue specimens are sometimes inconclusive in determining tumor viability^[40] and a thermal fixation effect following RFA. Thermal fixation can be considered as a different form of cell death in addition to coagulation necrosis^[41]. Thermal fixation seems to result from the denaturation of the tissue's structural and enzymatic proteins. This inhomogeneity needs attention and could be addressed in future randomized trials. Therefore, not only clinical studies are mandatory.

Accurate probe placement using laparoscopic ultrasound guidance is required to achieve complete tumor ablation. Not only the probe placement but also the continuous linear heat release from the applied needle is critical. A recently published study showed an image-guided surgery system for laparoscopic RFA (LapAssistant)^[42]. This technology is based on an electromagnetic tracking system that helped to navigate the laparoscopic ultrasound probe and a RFA needle^[42].

The success of laparoscopic ultrasound guidance is highly dependent on the localisation of the liver tumors to access. Whereas tumors in the ventral part of the liver are relatively easy to access, tumors in the posterior area of the liver are often technically not ideal to reach.

Together with technical advances in RFA-needle design, heat application and heat diffusion in tissue have to be studied intensely to overcome incomplete ablation of tumors due to the effect of vessel cooling and other limitations of RFA. Together with more advanced intraoperative imaging, e.g. CE-3DUS and 3D-navigation in laparoscopic RFA, more advanced needle and RFA technology and more refined histopathological assessment, one can potentially target more complex liver tumors.

INTRADUCTAL COOLING AND TISSUE PROTECTION ARE KEY FOR SUCCESSFUL RFA

Liver tissue contains parenchymal cells, bile duct structures, vascular tissue and, to some extent, matrix related tissue structures that are more prominent in cirrhotic liver disease and only marginally apparent in normal liver tissue. However, liver tumors are inhomogeneous tissues with an imbalance of parenchymal, bile duct, vascular and matrix tissue components. This heterogeneity of tumor tissue also changes the physical density of tissues and sensitivity to thermal ablation. Bile ducts are especially prone to thermal injury during RFA and consecutive complications after RFA treatment^[43]. Intraductal cooling during RFA^[43,44] has become an important tool to better control the unwanted thermal collateral damage. In general, a tumor location within 10 mm of a central bile duct is frequently considered a contraindication to RFA^[43,45,46]. It has been demonstrated that bile ducts within 6 mm of RFA are getting injured when not protected simultaneously with intraductal cooling^[47]. In a porcine model, it has further been shown that abscess formation and biliary obstruction occur more likely when bile ducts are within the area of RFA induced necrosis^[48]. In another porcine model it has been proven that the epithelial and bile duct damage became less prominent after intraductal cooling when analyzing histological tissue sections 48 h after RFA^[49]. Stenosis of the bile duct normally occurs when the duct is within the range of the ablation zone. Clinically, bile duct stenosis due to RFA generally becomes evident after 3 to 4 wk^[45,50]. The usage of cool-tip 10 mm RFA electrodes in order to increase the treatment potential of HCC lesions has suggested for hypovascular HCC lesions of less than 10 mm in diameter^[44]. To further prevent skin burns, it has been further suggested to only treat lesions at least 16 mm below the liver capsule^[44].

HOW TO OVERCOME LIMITATIONS OF RFA

Thermal ablation techniques are subjected to a dilemma: the tumor tissues have to be completely destroyed but at a price that no viable normal liver parenchyma and bile duct structures are being damaged unnecessarily. Therefore, the amount of thermal energy applied to the tumor mass has to be exactly regulated. This important regulation is further complicated by the often inhomogeneous tissue architecture and composition of liver masses and the inconsistent distribution of blood vessels. We developed a novel needle perfusion technique that has shown to enhance the efficiency of RFA in treating liver tumors^[51]. In this non randomized, retrospective study we studied the outcome of standard *vs* perfusion RFA needle technique in liver tumors of a median diameter of 2 cm. The group treated with the perfusion RFA needle displayed a significantly shorter RFA time of 8.0 min *vs* 18.9 min in the standard RFA protocol group^[51]. The rates of in-

complete ablations were comparable at around 3% in both groups whereas the local recurrence rate was 6.9% overall, 11.1% in the standard group compared to 4.8% in the perfusion group^[51]. We concluded that the perfusion of an expandable RFA needle with saline solution significantly accelerates the ablation procedure of liver tumors with less complications and no disadvantage regarding the oncosurgical outcome^[51]. The beneficial aspect of this needle perfusion technique may result from the better and more efficient thermal energy application without the regularly occurring charring effect. Saline-linked surface RFA has been described elsewhere and prevents charring and results in deeper coagulation of tumor lesions^[52]. Reports in pig livers showed that tissue destruction to 20 mm can be safely achieved with novel saline-linked RFA^[52]. A different bimodal electric tissue ablation-modified RFA technique has provided data that the modification of standard RFA technique with the addition of a direct electrical current (electric tissue ablation-modified RFA) helped to significantly enlarge the ablation radius in a liver pig model^[53].

CONCLUSION

Every new surgical technique faces hurdles, initial weaknesses and limitations. The development of refined instrumentation combined with a set of specific surgical skills has tremendously aided the implementation of RFA in clinical practice. Although the limitations and shortcomings of RFA are discussed and RFA is not considered a curative treatment in advanced hepatic tumors, it is a critical tool for patients awaiting liver transplantation (bridging therapy) as well as for patients with advanced central liver tumors where open surgery or other treatment modalities are of limited use. With the combination of potential future advances in imaging, diagnostic and modern treatment tools together with advanced probes and 3D-technology, we believe that RFA will extend its clinical position in the treatment of advanced end stage liver tumors in the near future.

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Multicentric occurrence of hepatocellular carcinoma with nonalcoholic steatohepatitis

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Abstract

AIM: To reveal the manner of hepatocellular carcinoma (HCC) development in patients with nonalcoholic steatohepatitis (NASH) focusing on multicentric occurrence (MO) of HCC.

METHODS: We compared clinicopathological characteristics between patients with and without MO of HCC arising from NASH background. The clinical features were implicated with reference to the literature available.

RESULTS: MO of HCC was identified with histological proof in 4 out of 12 patients with NASH-related HCC (2 males and 2 females). One patient had synchronous MO; an advanced HCC, two well-differentiated HCCs and a dysplastic nodule, followed by the development of metachronous MO of HCC. The other three patients had multiple advanced HCCs accompanied by a well-differentiated HCC or a dysplastic nodule. Of these three patients, one had synchronous MO, one had metachronous MO and the other had both synchronous and metachronous MO. There were no obvious differences between the patients with or without MO in terms of liver function tests, tumor markers and anatomical extent of HCC. On the other hand, all four patients with MO of HCC were older than 70 years old and had the comorbidities of obesity, type 2 diabetes mellitus (T2DM), hypertension and cirrhosis. Although these conditions were not limited to MO of HCC, all the conditions were met in only one of eight patients without MO of HCC. Thus, concurrence of these conditions may be a predisposing situation to synchronous MO of HCC. In particular, old age, T2DM and cirrhosis were suggested to be prerequisite for MO because these factors were depicted in common among two other cases with MO of HCC under NASH in the literature.

CONCLUSION: The putative predisposing factors and necessary preconditions for synchronous MO of HCC in NASH were suggested in this study. Further investigations are required to clarify the accurate prevalence and predictors of MO to establish better strategies for treatment and prevention leading to the prognostic improvement in NASH.

