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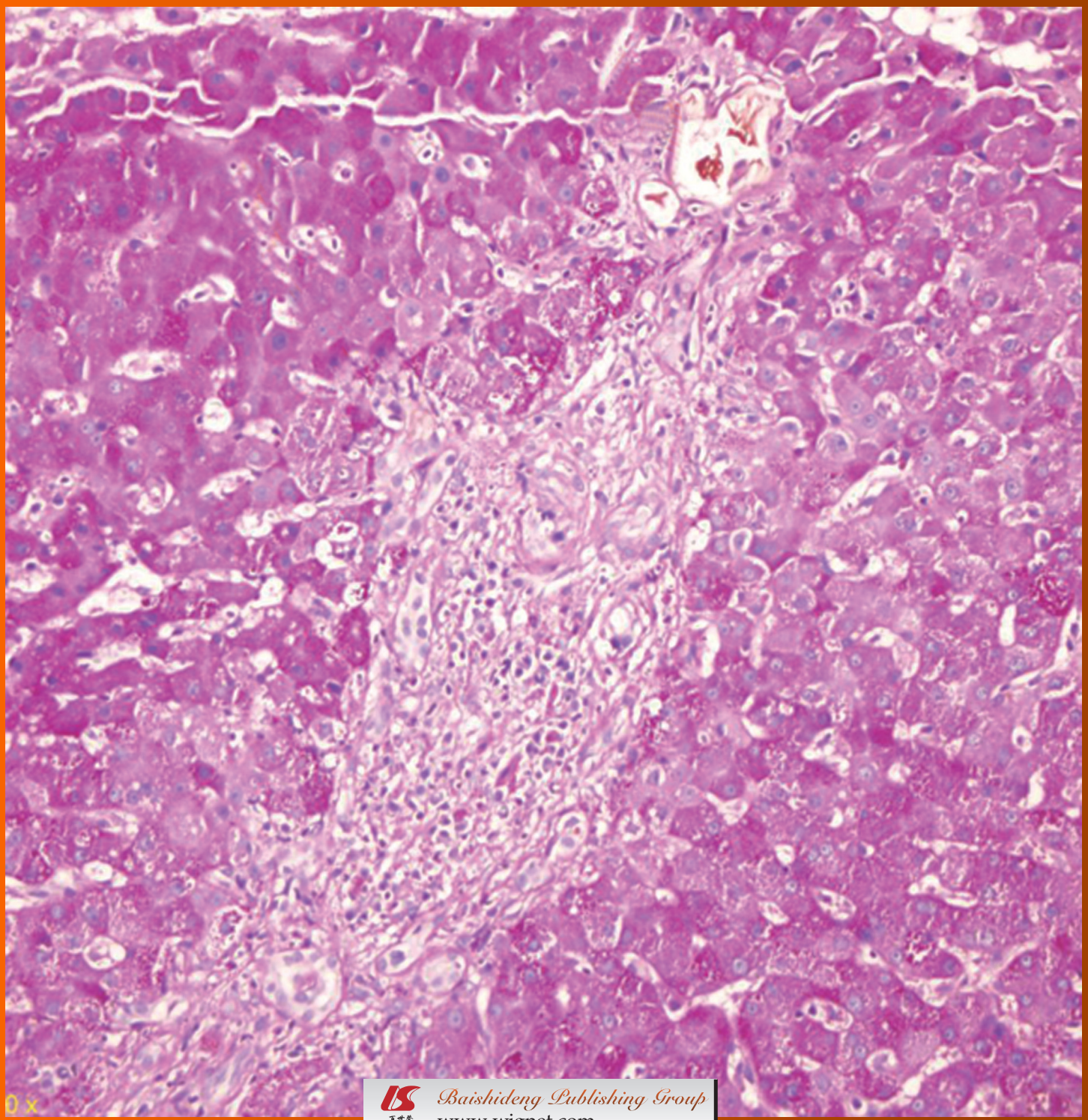

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AIM AND SCOPE

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

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REVIEW

Matching donor to recipient in liver transplantation: Relevance in clinical practice

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Abstract

Achieving optimum outcomes after liver transplantation requires an understanding of the interaction between donor, graft and recipient factors. Within the cohort of patients waiting for a transplant, better matching of the donor organ to the recipient will improve transplant outcomes and benefit the overall waiting list by minimizing graft failure and need for re-transplantation. A PubMed search was conducted to identify published literature investigating the effects of donor factors such as age, gender, ethnicity, viral serology; graft factors such as size and quality, recipient factors such as age, size, gender and transplant factors such as major or minor blood group incompatibility and immunological factors. We also report technical and therapeutic modifications that can be used to manage donor-recipient mismatch identified from literature and the authors' clinical experience. Multiple donor and recipient factors impact graft survival after liver transplantation. Appropriate matching based on donor-organ-recipient variables, modification of surgical technique and innovative peri-transplant strategies can increase the donor pool by utilizing grafts from marginal donors that are traditionally turned down.

Key words: Liver transplantation; Donor-recipient mismatch; Immunological mismatch; Viral serology mismatch

Core tip: Multiple donor and recipient factors impact graft survival after liver transplantation. In addition, interaction between donor, graft and recipient factors may significantly affect management and outcomes. Appropriate matching based on donor-organ-recipient variables can avoid wastage of liver grafts, improve outcomes and decrease graft loss. Modification of surgical techniques and innovative peri-transplant strategies can expand the donor pool by utilizing grafts from marginal donors that are traditionally turned down.

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INTRODUCTION

Transplantation involves the transfer of vascularized organs between genetically disparate individuals. Each individual has his/her distinct innate and acquired characteristics. Often combinations of these factors interact to affect outcomes in liver transplant recipients.

In view of the widening disparity between organ availability and demand, there is increasing pressure to accept all available liver grafts for transplantation. The only absolute contraindications at present are current or recent malignancy (other than some cutaneous and pri-

mary brain tumors) or infection with human immunodeficiency virus. Within the cohort of patients waiting for liver transplantation (LT), better matching of the donor organ to the recipient will improve transplant outcomes and benefit the waiting list by minimizing graft failure and need for re-transplantation. The method of choosing a recipient for a deceased donor graft varies amongst allocation systems. In most systems, patients with acute liver failure (ALF) are given priority over patients with chronic liver disease (CLD). Among patients with CLD, some systems prioritise based on MELD score while others prioritise based on waiting time. Blood group compatibility, graft-recipient size match are other factors considered in matching grafts.

This review attempts to highlight interactions between donor and recipient factors that may affect LT outcome. Ways to tailor operative technique and perioperative management to counteract these mismatched factors is also described. Use of donation after cardiac death (DCD) donor grafts and use of steatotic grafts is not discussed in this review.