Key words: Nonalcoholic steatohepatitis; Hepatocellular carcinoma; Multicentric occurrence

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INTRODUCTION

With the increasing prevalence of obesity and type 2 diabetes mellitus (T2DM), nonalcoholic fatty liver disease (NAFLD) has become pandemic, particularly in developed countries, causing public health problems. Within the broad spectrum of the pathophysiology of NAFLD, nonalcoholic steatohepatitis (NASH) is the most serious form because of its propensity to progress toward a fatal event. Although NASH was previously thought to be often indolent^[1,2], the following analyses revealed that NASH leads to fibrosis of the liver, cirrhosis and eventually hepatocellular carcinoma (HCC) in a substantial number of patients^[3-7]. HCC is currently regarded as a late complication of NASH according to a number of recent reports^[3-7]. In addition, it was recently shown that the development of HCC is associated with mortality in cirrhotic NASH in a prospective study in Japanese cohort^[5]. Thus, it is very important to elucidate the natural course of NASH in terms of the development of HCC for better management.

Multicentric occurrence (MO) and intrahepatic metastasis (IM) are characteristic in the development of HCC with chronic liver diseases that are caused by hepatitis B virus (HBV) or hepatitis C virus (HCV)^[8-13]. It is very important to distinguish MO from IM so that appropriate treatment options may be selected in a variety of clinical settings. Unfortunately, there have been few studies that focus on the characterization of MO in NASH-related HCC despite the increasing number of case reports on HCC based on NASH.

In this report, characteristic features of NASH cases that developed HCC with MO are discussed.

MATERIALS AND METHODS

Patients

From July 2002 to March 2010, we diagnosed and treated

40 adult patients with NASH at the Division of Gastroenterology and Hepatology, Niigata University Medical and Dental Hospital. Of these, HCC was observed in 12 cases including 4 with MO of HCC. The proportion of HCC patients is high in our series with NASH because our hospital is a tertiary referral center and most of the NASH patients who are referred to us are complicated cases.

Definitions

NASH was defined to satisfy all of the following requirements: (1) an absence of clinically significant alcohol intake (less than 20 g/d of ethanol consumption); (2) histological features showing steatosis with various combinations of ballooning liver cells, inflammatory infiltrate of neutrophils, pericellular fibrosis and Mallory bodies; and (3) no other liver diseases. All non-tumorous specimens were histologically scored according to the classification by Brunt *et al.*^[14].

MO of HCC was pathologically determined according to the classification of the Liver Cancer Study Group of Japan^[15] as follows: 2 or more separate lesions including an early HCC with a dysplastic nodule or no substantial destruction of the preexisting hepatic framework, or moderately and/or poorly differentiated HCCs with a margin of well-differentiated HCC. When histological specimens of HCC could not be obtained, MO was identified by particular findings that correspond to MO on imaging studies^[16,17].

Advanced HCCs were defined when they had a vascular pattern that was consistent with contrast enhancement in the arterial phase followed by rapid washout in the portal and/or equilibrium phases on dynamic computed tomography (CT) and/or dynamic magnetic resonance (MR) imaging. A nodule was also diagnosed with an advanced HCC when the lesion was depicted as a defect on CT during arterial portography (CTAP) and as a hyperattenuated lesion in the first phase of double-phase CT during hepatic arteriography (CTHA) followed by coronal-like enhancement in the second phase^[16,17]. A nodule-in-nodule appearance on dynamic CT, dynamic MR imaging or CTHA was regarded as a specific finding that indicated the emergence of a dedifferentiated component (moderately or poorly differentiated HCC) within a well-differentiated HCC, even without histological proof. Otherwise, well-differentiated HCCs or dysplastic nodules were diagnosed based on histological examinations.

The clinical stage of HCC was stratified according to the TNM classification of the Liver Cancer Study Group of Japan^[18].

Obesity was defined as body mass index (BMI) ≥ 25 kg/m² according to the criteria proposed by the Japan Society for the Study of Obesity^[19]. The definition was based on the fact that obesity-related diseases increase with BMI ≥ 25 kg/m² in the Japanese population. T2DM was diagnosed according to the criteria advocated by the Japan Diabetes Society as follows: fasting plasma glucose ≥ 126 mg/dL, random plasma glucose ≥ 200 mg/dL

Table 1 Clinical characteristics of patients with nonalcoholic steatohepatitis-related hepatocellular carcinoma with or without multicentric occurrence

Case	HCC with MO				HCC without MO							
	1	2	3	4	5	6	7	8	9	10	11	12
Age at Dx of NASH	73	81	78	71	68	75	71	80	75	78	81	66
Gender	F	M	M	F	M	F	M	F	F	M	M	M
History of BTF	-	-	-	-	-	-	-	+	-	-	-	-
BMI (kg/m ²)	31.0	26.3	25.5	32.0	26.8	23.0	27.0	32.1	34.0	23.1	24.5	25.2
T2DM	+	+	+	+	-	+	+	-	+	-	-	-
Hypertension	+	+	+	+	-	-	-	+	+	-	+	+
Dyslipidemia	-	-	-	+	-	+	+	+	-	+	+	-
Varices	-	-	-	-	+	-	+	-	+	-	-	-
Ascites	-	-	-	-	-	-	-	-	-	-	-	-
AST (IU/L)	91	67	43	33	34	43	36	34	43	25	83	78
ALT (IU/L)	46	48	49	21	17	32	22	33	40	20	37	75
γ-GTP (IU/L)	122	129	418	72	30	222	46	67	73	104	360	279
Total bilirubin (mg/dL)	1.0	0.7	0.8	1.1	1.4	1.1	1.5	0.6	1.2	0.6	0.6	0.8
Albumin (g/dL)	3.8	3.7	3.4	2.9	3.3	4.0	2.9	4.1	3.7	3.8	3.4	4.2
Prothrombin time (%)	53	72	97	62	69	NA ^a	59	69	86	75	84	NA ^a
Platelet (× 10 ⁴ /μL)	21.1	12.3	10.4	11.2	4.5	14.5	6.4	31.6	11.7	12.1	26.4	11.4
Child-Pugh score	A	A	A	B	B	- ^a	B	A	A	A	A	- ^a
HBsAg	-	-	-	-	-	-	-	-	-	-	-	-
Anti-HBc	+	-	-	+	-	-	-	-	+	+	-	+
Anti-HCV	-	-	-	-	-	-	-	-	-	-	-	-
Histological features (Classification by Brunt)												
Grade	2	2	2	2	1	2	2	1	1	1	2	2
Stage	4	4	4	4	4	4	4	4	4	1	2	4
Type of cirrhosis	Mixed	Mixed	Macro	Mixed	Micro	Mixed	Mixed	Mixed	Mixed	-	-	Macro
AFP (ng/mL)	11	26	15	25	7	9	3	8 757	12	2 963	900 100	8
DCP (mAU/mL)	18	24	20	116	24	NA ^a	14	35	907	131	10 700	NA ^a
Maximum size of HCC (mm)	35	50	40	28	22	17	50	26	40	40	110	70
Number of HCC	Mul	Mul	Mul	Mul	Mul	Sol	Mul	Sol	Mul	Mul	Mul	Sol
TNM stage	III	III	III	III	IVA	I	III	II	III	IVB	III	II
Initial treatment	Ope	TACE + RFA	Ope	TACE + RFA	TAI	RFA	TACE	TACE + Ope	Ope + RFA	Chemo	Ope	TAI + Ope
Outcome	Alive	Dead	Dead	Alive	Alive	Alive	Alive	Alive	Dead	Dead	Alive	Alive
Cause of death	-	LR	NLR	-	-	-	-	-	LR	LR	-	-
Follow-up period after Dx of HCC (days)	2 599	1 706	288	1 140	280	1 011	687	2 198	1 525	489	162	330