DEMOGRAPHICS FACTORS

AGE

Increasing donor age has been consistently identified as a factor affecting transplant outcome adversely[1,2]. The donor risk index (DRI)[3] is a mathematical formula that predicts the risk of liver graft loss. It is calculated using several donor and graft factors including donor age, ethnicity, donor cause of death, type of graft, *etc*. Donor age greater than 40 years is considered a risk factor with grafts from donors older than 70 years having a 65% increased risk of allograft failure.

Liver grafts from young adults are usually of excellent quality and should be split to provide grafts for two recipients. This maximizes graft utilization and ensures access for paediatric patients[4]. Grafts from paediatric donors are of good quality and should be prioritized to appropriately matched paediatric recipients. This ensures transplantation of these grafts into recipients who theoretically will need the graft to function for the longest duration. Full size graft paediatric transplantation into children is technically straightforward and minimizes the risks of biliary complications seen with split grafts[5]. Pediatric transplant outcomes using grafts from donors less than 6 years have similar graft survival when compared to older donors[6]. Their use for adult recipients may not provide an adequate liver mass and may be associated with vascular complications and potential risk of graft rotation and outflow problems[7].

Liver grafts from elderly donors are more fatty, with an element of fibrotic change. They may not tolerate long periods of cold ischemia. In a retrospective study of 772 adult transplants, Hoofnagle *et al*[8] reported increased incidence of initial graft dysfunction and early and late graft loss in transplants using grafts from donors older than 50 years. Atherosclerotic changes in the hepatic artery of grafts from elderly donors can increase the risk of arterial complications[9]. Modification of surgical techniques such as avoiding the use of the donor carrel patch for anastomosis, accessory right artery reconstruction to the gastroduodenal artery has decreased the incidence of hepatic artery thrombosis[10]. Older grafts are particularly associated with poorer outcomes in recipients with liver disease due to hepatitis C virus (HCV). In a retrospective review of 111 patients transplanted for hepatitis C related CLD, Rayhill *et al*[11] reported that grafts from donors over 60 years of age were associated with severe recurrent HCV. Several authors have suggested that these should be avoided in HCV recipients[12,13]. Despite these issues, older donors remain a valuable source of organs[14-16]. The risk can be minimised by careful visual assessment, routine pre-transplant biopsy to rule out fibrosis and keeping the cold ischemia time to the minimum[17]. Their use can be considered in patients with hepatocellular carcinoma who usually have stable liver disease and can tolerate a marginal graft better[18,19]. Similarly, these grafts will provide adequate graft function for elderly stable recipients who otherwise may be disadvantaged in an organ allocation system where graft placement may be biased by a utilitarian viewpoint of maximum life-years gained.

Gender

In a UNOS database analysis of over 34000 transplants, Rustgi *et al*[20] reported that grafts from female donors transplanted into male recipients have a 20% increased risk of graft loss as compared to gender matched male recipients. Other authors have reported no such difference in graft loss rates. Teodorescu *et al*[21] analyzed the differences in liver grafts from male and female donors using a dataset of 28000 transplants from the UNOS database. Their analysis suggested that female donors were shorter, older and more likely to die from cardiovascular disease as compared to their male counterparts. Once these factors were adjusted for, graft outcomes from female-to-male transplantation were similar to other gender based donor-recipient pairings.

Female recipient gender has been reported to be a risk factor in the setting of transplantation for HCV. Female recipient status increases the risk of advanced fibrosis and graft loss[22]. They are also at higher risk for treatment failure for recurrent HCV[23].

Ethnicity

Majority of studies investigating the effect of donor race on LT have originated from the United States. Studies have previously reported poorer outcomes with transplantation of non-Caucasian donor livers into Caucasian recipients. African-american (AA) race is a factor included in the calculation of the donor risk index[3]. Molenaar *et al*[24] investigated the effect of donor race on outcomes of liver transplant in AA race HCV recipients. They reported poorer outcomes when Caucasian donor livers were transplanted into AA recipients with HCV.

This effect disappeared in transplantation for non-HCV and in Caucasian HCV recipients irrespective of donor race. The authors reported that the effect was unrelated to donor factors such as age, cause of death, weight, cytomegalovirus (CMV) status and HLA mismatch.

Wallace *et al*[25] investigated the relevance of ethnicity in determining graft outcomes by analyzing OPTN data from 10874 transplants. They reported that while unadjusted data showed increased risk of graft loss in transplants with livers from AA, Asia-Pacific Indians (API) and "Others" donors, the difference disappeared in the former two racial groups when other confounding factors were adjusted. Only livers from "others" sub-group (*i.e.,* non-Caucasian, non AA, non-API donors) livers were associated with a higher risk of graft loss. The authors suggested that as over 80% of the "others" sub-group were of Hispanic ethnicity who have a higher prevalence of fatty livers; this could explain the worse post-transplant outcomes. Similarly, Chen *et al*[26] suggested that socio-economic parameters such as median household income were more influential than the ethnicity in affecting recipient outcomes.

Graft-recipient size mismatch

Space for the new liver graft is created by recipient hepatectomy. The musculoskeletal cage formed by the lower right hemithorax, diaphragm and vertebral column limits the size of liver graft that can be transplanted. Space consideration is particularly important in the antero-posterior dimension, where the graft right lobe will be positioned. In practice, the height and abdominal girth of the individual provide an approximate measure of the liver size.

Implantation of a large liver in a small recipient is technically difficult. Compression after wound closure can compromise graft perfusion. Presence of significant pre-operative ascites may ease the situation due to chronic stretching of the abdominal wall. Graft reduction by a right posterior sectionectomy can improve the space constraint by decreasing the antero-posterior dimensions of the graft. However, this leaves a large cut surface and is not appropriate for routine use. Implanting a large graft can also cause poor alignment in the position of the inflow structures for the graft and recipient. In these cases, it is preferable to site the caval anastomosis as high as possible on the recipient cava (piggyback) to bring the graft and recipient portal structures in alignment.

Large graft size is also an issue in transplantation of babies. Reduced left lateral segment grafts and mono-segmental grafts have been used[27,28]. Hyper-reduction of grafts to provide liver grafts for very small babies has been reported with satisfactory results[29]. Mismatch in size of hepatic arteries of the graft and paediatric recipient is another factor encountered in this situation and imaginative ways to deal with this problem have been described[30]. Wound closure may be difficult in these babies due to the relative size of the graft. Use of temporary closure with synthetic material followed by delayed closure is usually feasible[27].