HCC: hepatocellular carcinoma; MO: multicentric occurrence; Dx: diagnosis; BTF: blood transfusion; BMI: body mass index; M: Male; F: Female; T2DM: type 2 diabetes mellitus; AST: aspartate aminotransferase; ALT: alanine aminotransferase; γ-GTP: γ-glutamyltranspeptidase; NA: not available; AFP: alpha-fetoprotein; DCP: des-γ-carboxy-prothrombin; Mixed: mixed nodular cirrhosis; Macro: macronodular cirrhosis; Micro: micronodular cirrhosis; Mul: multiple; Sol: solitary; TACE: transcatheter arterial chemoembolization; TAI: transcatheter arterial infusion chemotherapy; RFA: radiofrequency ablation; Ope: operation; LR: liver-related death; NLR: non-liver-related death; ^aAn oral administration of warfarin prevented the evaluation.

or hemoglobin A_{1c} ≥ 6.5% on two separate occasions. The diagnosis of hypertension was made if the patient was on antihypertensive medication or had blood pressure ≥ 140/90 mmHg on at least two separate occasions. Dyslipidemia was defined as total cholesterol level ≥ 220 mg/dL and/or fasting triglyceride level ≥ 150 mg/dL on at least two separate occasions or continuously receiving lipid-lowering agents.

Laboratory examinations

The following laboratory tests were recorded at diagnosis of HCC in all patients: aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltranspeptidase (γ-GTP), total bilirubin, albumin, prothrombin time, platelet count, Child-Pugh score, hepatitis B surface antigen (HBsAg), hepatitis B core antibody (anti-HBc), HCV antibody (anti-HCV), alpha-fetoprotein (AFP) and des-γ-carboxy-prothrombin (DCP).

RESULTS

The clinical and laboratory data from 12 patients with NASH-related HCC are shown in Table 1. Of these, MO of HCC was observed in cases 1 to 4. Regarding common characteristics, all 4 patients with MO were over 70 years old, obese and had T2DM, hypertension and cirrhosis. Although these conditions were not limited to MO of HCC, all the conditions were met in only one (case 9) of eight patients without MO of HCC. There were no obvious differences between the patients with or without MO in terms of liver function tests, tumor markers and stages of tumor development. Although a few patients had positive anti-HBc, the titer of the antibody was low and no attribution of HBV infection to background liver disease was histologically ascertained.

The clinical courses of the 4 patients with MO of HCC are detailed below.

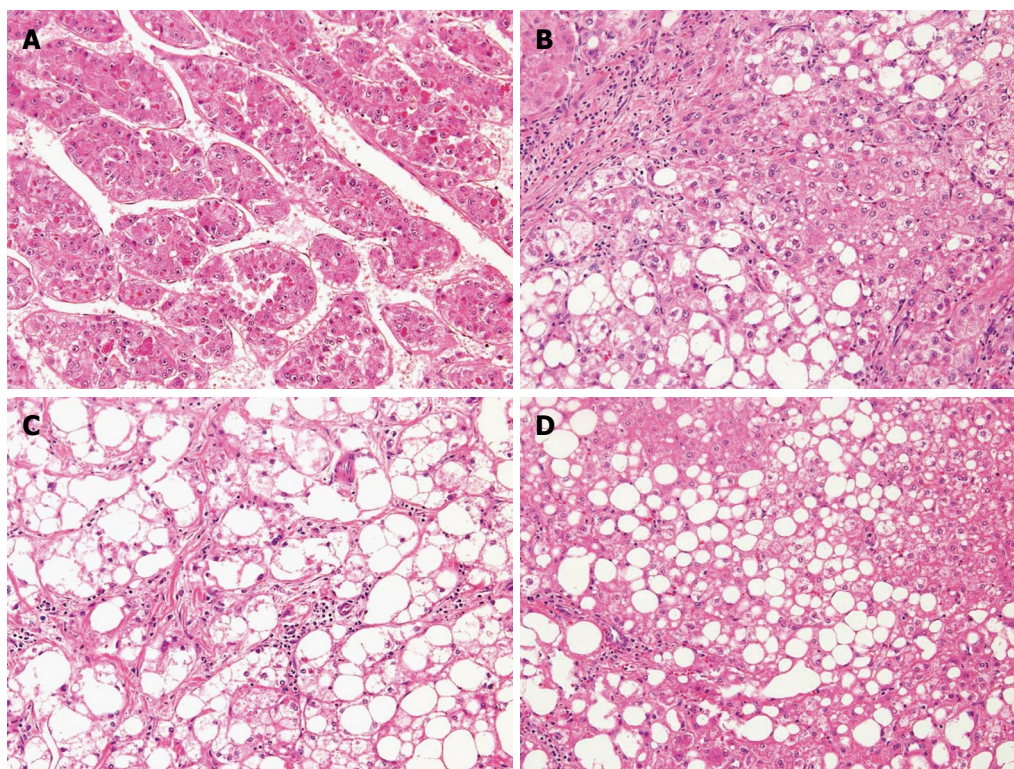


Figure 1 Histological examination of resected specimen in Case 1 showing four liver tumors of multicentric origin (HE stain, × 200). A: A moderately differentiated hepatocellular carcinoma (HCC); B: A well-differentiated HCC; C: A well-differentiated HCC; D: A dysplastic nodule.

Case 1

A 73 year old female had been diagnosed with hypertension at age 40 and T2DM at age 70. After the diagnoses, she was treated with an angiotensin II receptor blocker and an oral anti-hyperglycemic agent at our affiliated hospital. A liver tumor measuring 35 mm in diameter in segment 6 was found with a nodule-in-nodule appearance on a surveillance dynamic CT in January 2003. The finding was consistently shown on dynamic MR imaging, CTAP and CTHA. Near the tumor, another nodule 8 mm in diameter was depicted as a hyperechoic nodule on ultrasonography (US) and as a perfusion defect on CTAP without early enhancement on CTHA. A well-differentiated HCC or a dysplastic nodule was suspected. The patient was referred to our hospital and underwent surgical resection of segment 6. During the operation, multiple hyperechoic nodules measuring less than 10 mm in diameter in addition to the main tumor were detected in segment 6 and segment 8 by intraoperative US which led to an additional resection of segment 8. The largest tumor in segment 6 was histologically diagnosed with a moderately differentiated HCC (Figure 1A). In addition, two well-differentiated HCCs and one dysplastic nodule were observed among the other small nodules (Figure 1B, C and D). All of these tumors were considered to occur in a multicentric manner.

In October 2008, recurrence of HCC was found in segment 8 on dynamic CT with early enhancement and the patient underwent transcatheter arterial chemoembolization. In March 2009, one and two nodules were detected in segments 8 and 3, respectively, as hypovascular tumors measuring less than 15 mm in diameter on dynamic MR imaging that were suspicious for well-differen-

tiated HCCs or dysplastic nodules. The tumors subsequently increased in diameter and were compatible with well-differentiated HCCs on imaging studies which suggested metachronous MO. The patient was treated with percutaneous ablation therapy and has had no recurrence of HCC after the therapy.

Case 2

An 81 year old male had been diagnosed with hypertension at age 40 and T2DM at age 75. After the diagnoses, he was treated with a calcium channel blocker and an oral anti-hyperglycemic agent. When he was admitted to the Division of Dermatology at our hospital for the treatment of psoriasis vulgaris, dynamic CT and MR imaging depicted multiple tumors in the bilateral lobes of the liver. The classical pattern of contrast enhancement that indicated advanced HCCs was observed in those tumors with the exception of a nodule measuring 10 mm in diameter in segment 3. This distinct nodule was recognized as hypoattenuation in the arterial and equilibrium phases on dynamic CT. Dynamic MR imaging demonstrated hypointensity only in the equilibrium phase but isointensity in the arterial phase and other sequences. The nodule was also shown as isoattenuation on CTAP and CTHA. We regarded this nodule as an equivocal lesion and decided to follow it closely. The combination therapy with transcatheter arterial infusion chemotherapy and percutaneous radiofrequency ablation was repeated four times for the advanced HCCs. Approximately 1 year later, the follow-up study with dynamic MR imaging showed an altered appearance of the nodule in segment 3; it was hypointense in the arterial and equilibrium phases and on T1- and T2-weighted images (WIs) and was slightly

enlarged, 18 mm in diameter. This nodule was finally diagnosed with a well-differentiated HCC by tumor biopsy and the surrounding nontumorous parenchyma was histologically defined as cirrhosis derived from NASH. Thus, the development of this well-differentiated HCC was considered MO and the tumor was treated with local ablation therapy. Afterward, multiple HCCs recurred *via* intrahepatic metastases and transcatheter arterial chemoembolization therapies were repeated. Unfortunately, the patient died of hepatic failure with marked extension of HCCs at age 86.

Case 3

A 78 year old male was referred to our hospital for further workup and treatment of liver tumors detected on CT. Abnormal liver function tests had been found for 8 years. The patient had a 2 year history of treatment for hypertension and T2DM with oral drugs. Two tumors, 40 mm in diameter in the posterior segment and 20 mm in diameter in segment 8, respectively, were discovered on US, performed as part of the regular screening. Other imaging studies, including dynamic CT, MR imaging, CTAP and CTHA, indicated advanced HCCs. Other than those tumors, a nodule measuring 8 mm in diameter with different characteristics was detected in segment 5. The nodule was slightly hypointense in the arterial and equilibrium phases and isointense on T1- and T2-WIs of dynamic MR imaging. It was exhibited as isoattenuation on CTAP and hypoattenuation in both phases of CTHA. On the basis of these results, the tumor was suspicious for a well-differentiated HCC or a dysplastic nodule. The patient underwent surgical resection of the right hepatic lobe. The histological examination revealed cirrhosis of a nontumorous liver caused by NASH. The advanced tumors in the posterior segment and segment 8 were diagnosed with moderately differentiated HCCs and the distinct nodule in segment 5 was confirmed as a well-differentiated HCC which implied MO. After the surgery, the patient suffered from pneumonia, renal insufficiency and subsequent hepatic failure and he finally died of multiple organ failure approximately 2 mo later.

Case 4

A 71 year old female had received medical treatment for hypertension, dyslipidemia and T2DM at a clinic 7 years prior to presentation. At age 66, abnormal liver function tests were revealed on a routine examination and the patient was diagnosed with cryptogenic cirrhosis by liver biopsy at another hospital. Multiple liver tumors were detected on periodic US and the patient was referred to our hospital for further management. The definitive diagnosis of NASH was made as the cause of cirrhosis by a pathologist at our hospital with reevaluation of the previously obtained liver specimen. Two tumors in segment 8 and one tumor in segment 3, both less than 30 mm in diameter, were demonstrated as advanced HCCs on dynamic CT, MR imaging and CTHA. CTAP was not informative because of insufficient portal perfusion due to hepatofugal portal flow through a gastrosplenic shunt. Meanwhile,

a nodule in segment 1 with a diameter of 28 mm was shown as a nodule-in-nodule appearance on dynamic MR imaging. Therefore, this tumor was considered multicentric in origin. The patient was treated with transcatheter arterial chemoembolization and/or percutaneous ablation therapy. Furthermore, 18 mo after the treatment, another well-differentiated histologically confirmed HCC emerged in segment 7 and was treated with RFA. Thereafter, the patient remains alive without recurrence of HCC.

DISCUSSION

HCC occurs frequently through multicentric carcinogenesis in chronic liver diseases caused by HBV or HCV infection. In the present study, we found the prevalent occurrence of HCC with multicentric manner in patients with NASH as well. According to the previous studies based on pathological examinations, frequencies of synchronous MO in patients with HBV- or HCV-related HCC were 3.7%-16.5% and 11.9%-34.1%, respectively^[10-12]. As for metachronous MO, it was reported that the 1 year, 3 year and 5 year MO rates were also as high as 5.3%, 28.9% and 50.8%, respectively, in HCC patients, most of whom were associated with HCV infection^[9]. HCV infection that causes persistent active inflammation in the liver is believed to be one of the most important factors for MO of HCC^[8-13]. Contrary to the abundant clinical data on MO in HBV- and HCV-related HCC, there is no epidemiological study addressing MO in NASH-related HCC. However, informative observations were reported by Oikawa *et al.*^[12]. They evaluated the liver specimens surgically obtained from 94 cases with nonB-nonC HCC and detected synchronous MO of HCC in 12 cases (12.8%). They speculated that these cases might have occult HBV infection or other undefined hepatitis virus infection. Although the etiology of the underlying chronic liver disease in this group could not be further estimated because of the lack of clinical profiles and histological findings of noncancerous liver tissues, it is not difficult to suppose a causal attribution to NASH in some cases. In addition, Tokushige *et al.*^[20] revealed a high recurrence rate of HCC with NASH after two years or more of curative treatment. Those recurrent tumors in the later follow-up years were found in 9 of 16 patients (56.3%) and some of them were presumed to be of multicentric origin. Therefore, even in our cases without synchronous MO of HCC, it is possible that HCC may develop metachronously in the future. MO may be a frequent manner of HCC development in NASH as well and it is crucial to clarify the exact prevalence of MO with further studies.

Clinical features of patients with MO of HCC related to NASH are also quite obscure. There are only two case reports that have documented MO of HCC in NASH with histological proof. Zen *et al.*^[6] reported a 72 year old female with HCC arising multicentrically from cirrhotic NASH. The patient was diagnosed with T2DM. Three tumors developed synchronously and metachronously and were histologically defined as a moderately differentiated HCC, a well-differentiated HCC and a

Table 2 Clinical characteristics of patients with nonalcoholic steatohepatitis-related hepatocellular carcinoma with multicentric occurrence in previous and present reports

Author	Cases	Age older than 70 yr	Gender		Metabolic diseases				Histological features	
			M	F	OB	DM	DL	HT	LC	Non-LC
Zen <i>et al</i> ^[6]	1	1	0	1	0	1	0	0	1	0
Sasaki <i>et al</i> ^[7]	1	1	0	1	1	1	0	0	1	0
Present authors	4	4	2	2	4	4	1	4	4	0
Total	6	6	2	4	5	6	1	4	6	0
(%)	-	(100)	(33.3)	(66.7)	(83.3)	(100)	(16.7)	(66.7)	(100)	(0)

M: male; F: female; OB: obesity; DM: type 2 diabetes mellitus; DL: dyslipidemia; HT: hypertension; LC: liver cirrhosis; Non-LC: non-liver cirrhosis. All values represent number of cases.