Transplanting a small liver into a large adult may be technically easier as there is adequate space for the liver to be rotated during implantation. Difficulty may occur during portal and bile duct anastomosis due to wide gap between donor and recipient structures if the liver is implanted using piggyback technique. A side-to-side cavo-cavoplasty will enable the surgeon to tailor the level of venous anastomosis thus bringing the donor hilar structures down to an appropriate level for safe anastomoses. Smaller livers also have the risk of torsion in the roomier upper abdomen increasing the risk of vascular outflow complications. Caval replacement technique may be used to minimize outflow problems. Firm fixation of the falciform ligament to the diaphragm also helps in minimizing this risk.

MISMATCH IN VIRAL SEROLOGY

CMV

The world-wide prevalence of CMV IgG positivity suggestive of previous CMV infection is around 50%. The prevalence is highest in the developing world where seropositivity rates can reach 90%[31]. New infection or re-activation of previously acquired CMV infection is a significant cause of morbidity in the post-transplant setting.

In current clinical practice, donor-recipient pairs are classified as high-risk and low-risk groups based on the recipient's risk of developing CMV disease (Table 1). In addition, patients needing induction immunosuppression or steroid boluses for acute rejection are also considered high-risk. Use of CMV prophylaxis varies with some units favoring it for high-risk recipients only while others use it universally. Two strategies for CMV prophylaxis are available[32]. The more common strategy is oral prophylaxis for all high-risk transplants for three months. Use of universal CMV prophylaxis has been reported to decrease the risk of CMV disease in the early post-transplant period. While CMV infection and disease can occur beyond three months, the patient is on less intense immunosuppression and the risk of serious CMV disease is lower. An alternative means is monitoring of CMV titres using periodic assays for CMV viremia. Treatment for CMV disease is instituted when the titres reach a pre-determined level[33]. This is applicable in low risk recipients.

Hepatitis B virus

Hepatitis B virus (HBV) related liver disease (ALF, CLD or hepatocellular carcinoma) is an important indication for LT. Outcomes of transplantation for HBV have greatly improved over the years[34]. Post-transplant graft re-infection with HBV depends on donor hepatitis B core antibody status, pre-transplant HBV DNA titre, indication for transplant (ALF or CLD) and the use of oral anti-viral therapy and hepatitis B immunoglobulin (HBIG) after transplantation[35]. Almost all these patients

Table 1 Risk stratification and need for cytomegalovirus prophylaxis

Donor CMV IgG status	Recipient CMV IgG status	Risk type[1]	Need for CMV prophylaxis
Positive	Positive	Low risk	Low
Positive	Negative	High risk	High
Negative	Positive	Low risk	Low
Negative	Negative	Low risk	Low

[1]Patients needing induction with antithymocyte globulin and OKT3 or steroid boluses for acute rejection are considered high-risk irrespective of their CMV mismatch status. CMV: Cytomegalovirus.

Table 2 Risk of *de novo* hepatitis B in recipients receiving grafts from hepatitis B core antibody positive donors

Recipient status	Recipient HBV antibody status	Risk of de novo hepatitis	
		No prophylaxis	With prophylaxis
HBV naive	HBcAb-, HBsAb-	58%	11%
No past infection, immunized	HBcAb-, HBsAb+	18%	2%
Past infection, immune	HBcAb+, HBsAb+	4%	3%
Past infection, not immune	HBcAb-, HBsAb+	14%	3%

Incidence in patients with or without prophylaxis is shown (Modified from Skagen *et al*[40]). HBV: Hepatitis B virus.

need life-long anti-viral therapy in the post-transplant period. HBIG is now primarily indicated for patients with high pre-operative HBV DNA titres[36].

Individuals who recover from an acute HBV infection develop antibodies for the hepatitis B core antigen. Use of the core antibody donor livers was previously avoided except in HBsAg positive recipients. With increasing demand for donor organs, grafts from core antibody positive donors are being used in many centres. This is particularly pertinent in countries where more than 50% of living donors are core antibody positive[37]. Safety of using core antibody positive grafts has been confirmed by several retrospective studies[38,39]. Development of *de novo* HBV infection is the main concern in this situation and the risk depends on the recipient's prior exposure to HBV, immunization status and use of prophylaxis[40] (Table 2). The ideal prophylactic therapy is unclear with some centres using HBIG based regimens[41] while others have used oral antiviral-based regimens[42,43]. We maintain our patients on anti-viral therapy alone due to cost considerations. Use of these grafts needs a careful discussion with the potential recipient regarding the risk of *de novo* HBV infection and the cost of additional prophylaxis.

Another strategy to decrease the risk of *de novo* HBV is by active immunization with HBV vaccine[44]. Prospective studies have shown that both pre-transplant and post-transplant vaccination are effective in preventing *de novo* HBV infection, though additional doses of the vaccine may be required to induce an effective immune response[44,45]. Our current recommendation for patients with non-HBV related liver disease is to be immunized for HBV. This provides protection against new HBV infection before transplant and decreases the risk of *de novo* HBV infection if the patient receives a core antibody positive graft.

HBsAg positive donor grafts are not routinely used even in recipients with HBV related liver disease due to the risk of early graft damage. It is also contraindicated in individuals who have concurrent Hepatitis Delta virus infection. However these may be used in life threatening situations such as ALF or HBV related HCC where delay may make these cases untransplantable. Two small retrospective studies have reported the safe use of HBsAg positive liver grafts in patients with HBV related CLD[46] and HBV unrelated CLD[47] with satisfactory results. Both groups have suggested that long-term HBIG prophylaxis may not be effective and advised institution of double anti-viral therapy as prophylaxis. Careful assessment of graft quality (fibrosis and inflammation on biopsy and serum enzyme levels) is essential to avoid transplanting chronically damaged grafts in this setting.

Hepatitis C virus

HCV infection of the new liver graft after transplantation for HCV related liver disease is nearly universal. The rate and severity of graft damage due to HCV is however variable and has been found to depend on several donor and graft related factors. High viral titres, older donors, inflammation and fibrosis on graft biopsy, prolonged cold ischemia time and more intense immunosuppression have been associated with poorer outcomes in HCV patients[48,49].

Grafts from HCV seropositive donors are not routinely utilized due to concern regarding transmission of the infection to recipients. Use of grafts from HCV antibody positive donors has been suggested as a way to improve access to transplantation for HCV related liver disease patients. Several case-control studies have suggested

equivalent results in terms of frequency and severity of HCV recurrence, graft and patient survival[50,51]. Though the patient numbers in these studies are small and they are all retrospective studies, the evidence is promising. It is unlikely that a clinical trial comparing outcomes with HCV infected or uninfected grafts can ever be organized for ethical reasons. When a HCV positive graft is considered for transplantation, a pre-transplant biopsy is necessary to ensure no significant hepatitis or fibrosis. A detailed discussion with the potential recipient is also mandatory. The significance of donor viral load and co-infection with two different genotypes of HCV on transplant outcome is presently unclear[52].