Table 3 Clinical characteristics of patients with solitary hepatocellular carcinoma related to nonalcoholic steatohepatitis in previous and present reports

Author	Cases	Age older than 70 yr	Gender		Metabolic diseases				Histological features	
			M	F	OB	DM	DL	HT	LC	Non-LC
Cotrim <i>et al</i> ^[21]	1	0	1	0	1	1	0	0	1	0
Orikasa <i>et al</i> ^[22]	1	0	0	1	0	1	0	0	1	0
Shimada <i>et al</i> ^[23]	4	1	2	2	3	2	1	2	4	0
Mori <i>et al</i> ^[24]	1	1	1	0	1	1	0	0	1	0
Bullock <i>et al</i> ^[25]	2	1	2	0	2	2	0	2	0	2
Cuadrado <i>et al</i> ^[26]	2	1	2	0	2	2	0	0	1	1
Sato <i>et al</i> ^[27]	1	0	1	0	1	0	1	0	0	1
Ichikawa <i>et al</i> ^[28]	2	0	1	1	1	0	1	1	0	2
Ikeda <i>et al</i> ^[29]	1	0	1	0	1	1	0	1	1	0
Hai <i>et al</i> ^[30]	2	1	2	0	2	2	0	1	1	1
Hashizume <i>et al</i> ^[31]	7	5	4	3	5	5	6	5	5	2
Maeda <i>et al</i> ^[32]	3	0	2	1	NA	NA	NA	NA	3	0
Kawada <i>et al</i> ^[33]	6	4	3	3	2	3	1	4	0	6
Malik <i>et al</i> ^[34]	8	2	6	2	5	6	NA	5	8	0
Chagas <i>et al</i> ^[35]	4	3	2	2	4	3	1	NA	4	0
Takuma <i>et al</i> ^[36]	8	5	4	4	4	6	3	6	3	5
Present authors	3	2	1	2	2	1	2	2	3	0
Total	56	26	35	21	36	36	16	29	36	20
(%)	-	(46.4)	(62.5)	(37.5)	(67.9)	(67.9)	(35.6)	(59.2)	(64.3)	(35.7)

M: male; F: female; OB: obesity; DM: type 2 diabetes mellitus; DL: dyslipidemia; HT: hypertension; NA: not available; LC: liver cirrhosis; Non-LC: non-liver cirrhosis. All values represent number of cases.

dysplastic nodule, respectively. Sasaki *et al*^[7] described a 73 year old female with MO of HCC based on cirrhotic NASH. The patient was complicated by T2DM and obesity. Two liver tumors were detected metachronously and both were diagnosed with well-differentiated HCCs.

In the present report, the clinical profiles of old age, obesity, T2DM, hypertension and cirrhosis are identified as common characteristics of the 4 patients with MO of HCC (Table 1); these may be predisposing factors to MO of HCC. Moreover, of these characteristics, old age, T2DM and cirrhosis are recognized as common conditions among the 2 patients with MO of HCC in previous reports (Table 2). Although these conditions are also observed in some patients with solitary HCC which is unaccompanied by MO as described in the present report and previous literature (Table 3)^[21-36], they do not always satisfy these conditions. A literature search shows that the proportion of patients with age older than 70 years, T2DM and cirrhosis with solitary HCC is 46.4% (26 of 56 cases), 67.9% (36 of 53 cases) and 64.3% (36 of 56 cases), re-

spectively. Thus, it is appropriate to consider that those conditions (old age, T2DM and cirrhosis) are necessary preconditions of synchronous MO.

The role of each factor in the pathogenesis of MO of HCC is not understood. Old age and cirrhosis have been shown to be the strongest risk factors for the development of HCC in NASH with a prospective cohort study^[37]. Obesity has been confirmed as an independent risk factor for the development of HCC in several studies^[38-42], as has T2DM^[43-45]. With regard to the association of T2DM, it was postulated that chronic hyperinsulinemia and insulin-like growth factor 1 might be involved in carcinogenesis^[46]. Although MO of HCC may be attributable to independent factors, a concurrence of the factors of old age, metabolic abnormalities and cirrhosis perhaps significantly raises the malignant potential in the liver and probability of subsequent synchronous and multifocal development of HCCs. The exact mechanism of MO of HCC under those conditions needs to be elucidated.

It is unclear whether the same conditions can be

adapted to cases with metachronous MO because it may not be long enough to observe metachronous MO of HCC in the present study. Even in patients without MO of HCC at diagnosis, HCC of multicentric origin might occur metachronously after longer periods of follow up. Moreover, the number of subjects is too small to draw a clear conclusion. These are limitations of this study and warrant further exploration with a larger scale and longer duration.

Discrimination of MO from IM is critical for selecting an appropriate method of treatment for HCC caused by HCV infection. The prognosis of HCV-related HCC patients with MO is thought to be better than that with IM because of the low rate of recurrence by IM in the MO group^[13]. Therefore, it is reasonable to adopt locoregional therapy such as surgical removal or radiofrequency ablation which is more effective than transcatheter arterial chemoembolization or systemic chemotherapy, even in cases with multiple HCCs when they are considered of multicentric origin. However, whether the same strategy can be adapted to NASH-related HCC remains unclear because outcomes of HCC with or without MO in NASH have not been clarified. To establish an adequate strategy against HCC with NASH, their outcomes should be analyzed with stratification on the basis of not only tumor staging, hepatic reserve and kinds of treatment but also the manner of HCC development.

Furthermore, it is important to prevent MO of HCC. In patients with HCV-related HCC, eradication of HCV by interferon therapy after curative treatment for HCC provided promising effects on the prevention of HCC recurrence in several reports^[47-49]. Adjuvant therapy using interferon reduced late recurrence of HCC after two or more years of curative treatment. This fact may be attributable to the suppression of multicentric carcinogenesis through the inhibition of chronic inflammation. Even in NASH, it would be possible that anti-inflammation or anti-fibrosis therapy for the liver leads to the prevention of HCC development with multicentric manner. Based on this, it may be worth attempting to intensely treat underlying liver disease as well as HCC to produce consequent improvement in the prognosis of NASH.

In summary, synchronous and/or metachronous MO was recognized in 4 of 12 patients with NASH-related HCC. MO may be frequently provoked in NASH-related HCC as well as in chronic liver diseases caused by HBV or HCV infection. The clinical status of age older than 70 years, obesity, T2DM, hypertension and cirrhosis was identified as common characteristics of the patients with MO of HCC and they might be predisposing factors to at least synchronous MO. Of these characteristics, old age, T2DM and cirrhosis are also common features of the other 2 patients from the previous case reports so these confined conditions may be necessary preconditions for synchronous MO. Adequate methods of treatment and prevention for MO of HCC are necessary and may lead to a consequent improvement in the prognosis of NASH.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is an often fatal event of nonalcoholic steatohepatitis (NASH). Although multicentric occurrence (MO) is frequently observed characteristics in the development of HCC caused by HBV or HCV infection, the manner of HCC development in NASH remains unclear.

Research frontiers

Although many clinicopathological and epidemiological studies on NASH have been conducted to define the incidence of HCC and the predisposing factors to HCC, there has been no study that focuses on MO of HCC with NASH.

Innovations and breakthroughs

The authors demonstrated that MO of HCC was found in 4 of 12 patients with NASH-related HCC. The common characteristics among the patients with MO of HCC were old age, obesity, type 2 diabetes mellitus (T2DM), hypertension and cirrhosis, suggesting putative predisposing factors to synchronous MO of HCC with NASH. Of these characteristics, old age, T2DM and cirrhosis are also common features of the other 2 patients from previous case reports so these confined conditions may be necessary preconditions for synchronous MO.

Applications

Understanding the manner of HCC development in NASH may be helpful for developing an adequate treatment strategy for HCC.

Terminology

MO of HCC means development of multiple HCCs with independent origins. When multiple HCCs include an early HCC with a dysplastic nodule or a moderately and/or poorly differentiated HCC with a margin of well-differentiated HCC, they are referred to as multicentric in origin.

Peer review

The present manuscript reports four cases of NASH patients that developed HCC with multicentric occurrence. The results showed are potentially interesting, and the conclusions are, in general, adequately supported by the experimental findings. All these considerations plus the interest of the data reported make the paper to be considered worth publishing.

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First multicenter study for risk factors for hepatocellular carcinoma development in North Africa

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Abstract

AIM: To assess the role of the major risk factors for hepatocellular carcinoma (HCC) development in the western part of North Africa.

METHODS: A multicenter case control study was conducted in Tunisia, Morocco and Algeria in collaboration with Pasteur Institutes in these countries. A total of 164 patients with HCC and 250 control subjects without hepatic diseases were included. Prevalences of HBsAg, anti-hepatitis C virus (HCV) and diabetes were assessed. HCV and HBV genotyping were performed for anti-HCV and HBsAg positive patients.

RESULTS: The mean age of patients was 62 ± 10 years old for a 1.5 M:F sex ratio. Sixty percent of HCC patients were positive for anti-HCV and 17.9% for HBsAg. Diabetes was detected in 18% of cases. Odd ratio (OR) and 95% confidence intervals (CI) were 32.0 (15.8 - 65.0), 7.2 (3.2 - 16.1) and 8.0 (3.1 - 20.0) for anti-HCV, HBsAg and diabetes respectively. Multivariate analysis indicated that the three studied factors were independent. 1b HCV genotype and D HBV genotype were predominant in HCC patients. HCV was the only risk factor significantly associated with an excess of cirrhosis (90% vs 68% for all other risk factors collectively, $P = 0.00168$). Excessive alcohol consumption was reliably established for 19 (17.6%) cases among the 108 HCC patients for whom data is available.

CONCLUSION: HCV and HBV infections and diabetes are the main determinants of HCC development in North Africa. An active surveillance and secondary prevention programs for patients with chronic hepatitis and nutrition-associated metabolic liver diseases are the most important steps to reduce the risk of HCC in the region.

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Key words: Hepatocellular carcinoma; Hepatitis B virus; Hepatitis C virus; Non-insulin-dependent diabetes mellitus; North Africa

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the 130 major causes of morbidity and mortality in the world^[1]. It represents the third leading cause of cancer death in males and the fourth in females with more than 600 000 deaths per year^[2]. Geographical distribution of HCC varies throughout the world with an incidence ranging from 2.1 in Central America to 35.5 in Eastern Asia^[3]. Globally, three epidemiological zones have been defined according to the age-adjusted HCC incidence per 100 000 habitants per year: low (with less than 5), intermediate (between 5 and 15) and high (higher than 15)^[4]. There is a geographic correlation between the incidence of HCC and the prevalence of chronic hepatitis B and C, suggesting that these two viral infections are the most important risk factors of HCC worldwide^[5]. In countries where hepatitis C virus (HCV) infection is endemic such as Japan and Egypt, high prevalence of HCV infection is reported among people with HCC. On the other hand, hepatitis B virus (HBV) infection is the major risk of HCC in regions with large populations like China and southern Asia because of the high endemicity of this virus there^[1].

In addition to the viral infections largely implicated in HCC development, other factors associated with HCC are well documented. They include toxins and drugs (e.g. alcohol consumption, aflatoxins and anabolic steroid use), cigarette smoking, metabolic liver diseases (e.g. hereditary haemochromatosis, alpha1-antitrypsin deficiency) and steatosis^[6,7]. Some of these factors have a direct carcinogenic role while others interact by promoting fibrosis and cirrhosis^[8]. The significant association found recently between non-insulin-dependent diabetes (NIDD or type II diabetes) and HCC suggests that diabetes is a potential risk factor for HCC development^[6,9].

In the western part of North Africa (also called Maghreb), HCC incidence is lower than in sub-Saharan Africa

and southern Europe with approximately 1 to 2 cases per 100 000 habitants per year (Globocan 2002, <http://www-dep.iarc.fr/>). HCC represents 5.9% of the total tumor burden in Morocco and is responsible for 1.1% of cancer deaths in Tunisia^[10]. This region belongs, culturally, to the broader Arabo-Muslim world which is different from Europe, Sub-Saharan Africa or Eastern Asia for the characteristics of HCC risk factors. It is characterized by a low consumption of alcoholic drinks, an intermediate endemicity for chronic hepatitis B, low rate of HCV carriage (with Egypt as a major exception) and a recent rise of obesity and NIDD incidences due to lifespan expansion and nutritional transition in populations^[11-17]. To our knowledge, the only recent study on HCC in the Maghreb region is a genetic one which demonstrated a lower rate of p53 mutation in comparison with Egypt and Sub-Saharan Africa. No report has yet detailed the respective implications of viral infections or other risk factors such as NIDD in HCC genesis in countries from North Africa. Differently from other parts of the Arabo-Muslim world such as Iran, Egypt and Saudi Arabia, HCC in the Maghreb region has not been described since the 1980s^[10-18]. We therefore conducted a multicenter case control study on risk factors for HCC in Tunisia, Morocco and Algeria with the collaboration of Pasteur Institutes in these countries. The most important factors examined were diabetes and HBV or HCV infection.

MATERIALS AND METHODS

Study design and sample size justification

We conducted a case-control study matched by age (\pm 5 years) and gender in Tunisia, Morocco and Algeria from January 2002 to January 2005. The same protocol was used for recruitment of patients in the three countries. Investigations were approved by the Ethics Committee of the Faculty of Medicine of Casablanca as well as the Algerian and Tunisian Ministries of Health. Informed consent was obtained from each patient. During the study period, 164 cases were recruited from the three countries and we attempted to recruit two matched controls. With this sample size, we determined we would have 90% power, assuming a 7.0% of prevalence of HBV in the general population^[11] and an OR of 3 with a 5% significance level. Assuming that the matching was effective, we expected the power to be higher. This level of power would allow for some failure in the recruitment of the targeted number of controls. Power calculations were performed with EPI INFO (version 6.04).

Diagnostic criteria

Diagnosis of HCC was based on imaging showing the characteristic features of HCC and/or, when possible, histological assessment of tissues samples and serum alpha-fetoprotein levels. The presence or not of cirrhosis in the non-tumor liver was considered. A diagnosis of cirrhosis was based on morphological and clinical criteria, ultrasound or computed tomography. For patients with diabetes mellitus, data on onset of the disease, the evolution of

Table 1 Distribution of hepatocellular carcinoma cases and control according to studied risk factors

Risk factor	HCC cases (<i>n</i> = 164)	Controls (<i>n</i> = 250)	Odds ratio
NIDD	18.00% (<i>n</i> = 139)	2.70% (<i>n</i> = 225)	8.0 (3.1 - 20.0)
HBV status			
HBsAg +/anti-HBc +	17.90%	4.00%	7.2 (3.2 - 16.1)
Anti-HBc + alone	15.40%	11.50%	2.1 (1.1 - 4.0)
Anti-HBc +/anti-HBs +	23.50%	14.60%	2.5 (1.5 - 4.4)
Anti-HCV			
Anti-HCV +	60.00%	4.40%	32.0 (15.8 - 65.0)

HCC: hepatocellular carcinoma; HCV: hepatitis C virus; HBV: hepatitis B virus; NIDD: non-insulin-dependent diabetes.

glycemia rates and the type of treatments received were recorded. History of dysmetabolic syndrome or excessive alcohol consumption was also collected from the medical records of each patient. Control subjects were patients admitted during the same period with no hepatic diseases.

Serology and molecular tests

The presence of HBsAg and other serological markers (antibodies to HBs and HBc) was assessed in all patients and controls using commercial kits for enzyme-linked immunosorbent assay (ELISA). Antibodies to HCV were assessed using a fourth generation ELISA test. Subjects positive for anti-HCV were tested for the presence of HCV RNA by PCR-hybridization (HCV 2.0 Roche); HCV RNA was then genotyped by a commercial kit (Inno-Lipa, Innogenetics) or by partial sequencing in the 5' non-codant region. For HBV infected patients, virus genotyping was performed by PCR - RFLP in the pre-S region as previously described (Bahri *et al* 2006, Ezzikouri *et al* 2008). Ongoing HBV infection was defined on the basis of the presence of both HBsAg and anti-HBc in serum. HCV infection was retained for all patients positive for anti-HCV.