IMMUNOLOGICAL MISMATCH

ABO incompatible liver transplants

Most liver transplants are either between ABO identical or ABO compatible donor-recipient pairs. Earlier studies had reported increased risk of humoral and cellular rejection, arterial thrombosis and biliary complications after ABO incompatible liver transplants (ABOiLT)[53].

Measures such as peri-operative plasmapheresis[54], and rituximab[55] have been used to lower peri-operative recipient antibody levels and thereby decrease the risk of these complications. ABOiLT in the pediatric population has been used more frequently and outcomes similar to ABO compatible LT have been achieved[56]. Its role in adult transplantation is still unclear though it remains an option in desperate situations like fulminant hepatic failure when an ABO compatible organ is unavailable[57,58]. Re-transplantation will be required in some of these patients.

Use of blood group A2 donors

Around 10% of all blood group A individuals can be sub-typed as A2. A2 sub-group patients have lower expression of A antigen on their RBCs and are hence less likely to undergo immune mediated haemolysis on coming in contact with serum containing anti-A antibodies (present in serum of blood group B and O patients). This fact has been exploited in the use of A2 grafts for O and B recipients and use of A2B grafts for B recipients. While this strategy has been regularly used in kidney transplantation, the first large series in deceased donor LT has recently been published[59].

Minor ABO incompatibility

Blood group-O is considered as universal donor and O-group grafts are considered for patients of all blood groups in acute situations. Transplantation of grafts from O donors to A, B, AB blood groups has been reported to cause haemolysis. This occurs due to the passenger lymphocyte syndrome where donor lymphocytes transferred via the graft produce antibodies against A and B antigens on the recipient red cells[60]. These antibodies cause recipient RBC haemolysis by fixing complement. This is a self-limited phenomenon as the donor lymphocytes gradually die out and the haemolysis stops. This has been most commonly reported in O to A transplants though it can occur in O to B, O to AB or minor blood group incompatibilities[61].

Rhesus factor mismatch

Rh factor is usually not considered significant in matching organs for transplantation. Bryan *et al*[62] investigated the effect of Rh mismatch on the outcome of kidney transplantation. They reported poorer 7-year graft survival in cases of Rh mismatched transplantation. Ashkenazi *et al*[63] reported Rh mismatched transplantation as a significant risk factor for biliary complications after LT. Anecdotal reports of severe haemolysis or graft versus host disease have reported[64,65]. However, Rh-mismatch in LT is not taken into consideration for organ matching in most centres.

HLA matching

The role of HLA matching across the A, B, and DR loci in kidney transplantation is well established. Lymphocytic cross-match prior to LT is not routinely used. One reason is that cross-match takes 4-5 h to complete, which can increase the cold ischemia time of the graft.

Balan *et al*[66] investigated the effect of HLA mismatching on outcomes in 799 patients undergoing LT. They reported poorer 10-year survival for recipients receiving grafts with HLA-A locus mismatch. Similarly a mismatch at HLA-DR locus was found to increase recurrence of auto-immune liver disease. Lan *et al*[67] conducted a meta-analysis investigating the role of HLA matching in LT. They found that increasing number of mismatches was associated with increased risk of acute rejection though the graft survival rates were similar. In contrast, Muroetal[51] analysed data from 242 liver transplants and reported that matching at HLA-A locus increased the risk of graft failure. There is hence a lack of clarity regarding the relevance of HLA matching in LT. There is no current recommendation regarding its routine use in matching liver grafts either in the DDLT or LDLT settings.

Immunological pre-sensitisation

Liver graft has the capacity to absorb large quantities of antibodies and hence pre-sensitization, which is a risk factor for poor outcome in kidney transplantation is not relevant in LT. Hyper-acute rejection caused by preformed antibodies against donor antigens is very uncommon in LT. In fact, simultaneous liver or split LT along with a kidney transplant in a highly sensitized recipient protects the renal graft from immune damage[68].

HLA matching and graft versus host disease

Graft versus host disease (GVHD) is a rare complication after LT occurring in around 0.5%-1% of recipients[69]. It is associated with high mortality due to complications of bleeding, sepsis and multiple organ failure. Close HLA matching has been reported as a risk factor

for GVHD in LT. Soejima *et al*[70] described six cases of GVHD after LT where the donor was homozygous at HLA A,B and DR loci with one haplotype match with the recipient leading HLA mismatch of 1000. This association of donor-dominant one-way HLA matching in the 3 loci of HLA-A, -B, and -DR with GVHD has also been confirmed from other studies[71]. This is of particular relevance in the setting of paediatric living donor LT where a parent might be donating to the child.

Glutathione S-Transferase T1 genotype mismatch and de novo auto-immune liver disease

Glutathione S-Transferase (GST) is an enzyme present in the liver and kidneys and is involved in drug metabolism. T1 genotype of this enzyme is absent in around 20% of Caucasian population. Patients who do not have the *GSTT1* gene (GSTT1 null) can develop antibodies against GSTT1 when a GSTT1 positive donor liver is transplanted. In 2004, Aguilera *et al*[72] reported the relation between GSTT1 donor-recipient mismatch and the incidence of *de novo* auto-immune hepatitis (AIH). Further work by this group has found an association between high titres of anti-GSTT1 antibodies and development of *de novo* hepatitis. They reported that while anti-GSTT1 antibodies are present in 6.9% of patients with a mismatched graft, the incidence of *de novo* AIH in patients with antibodies was 60% at 36 mo post-transplant. The incidence of antibodies is lower in patients maintained on tacrolimus[72]. Its clinical significance in the pre-transplant setting is controversial. Knowledge of the mismatch may help in management of post-transplant immunosuppression as these patients may benefit from tacrolimus and long-term low dose steroids as part of their immunosuppression protocol.

CONCLUSION

Multiple donor and recipient factors impact graft survival after LT. Appropriate matching based on donor-organ-recipient variables can improve outcomes and decrease graft loss. Modification of surgical techniques and innovative peri-transplant strategies can increase the donor pool by utilizing grafts from marginal donors that are traditionally turned down.

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MINIREVIEWS

Hepatitis B virus reactivation with rituximab-containing regimen

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Core tip: The deleterious effects of hepatitis B virus (HBV) reactivation in rituximab-containing chemotherapy regimens have been reported and the effect of lamivudine treatment in the prevention of HBV reactivation is also well documented. Once reactivated, HBV may lead to death due to hepatitis. In this review, we discuss the factors of preventive lamivudine treatment (especially in the course of HBV antibody), including to whom and for how long the drug should be given, based on case studies and reports that span rituximab's debut in 2002 on the Japanese market to June 2013.