Statistical analysis

Data entry was checked for consistency and accuracy using Epi-data, version 3.0 and analysis was conducted with the Statistical Package for the Social Science (SPSS, version 13.0). The prevalence of HBsAg, anti-HCV and diabetes mellitus was estimated with corresponding 95% confidence intervals (95% CIs). Matched odds ratios (OR) and their 95% were calculated by univariate logistic regression analysis. For each risk factor, those with $P < 0.2$ were considered as statistically significant for further evaluation in the multivariate analysis. The model also included age and gender; participants were matched by these factors. To identify variables independently associated with HCC development, conditional logistic regression analysis (backward logistic regression method) was conducted. The adjusted ORs (AORs) and their 95% CIs based on the final model were used to interpret the results. The statistical significance of the associations was based on a P value of ≤ 0.05 .

RESULTS

Demographic characteristics

A total of 164 HCC patients and 250 controls were included in the study; 252 were men (100 HCC patients and 152 controls) and 162 women (64 HCC patients and 98 controls). The sex ratio M/F was 1.53 for HCC cases. The mean age was 62 ± 10.43 years among patients and 58 ± 10.60 among controls. However, it should be noted that the four year difference between mean age of patients and controls may marginally affect the extent of calculated odds ratio.

Prevalence of NIDD and viral markers in studied population

The prevalence of NIDD, HBV markers and anti-HCV in the population studied is shown in Table 1. History of diabetes could be assessed in 139 HCC and 225 control cases. This particular risk factor was present in 27 patients (18%), a rate significantly higher than in controls (2.7%) [odds ratio of 8.0, 95% CI (3.1-20.0)]. Of the 164 HCC patients, 127 were positive for HBsAg and/or anti-HCV (77.7%). Twenty-nine (17.9%) were HBsAg-positive *vs* 9 (4%) of controls ($P < 0.001$). Anti-HBc positivity was observed more frequently among HCC patients than among controls with respectively 56.9% and 30.1% of prevalence ($P < 0.001$). Resolutive HBV infection as defined by the presence of both antibodies to HBs and HBc was found in 32.5% of HCC patients and 14.6% of controls. Ninety-eight cases (60%) and 10 controls (4.4%) were positive ($P < 0.001$) for anti-HCV. Nine patients were positive for the two markers anti-HCV and HBsAg whereas none of the controls had co-infection. Thirty-six HCC patients (22%) had no history of diabetes and had negative serological markers for HBV and HCV. Excessive alcohol consumption was reliably established for 19 (17.6%) cases among the 108 HCC patients for whom data is available. In all cases, no metabolic diseases (haemochromatosis, alpha1-antitrypsin deficiency) or primary biliary cirrhosis were observed. Of the 98 anti-HCV positive patients, HCV RNA was detected in 91 (93%) cases and in the remaining 7 patients was undetectable. Genotypes 1a, 1b and 2 were determined in 17.3%, 63.7% and 14.5% of patients positive for HCV RNA respectively. For the 29 HBsAg positive patients, HBV DNA was detected in 11 (38%) cases; almost all patients were infected by genotype D; genotype A was detected in only one patient.

Multivariate analysis

Table 2 shows the risk factors considered in the multivariate model based on univariate results. The OR for developing HCC in patients with dual infection was estimated to 84.7 [95% IC (4.3-366.9)]. After adjustment for age and gender, there was an association between development of HCC and NIDD, HBV and HCV infection and dual co-infection. No significant interaction between these three risk factors was observed.

Table 2 Risk of hepatocellular carcinoma related to hepatitis B virus and/or hepatitis C virus infections and non-insulin-dependent diabetes

Risk factor	Adjusted odds ratio	95% IC
NIDD	5.9	1.7 - 19.7
HBsAg +/anti-HBc +	10.6	3.9 - 28.8
Anti-HCV +	33.3	14.1 - 78.8
B/C co-infection	84.7	4.3 - 366.9

HCV: hepatitis C virus; NIDD: non-insulin-dependent diabetes.

Association with cirrhosis

Conclusive evidence on the presence or absence of cirrhosis was reached in 133 HCC patients. Cirrhosis was present in 108 patients (81%). Table 3 shows the prevalence of viral markers and diabetes in HCC patients according to the presence of cirrhosis. Of the 108 HCC patients with cirrhosis, 74 (68.5%) were positive for anti-HCV and 21 (19.5%) were positive for HBsAg; 7 of them had both. NIDD was found in 20 (18.5%) patients: 18 patients were also positive for HBsAg and/or anti-HCV; two patients were negative for both. Nineteen (17.5%) HCC cases with cirrhosis were negative for all factors analysed. HCV was the only risk factor significantly associated with cirrhosis (90% *vs* 68% for all other risk factors collectively, $P = 0.00168$).

DISCUSSION

WHO estimated that approximately three-quarters (78%) of HCC worldwide are attributable to HBV or HCV^[1,19,20]. HBV infection remains the main cause of HCC development, especially in regions with high endemicity of the virus such as China or sub-Saharan Africa where almost 75% of HCC patients are positive for HBsAg^[21]. HCC associated with chronic HCV infection is observed more frequently in some countries of Asia and the Near East^[22]. However, to date, their association with HCC has not been documented in the developing region of North Africa.

This is the first study on the association between various etiological factors and the risk of development of HCC in North African countries. We detected viral markers for HBsAg and anti-HCV in more than 70% of HCC patients.

Anti-HCV were found in 60% of HCC cases; similar levels were found in North America, Europe (44% to 66% in Italy, 27% to 58% in France, 60% to 75% in Spain) and Asia (over 70% in Japan)^[19]. Similar rates were also reported in Egypt despite the marked difference in HCC incidence and viral genotypes^[20,21].

In contrast, we observed HBsAg in only 17.9% of our HCC cases whereas the reported rates were higher in other parts of the Near- or Middle-East, like Saudi Arabia, Lebanon, Iran and Turkey where HBV infection remains the most important risk factor of HCC^[22-25]. This finding was also markedly different from that reported by the only study conducted in the North African region - a small Tu-

Table 3 Prevalence of viral markers and non-insulin-dependent diabetes in hepatocellular carcinoma patients with cirrhosis

	Presence of cirrhosis (<i>n</i> = 108)	Absence of cirrhosis (<i>n</i> = 25)	<i>P</i>
Anti-HCV positive	51	5	0.01
HbsAg positive	12	2	0.48
HBV and HCV	6	1	0.60
Presence of NIDD			
NIDD alone	2	0	0.81
HCV and NIDD	16	1	0.12
HBV and NIDD	2	1	0.46
HBV, HCV and NIDD	1	1	0.34
Negative for HBV, HCV and NIDD	19	14	< 10 ⁻³

HCC: hepatocellular carcinoma; HCV: hepatitis C virus; HBV: hepatitis B virus; NIDD: non-insulin-dependent diabetes.

nisian HCC group in the early 1990s with 29 cases - that estimated HBsAg prevalence at 60%^[26]. This discrepancy may be explained by the probable decline of HBV infection in recent years due to improvements in hygiene and health standards and the widespread introduction of vaccination against HBV in the region. In addition, we used a fourth generation enzyme assay to detect anti-HCV, a test far more sensitive than first generation tests previously used.

We observed 33-fold and 10-fold increase in HCV and HBV infections respectively compared to the control group. This risk increased 84-fold when patients were co-infected by the two viruses, suggesting a synergy between the two infections. Such positive interactions between HBV and HCV infections have been previously reported^[4] but are not constantly present in the Middle-East^[22]. The causes of such heterogeneity, presumably linked to the timing of viral infections in a patient's lifespan, are yet to be defined. In a case-control study conducted in Italy, concomitant infection with HBV and HCV was associated with an OR of 165 (95% CI, 81 to 374), while ORs of 17 and 23 were observed with HCV and HBV single positivity^[19].

The mechanisms of carcinogenesis in HBV and HCV infection differ. HBV is known to play a direct role in liver cell transformation through direct interactions between viral and cellular proteins or by integration of HBV genome into the host genome^[27-31]. We found no significant association between cirrhosis and HBsAg (+) in HCC patients confirming prior research. HCV, in contrast, is thought to promote a fibrotic process progressing to cirrhosis and ultimately to HCC^[18,31]. Moreover, it has been recently suggested that several aspects of the HCV life cycle are important in the mechanism of carcinogenesis.

The impact of active replication of the virus and the presence of HCV genotypes 1 and 2 was associated to the development of tumor^[32]. In the present work, more than 90% of anti-HCV positive patients were positive for viral RNA and were infected with Subtype 1b (63.7%) or Genotype 2 (14.5%). Both genotypes are prevalent in the region: In Tunisia, Subtype 1b is largely predominant

(79%); Subtype 1a and Genotype 2 are less frequent (5% and 10% respectively)^[33]. In Morocco, Subtype 1b and Genotype 2 have been found with a prevalence of 68.4% and 15.8% respectively^[34]. For HBV genotypes, our data indicate that genotype D is dominant in HCC patients from the western part of north-Africa, similarly to what was previously reported in HCC patients from other countries of Near- and Middle-East^[35,36]. Genotype D was also detected in 80% and 86% of HBV-infected patients from Tunisia and Morocco respectively^[37,38].

In addition to HCV and HBV infections, NIDD appears to be associated with HCC in the countries where our study was carried out. After adjusting for age, the association of each risk factor with HCC development remains significant. These findings which confirm previous studies suggest that HCV, HBV and NIDD play an independent role in liver tumorigenesis^[39,40]. One may question if diabetes is a risk factor for HCC development or was caused by HCC. However, almost all our patients had onset of diabetes several years before HCC development, suggesting that NIDD would act more as risk factor than a consequence of this tumoral disease. NIDD was frequently associated with cirrhosis in our HCC series and likely plays an indirect role in HCC pathogenesis through predisposing the liver parenchyma to Non-Alcoholic Fatty Liver Disease (NAFLD). This chronic liver disease, frequently diagnosed in western countries^[42], is characterized as a chronic necroinflammatory condition that can lead to liver fibrosis, cirrhosis and subsequently to HCC^[41]. Recent studies performed in the Middle-East or North-Africa indicate that NIDD, early obesity, metabolic syndrome and NAFLD represent serious public health problems^[42-46]. However, the roles of NIDD or NAFLD are still not clear in the development of HCC. Some authors reported NIDD as a risk factor of HCC in some cases from Saudi Arabia and Egypt; this association is not confirmed by studies conducted in Lebanon^[47,48]. NIDD has been frequently associated with other risk factors for HCC by promoting cirrhosis^[49]. Corroborating these previous results, our study detected anti-HCV among 17 out of the 20 patients, thereby associating NIDD and cirrhosis.

Alcohol consumption was found in only 17.9% of our HCC patients. Similar proportions were reported in other Muslims countries such as Turkey where alcohol abuse was found in only 10% to 16% of HCC patients^[20,23]. In countries like Lebanon where an important proportion of the population is not Muslim, excessive alcohol consumption is observed in more than 20% of HCC^[23]. However, this risk factor is certainly underestimated because it is not easily revealed by Muslim patients. It is actually admitted that in the Maghreb region alcohol intake plays an important role in different pathologies like psychiatric disorders^[50].

In conclusion, our results suggest that HCV and HBV infections and NIDD are the main determinants of HCC development in individuals at risk in North Africa. Preventing the transmission of hepatitis viruses is the most important step to reduce the risk of HCC. However,

approximately one fourth of the HCC patients described in our current study were negative for these three risk factors. Other studies should be conducted in the region to estimate the impact of factors such as occult hepatitis B in HCC which are frequently associated with HCC development throughout the world.

COMMENTS

Background

The western part of North Africa (also called Maghreb) is known for a low incidence of hepatocellular carcinoma (HCC), a low consumption of alcoholic drinks, an intermediate endemicity for chronic hepatitis B, a low rate of hepatitis C virus (HCV) carriage and a recent rise of obesity and non-insulin-dependent diabetes (NIDD) incidences. HCC in the Maghreb region has not been described since the 1980s; no report has yet detailed the respective implications of viral infections or other risk factors such as NIDD in HCC genesis in these countries.

Research frontiers

The current study was performed to evaluate the role of hepatitis C, hepatitis B and Diabetes Mellitus in HCC development in the Maghreb region.

Innovations and breakthroughs

This is the first study on the association between various etiological factors and the risk of development of HCC in North African countries.

Applications

This report shows the high implication of HCV and HBV infections and diabetes in HCC development in North Africa. Obtained results incite active surveillance and prevention programs for patients with chronic hepatitis and nutrition-associated metabolic liver diseases, the most important steps to reduce the risk of HCC in the region.

Peer reviews

It is a valuable study that assessed the major risk factors for HCC development in the western part of North Africa. It has an important impact on epidemiology of HCC in the world, giving information about the implications of chronic hepatitis and diabetes for HCC genesis in countries where, so far, no data are available. The article is well written and easy to read. The authors used a matched case-control study with good epidemiological tools to perform their analysis. This study should be completed by others in the region to evaluate other risk factors and to study molecular aspects of HCC.

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Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011
Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium
2011
San Francisco, CA 94143, United
States

January 27-28, 2011
Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11
Regensburg 93053, Germany

January 28-29, 2011
9. Gastro Forum München
Munich, Germany

February 13-27, 2011
Gastroenterology: New Zealand
CME Cruise Conference
Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week
2011
Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases
2011-6th Congress of the European
Crohn's and Colitis Organisation
Dublin, Ireland

March 3-5, 2011
42nd Annual Topics in Internal
Medicine
Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in
the Outpatient and Inpatient Settings
Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology
Annual Meeting 2011
Birmingham, England, United
Kingdom

March 17-20, 2011
Mayo Clinic Gastroenterology &
Hepatology 2011
Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform
Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011
Good Medical Research, Istanbul,
Turkey

March 26-27, 2011
26th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 94143, United States

April 25-27, 2011
The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition
Riyadh, Saudi Arabia

May 7-10, 2011
Digestive Disease Week
Chicago, IL 60446, United States

May 19-22, 2011

1st World Congress on Controversies
in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course
Venice, Italy

May 25-28, 2011
4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease
Forum 2011
Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV
SPIGC, II ESYS
Napoli, Italy

June 22-25, 2011
ESMO Conference: 13th World
Congress on Gastrointestinal Cancer
Barcelona, Spain

October 19-29, 2011
Cardiology & Gastroenterology |
Tahiti 10 night CME Cruise
Papeete, French Polynesia

October 22-26, 2011
19th United European
Gastroenterology Week
Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting &
Postgraduate Course
Washington, DC 20001, United
States



Instructions to authors

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The columns in the issues of *WJH* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in hepatology; (9) Brief Article: To briefly report the novel and innovative findings in hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJH*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of hepatology; and (13) Guidelines: To introduce consensus and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice in hepatology.

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Instructions to authors

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Format

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- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 ± 24.5 μ g/L; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

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