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Abstract

Rituximab is recognized as a useful drug for the treatment of B-cell non-Hodgkin's lymphoma and its use has been extended to such diseases as idiopathic thrombocytopenic purpura, thrombotic thrombocytopenic purpura, chronic rheumatoid arthritis and ANCA-associated vasculitides. One serious complication associated with its use is the reactivation of hepatitis B virus and the search for methods to prevent this occurrence has resulted in the rapid accumulation of knowledge. In this review, we discuss case analyses from our department and other groups and outline the current knowledge on the topic and the remaining issues.

Key words: Rituximab; Hepatitis B virus; Reactivation; Chemotherapy; Lamivudine; Non-Hodgkin's lymphoma

INTRODUCTION

Rituximab, which is a mouse-human chimeric antibody that targets CD20, was introduced to treat B-cell non-Hodgkin's lymphoma and has improved outcomes in this patient group[1-3]. Reports, however, indicate that it may be associated with such complications as several serious viral infections and work is currently underway to understand and deal with this problem[4-7]. One such complication is the reactivation of hepatitis B virus (HBV), an important problem that was sometimes observed with chemotherapy treatments even before the introduction of rituximab[8-12]. The deleterious effects of HBV reactivation in rituximab-containing chemotherapy regimens have been reported and the effect of lamivudine treatment in the prevention of HBV reactivation is also well documented[13-17]. Several issues remain, including the optimal timing and the treatment length of preventive lamivudine and the follow-up range of patients who are

responsive to this treatment. Once reactivated, HBV may lead to death due to hepatitis in some patients[18-22]. Even in cases where hepatitis has been overcome, HBV reactivation can disrupt the optimal treatment schedule for lymphomas and lead to relapse and shortened survival. In this review, we discuss factors in preventive lamivudine treatment, including to whom lamivudine should be given and for how long, based on case studies and reports that span from rituximab's debut on the Japanese market in 2002 to June 2013.

HBV REACTIVATION FOLLOWING RITUXIMAB TREATMENT

Upon HBV infection, HBV-DNA synthesis is initially suppressed by cytokine production by NK and other cells. A subsequent cytotoxic T cell (CTL) reaction occurs due to the CD8-positive T lymphocyte. Because hepatitis is triggered by CTLs, a time lag probably exists between HBV infection and hepatitis's manifestation[23,24]. Hepatitis that stems from HBV reactivation is thought to progress in a smaller time period than the initial infection because the virus is induced when immunosuppression is engaged under conditions where CTLs are being induced and HBV has been reactivated and has replicated. This system, which leads to accelerated hepatitis progression, might be linked to the number of deaths that occurred despite the administration of such drugs as lamivudine upon HBV reactivation when using chemotherapy or immunosuppressive agents.

We previously reported the occurrence of HBV reactivation following rituximab therapy as well as rituximab-combined chemotherapy treatments. Despite some variations, the prevalence of HBV reactivation is estimated at between 20%-55%[25-28]. However, there are reports of a 3% prevalence rate in HBsAg-negative cases[29]. Reactivation is often associated with the chemotherapy given for lymphomas and is probably influenced by steroids[26,30]. Upon the introduction of rituximab, it was initially debated whether rituximab alone or combined with chemotherapy could induce HBV reactivation and our subsequent study, as well as work by Yang *et al*[22], reported HBV reactivation after rituximab alone, suggesting that rituximab itself, without chemotherapy, can induce HBV reactivation[14,22]. Although rituximab is more likely to induce HBV reactivation in combination with chemo- or steroid-therapies, since it alone can induce HBV reactivation, caution must be exercised in its use[14]. Debate continues over whether the addition of rituximab to chemotherapy increases the risk of HBV reactivation. Our results involving a survey at a hematology institute in Hokkaido showed that reactivation only developed when rituximab was used. This result is consistent with another study by Yeo *et al*[31] that suggested that rituximab increases the chance of HBV reactivation more than chemotherapy alone[14,31]. Rituximab is more likely to induce HBV reactivation in combination with chemo- or steroid- therapies, since it alone increases the chance of HBV reactivation.

Risk factors for HBV reactivation

Reports have identified the risk factors for HBV reactivation and they include being male, a lack of anti-HBs antibodies, HBV-DNA level, presence of lymphomas, anthracycline/steroid use, second/third line anticancer treatment and youth. These risk factors were reviewed by Yeo *et al*[32], who concluded that being male, young and liver function prior to chemotherapy are associated with risk factors. When rituximab is used, the identified risk factors for HBV reactivation include being male, a lack of anti-HBs antibodies and using rituximab[28,31,32]. Huang *et al*[33] recently reported that the lack of entecavir administration is the most important factor of HBV reactivation in rituximab-associated therapy. This report concluded that the most important treatment to prevent HBV reactivation was the preventive prophylactic administration of preventive nucleoside analog therapy, not only for HBe antigen-, HBs antigen- and anti-HBc-positive cases but also for anti-HBs-positive cases. A lack of prophylactic nucleoside analog therapy is the most important risk factor of HBV reactivation.

HBs antigen-positive, anti-HBc-positive and HBV-DNA-positive cases

HBV reactivation has been reported in HBs antigen-positive patients after chemotherapy and rituximab plus chemotherapy[8,17,22,34,35]. In these patients, caution is advised to prevent HBV reactivation, with or without rituximab. Such preventive nucleoside analog approaches as lamivudine or entecavir administration are currently recommended and a combination of lamivudine and chemotherapy has been suggested[36-40]. These reports indicate that HBV reactivation during chemotherapy is markedly suppressed in groups given preventive nucleoside analog administration and that chemotherapy can proceed as scheduled. There are few systematic studies on the concomitant usage of lamivudine and rituximab; however, some, including He *et al*[41], suggest the efficacy of preventive lamivudine[41-44]. Recently, Huang *et al*[33] also reported the efficacy of the preventive administration of entecavir. Studies using lamivudine to treat HBV hepatitis have reported an annual increase of approximately 15%-20% in HBV lamivudine resistance[45,46], indicating the problem of the emergence of drug-resistant HBV strains during preventive lamivudine administration. Pelizzari *et al*[47], however, showed that for lamivudine treatment during chemotherapy for hematological malignancies, the frequency of drug resistance may be lower than what was seen in hepatitis B treatment, suggesting that long-term lamivudine treatment might be possible. But the study's observation period was short and the number of cases was limited. Perhaps resistance was also difficult to acquire because the nucleoside analogs were administered for cases initially negative for HBV-DNA.

Picardi *et al*[48] reported a high prevalence of HBV genomic mutations after fludarabine-based chemotherapy, arguing that strong immunosuppression might induce HBV resistance to lamivudine. Similar reports

exist with combined rituximab chemotherapy, suggesting that adding steroids or fludarabine to rituximab may result in a high frequency of drug resistance[49]. We believe that the relationship of immunosuppression and HBV genomic mutations requires further study because it remains undetermined whether long-term preventive lamivudine treatment combined with strong immunosuppression treatment is possible. Perhaps preventive methods concerning HBV-DNA levels among HBsAg-positive cases will change. In each guideline, for cases that require a year or more of long-term administration of nucleoside analogs against HBV-DNA, switching to entecavir is recommended. This is because in patients with high HBV-DNA levels, using entecavir is desirable based on its relationship to YMDD mutations[50-52]. In referring to the guideline treatments against the chronic hepatitis of HBsAg, entecavir use is desirable when HBV-DNA exceeds 20000 IU/mL and lamivudine use is adequate if HBV-DNA falls under 20000 IU/mL. In addition, in HBV-DNA-positive cases, we must examine the YMDD mutations beforehand. If they are detected, using tenofovir or the combined use of two nucleoside analogs might become necessary[50-52]. (1) The prevention of nucleoside analog approaches was necessary in HBs antigen-positive, anti-HBc-positive and HBV-DNA-positive cases; (2) HBV genomic mutations were observed in the regimens that used fludarabine but it remains unclear whether strong immunosuppression caused the HBV genomic mutations; (3) YMDD mutations are desirable to select the prophylactic administration of nucleoside analogs; and (4) Entecavir use is desirable when HBV-DNA exceeds 20000 IU/mL and lamivudine use is adequate if HBV-DNA falls below 20000 IU/mL.

Anti-HBc-positive, anti-HBs-negative and HBsAg-negative cases

Anti-HBc-positive cases indicate the occurrence of a prior HBV infection. Some cases fall into the window period or they are anti-HBs and HBs antigen-negative, but anti-HBc and HBV-DNA positives (occult HBV infection)[53] require caution when using chemotherapy with rituximab and anti-cancer agents[29]. Additionally, there are reports of HBV reactivation following chemotherapy in anti-HBs-positive and anti-HBc-positive patients[16,25,30]. Furthermore, Hui *et al*[29] reported hepatitis that originated from HBV reactivation in anti-HBc-positive and anti-HBs-negative cases, even when HBV-DNA is negative. This shows that in anti-HBc-positive cases, hepatitis can develop from HBV reactivation regardless of the HBV-DNA status. The guidelines recommend strict observation of HBV-DNA levels for these groups, since hepatitis due to HBV reactivation is infrequent and the treatment costs of HBV prevention are high[50-53]. Although HBV reactivation in these patients is infrequent, it may lead to prolonged use of chemotherapy, less chemotherapeutic efficacy against lymphoma, and even death from HBV hepatitis. In particular, the lethality rate is 30%-38% in cases where hepatitis occurs from HBV reactivation[29,54]. When considering cost, however, it is desirable to identify a subgroup of patients within the anti-HBc-positive group that is especially prone to HBV reactivation. Previous analysis showed that the only risk factor of HBV reactivation was without the prevention of a HBV reactivation drug[33]. Therefore, preventive nucleoside analog in all anti-HBc-positive patients is recommended; (1) In anti-HBc-positive, anti-HBs-negative and HBsAg-negative cases, one idea is the strict observation of HBV-DNA levels since hepatitis due to HBV reactivation is infrequent; (2) In these patients, HBV reactivation is infrequent and the lethality rate is 30%-38% in cases where hepatitis occurs from HBV reactivation; and (3) Although HBV reactivation in these patients is infrequent, it is desirable to use a preventive nucleoside analog in all anti-HBc-positive patients because of the lethality rate or HBV reactivation.

Anti-HBs-positive, anti-HBc-positive and HBsAg-negative cases

Hepatitis from HBV reactivation has been reported in anti-HBs-positive, anti-HBc-positive and HBsAg-negative cases[16,25,29,30]. Few reports exist of HBV reactivation following rituximab treatment in patients positive for anti-HBs alone, but reactivation may occur, and these patients require careful observation[17,29,55]. Perhaps in these cases, antibody production may have declined with age, only anti-HBs remain and the specific details are unknown. However, since perhaps even anti-HBs-positive cases are due to HBV reactivation, caution is warranted. We previously studied the changes in anti-HBs titers during rituximab chemotherapy[13,20,21]. In these cases, perhaps because the initial antibody titer was relatively low, we observed a linear decrease in the titer in correlation with the amount of rituximab. We also reported a patient in whom anti-HBs and anti-HBc titers decreased, while HBV-DNA increased and HBV reactivation occurred[20,21]. These results show a correlation between anti-HBs antibodies and HBV reactivation and suggest that monitoring their titers can provide important clues about HBV reactivation. Additionally, Onozawa *et al*[56] reported that hepatitis from HBV reactivation occurs from a decline in HBs antibody titer levels during bone marrow transplantation. Since HBs antibody is a humoral immune response that monitors HBV, a change in the HBs antibody titer could predict hepatitis that occurs from HBV reactivation. In this report, we continued to analyze the titer in an anti-HBs-positive patient who was treated with rituximab alone or rituximab plus chemotherapy between January 2002 and July 2013. The 35 subjects (18 males and 17 females) ranged from 42 to 87 years of age (Table 1) and included 17 cases of diffuse large B-cell lymphoma and nine cases of follicular lymphoma. In almost all the patients, the initial treatment consisted of two to six rounds of CHOP (cyclophosphamide, 750 mg/m^2, vincristine, 1.4 mg/m^2, adriamycine, 50 mg/m^2 on day 1, and prednisolone, 60 mg/m^2 on days 1-5) that was mainly combined with rituximab

Table 1 Analyzed patients were 18 males and 17 females, with 17 cases of diffuse large B-cell lymphoma and nine cases of follicular lymphoma

Characteristics of anti-HBs Ab positive patients	
Age (yr)	67 (42-87)
Males/Females	18/17
Disease	
DLB	17
FL	9
MCL	1
MALT	3
Burkitt lymphoma	1
EBV associated LPD	1
WM	1
CLL	2
Stage	
Ⅰ	3
Ⅱ	3
Ⅲ	7
Ⅳ	21
Chemotherapy	
R-CHOP	10
R-THP-COP	20
R+VP16	1
R+MVP	1
R+TEOP	1
R+bendamustine	1
R	2
R-Course	10 (1-30)

In these patients, 21 were clinical stage Ⅳ. In 30 patients, the initial treatment consisted of 2 to 6 rounds of CHOP mainly combined with rituximab or THP-COP. DLB: Diffuse large B cell lymphoma; FL: Follicular lymphoma; MCL: Mantle cell lymphoma; MALT: Mucosa-associated lymphoid tissue; EBV: Epstein-Barr virus; LPD: Lymphoproliferative diseases; WM: Waldenstrom macroglobulinemia; CLL: Chronic lymphocytic leukemia.

or THP-COP (cyclophosphamide, 500 mg/m^2, vincristine, 1.0 mg/m^2, and pinorubine, 30 mg/m^2 on day 1, and prednisolone, 30 mg/m^2 on days 1-5). The clinical course of the anti-HBs antibody is shown in Figure 1. In five of 35 cases, the antibody titer slightly increased, and in three cases, it declined. In nine of 45 cases, the antibody titer was the same, and in 21 of 35, it declined after rituximab and chemotherapy. In six of nine cases with anti-HBs titers > 1000 mIU/mL at the time of the initial treatment, the titer did not fall below 1000 mIU/mL. However, in one particular case, the initial titer was > 1000 mIU/mL, and the anti-HBs titer fell to 71.1 mIU/mL after three rounds of treatment. This demonstrates that even in patients with initial anti-HBs titers > 1000 mIU/mL, HBV reactivation can nonetheless occur, indicating that caution must be exercised. Among these 35 patients, the antibody titers in ten were the same or elevated compared with the titer before the treatment. In 24 patients whose anti-HBs titer levels finally decreased, 16 patients had initial titers < 300 mIU/mL (16/18 patients of < 300 mIU/mL), and 11 had initial titers < 100 mIU/mL (11/12 patients of < 100 mIU/mL), demonstrating the need for preventive nucleoside analog administration. Six patients with anti-HBs titers > 1000 mIU/mL did not show a titer decrease. Although these cases are not completely accurate since we cannot measure titers above 1000 mIU/mL, in these patients, the titers probably did not drop below 1000 mIU/mL. Pei *et al*[57] reported anti-HBs antibody titers after rituximab therapy and concluded that the risks of HBV reactivation are the reduction of anti-HBs titers, especially low pretreatment anti-HBs titers and the loss of anti-HBs. These results on titer changes might provide an index for preventive lamivudine or entecavir administration in patients who are only anti-HBs-positive. Consistent with our results, Westhoff *et al*[16] reported HBV reactivation in a patient with an anti-HBs titer of approximately 868 mIU/mL, demonstrating the possibility of HB ion may occur. Even when anti-HBs-positive cases are due to HBV reactivation, caution is warranted; (2) Anti-HBs titer decreased in correlation with the amount of rituximab. Anti-HBs and anti-HBc titers decreased, while HBV-DNA increased and HBV reactivation occurred. The reduction of anti-HBs titers, especially low pretreatment anti-HBs titers, and the loss of anti-HBs are risk of HBV reactivation; (3) For anti-HBs titers > 1000 mIU/mL at the time of initial treatment, the titers of most patients did not fall below 1000 mIU/mL. In almost all cases where the initial anti-HBs titer was < 300, titers decreased; and (4) Monitoring HBV-DNA and anti-HBs titers is useful to prevent HBV reactivation.

Nucleoside analog treatment for the prevention of HBV reactivation

As mentioned above, HBe and HBs antigen-positive patients can be treated with preventive nucleoside analogs. In HBs antigen-negative and anti-HBc-positive patients, the frequency of HBV reactivation is not high; however, since it can result in death from hepatitis, preventive nucleoside analog should be considered for them as well. We previously reported the relatively high frequency of HBV reactivation in anti-HBc-positive and HBsAg-negative patients with rituximab and bendamustine treatment[58]. These results also support the preventive nucleoside analog for HBV reactivation in the new agents that have cytotoxic and immunosuppressive reactions.

On the other hand, in HBs antigen-negative and anti-HBs-positive patients, preventive nucleoside analog treatment may be considered when the anti-HBs titer is < 300 mIU/mL, especially when the titers are < 100 mIU/mL. In cases where the titer is between 300 and 1000 mIU/mL or higher, the titer levels should be closely examined, and when the titer drops below 300 mIU/mL, HBV-DNA monitoring or preventive nucleoside analog treatment should be performed. Periodic examination of HBV-DNA is also recommended to predict HBV reactivation[59,60]. The emergence of antibodies may also be slow in cases where a mutation occurred and thus HBV-DNA monitoring is essential[59]. However, in patients where only HBV-DNA monitoring was performed, the frequency of HBV reactivation was higher than those who were treated with preventive lamivudine, demonstrating the importance of identifying a subgroup of patients for which preventive lamivudine is recommended[37]. In evaluating methods to predict

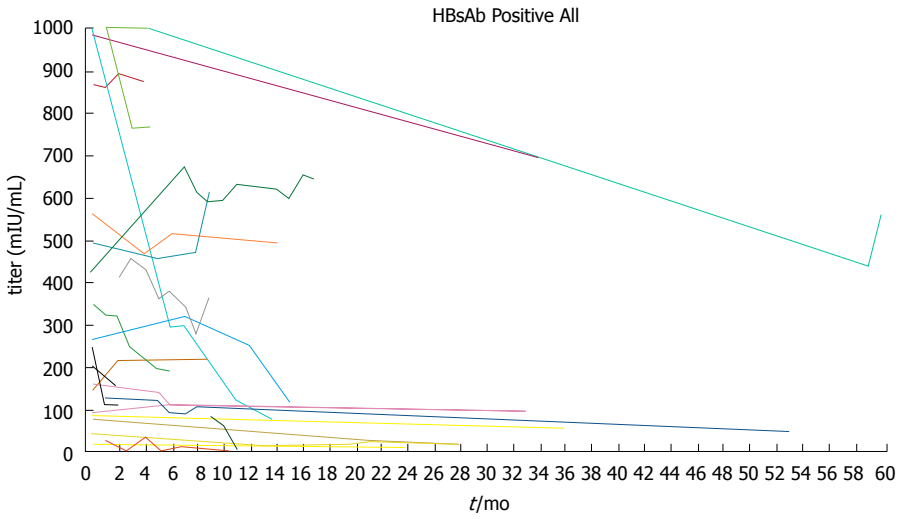

Figure 1 Antibody titer slightly increased and in three cases the titer declined. In nine cases, the antibody titer was the same and in 21 cases, it declined after rituximab and chemotherapy. In six of nine cases with anti-HBs titers > 1000 mIU/mL at the time of initial treatment, the titer did not fall below 1000 mIU/mL. In almost all cases where the initial anti-HBs titer was < 300 mIU/mL, the titer decreased. Of all cases where the initial anti-HBs titer was > 1000 mIU/mL, it dropped below 1000 mIU/mL in three of six patients.

HBV reactivation, HBV-DNA monitoring alone is insufficient and other pieces of information, such as shifts in the anti-HB titer levels (before and during the rituximab treatment), should be utilized to assist the early detection of HBV reactivation. Preventive nucleoside analog against HBV is currently recommended for 4-6 mo after chemotherapy completion[46,50,52,61]. However, reports of HBV reactivation 4 to 6 mo after chemotherapy[62-65] suggest that this number should be revised. The current 6 mo value probably reflects our knowledge about the changes in B-cell numbers[60,64,66-68]. The 2007 guidelines by Lok *et al*[50] are more specific than past guidelines and include a recommendation to extend the period of preventive lamivudine treatment, depending on HBV-DNA monitoring results. Some research reported a delayed onset of the HBV reactivation with rituximab therapy[69-71]. We also observed a case in which HBV reactivation was detected 4 years after chemotherapy and preventive lamivudine administration had been completed. The patient was a precore mutant case positive for HBe antibody, HBs antigen, and negative for HBe antigen. Due to declining blood cell counts, lamivudine therapy had been terminated and the patient was under observation for progression since entecavir had not yet been approved for HBV treatment in Japan. After initially administering lamivudine in 2002 and terminating it a month after completion of the treatment (2003), HBV-DNA was detected only sporadically in the patient. In 2003, HBs antibodies appeared with a natural progression and HBV-DNA disappeared in 2004. However, HBs antibody could not be detected, and from the latter half of 2004 to the beginning of 2006, detection was sporadic. From 2007, HBV-DNA was positive in every reading and in 2008, the individual died after being hospitalized for hepatitis from HBV reactivation. We believe that for anti-HBs-negative HBe or HBs antigen-positive patients, preventive lamivudine should be included when initiating treatment and continued indefinitely. On the other hand, anti-HBs-positive HBV-DNA became negative in this patient, suggesting that in anti-HBs-positive patients, nucleoside analog treatment should be continued until the anti-HBs titer returns to the pre-treatment level. We believe that 6 mo of preventive lamivudine treatment is too short; its length should be based on the recovery of the immune system, as judged by such criteria as anti-HBs levels. Long-term administration of such drugs as lamivudine or entecavir is problematic in terms of cost and cases that require long-term preventive administration must be clarified in the future by longitudinal surveys. In Japan, lamivudine is the only drug currently approved for preventive administration. Entecavir is also used to treat HBV infection; however, it is currently not allowed for preventive administration. For treatment regimens, 100 mg of lamivudine and 0.5 mg of entecavir are used. However, it is recommended that entecavir be increased to 1 mg to counter lamivudine resistance[50-52]. Telbivudine may also be used in the prophylaxis of HBV reactivation. Compared to lamivudine and telbivudine, entecavir is less likely to induce drug resistance in HBV, which has a treatment advantage and the preventive administration of HBV reactivation[65]. For this reason, entecavir administration is recommended for cases in which preventive administration against HBV will last 12 mo or more[50,52]. Additional effective drugs that combat HBV include 10 mg of adefovir, 600 mg of telbivudine, and 200 mg of tenofovir. With respect to lamivudine resistance, some recommend combining entecavir, adefovir or tenofovir with lamivudine[52], and others recommend switching[51]. However, it has also been reported that for lamivudine-resistant HBV strains, switching to adefovir alone quickly produces resistance. Thus, it may be desirable to use adefovir in conjunction with lamivudine[72]. Tenofovir is especially effective against lamivudine- and adefovir-resistant HBV and can be used to treat

lamivudine-resistant HBV[45,60,73-75]. Also, using HBV vaccines is recommended for HBV-seronegative cases during the use of immunosuppressive or anti-cancer agents[52]. However, as mentioned above, just as anti-HBs decline and disappear when using rituximab, antibodies might not be produced after the pre-administration of a vaccine and the vaccine must be administered after completion of the treatment. On the other hand, cases may also exist in which hepatitis arising from HBV reactivation cannot be suppressed by a vaccine[55]. Perhaps HBV reactivation cannot be prevented solely by a vaccine. (1) Preventive nucleoside analog treatment may be considered when the anti-HBs titer is < 300 mIU/mL, especially when the titers are < 100 mIU/mL. For other cases, the titer levels should be closely examined, and when they drop below 300 mIU/mL, HBV-DNA monitoring or preventive nucleoside analog treatment should be performed. Periodical examination of HBV-DNA is also recommended to predict HBV reactivation; (2) Although preventive nucleoside analog against HBV is currently recommended for 4-6 mo after chemotherapy completion, delayed onset of the HBV reactivation was observed. The length of the treatment should be based on the recovery of the immune system, as judged by such criteria as anti-HBs levels; (3) Lamivudine and entecavir are used for the prevention of HBV reactivation in Japan. Telbivudine may also be used in the prophylactic administration of HBV reactivation. Compared to lamivudine and telbivudine, entecavir is less likely to induce drug resistance in HBV, which has a treatment advantage, and the preventive administration of HBV reactivation; and (4) Tenofovir is especially effective against lamivudine- and adefovir-resistant HBV and can be used to treat lamivudine-resistant HBV.

CONCLUSION

Chemotherapy-induced HBV reactivation is thought to be caused by HBV replication in hepatocytes due to immunosuppression by anti-cancer agents, followed by a decline in the immunosuppressive effect, triggering the immune system to attack HBV-infected hepatocytes[30]. Decreased antibody titer levels resulting from decreased numbers of B-cells may be one factor that causes rituximab-induced HBV proliferation. Additionally, rituximab can indirectly alter the T-cell population in the body and stimulate HBV replication, and during immune reconstitution, the targeting of HBV may be intensified[76]. As reported by Umemura *et al*[35], chemotherapy-induced HBV reactivation results in lower survival rates than acute HBV hepatitis and thus prevention of HBV reactivation is essential. However, the screening of HBV serology is not performed routinely (36.6%). Some HBV reactivation was developed by the lack of HBV screening[77]. We hope that in the future, HBV screening will be performed routinely so that we can better understand the effect of rituximab on the immune system as well as the mechanism of HBV reactivation for improved treatment of malignant lymphomas.

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BRIEF ARTICLE

Association between nonalcoholic fatty liver disease and acute ischemic stroke severity and outcome

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Abstract

AIM: To evaluate the association of nonalcoholic fatty liver disease (NAFLD) with acute ischemic stroke severity and in-hospital outcome.

METHODS: We prospectively studied all patients who were admitted in our Department with acute ischemic stroke between September 2010 and August 2012 (n = 415; 39.5% males, mean age 78.8 ± 6.6 years). The severity of stroke was assessed with the National Institutes of Health Stroke Scale (NIHSS) score at admission. NALFD was defined as serum alanine aminotransferase and/or aspartate aminotransferase levels above the upper limit of normal in the absence of other causes of elevated aminotransferases levels [chronic hepatitis B or C, drug toxicity, increased alcohol consumption (> 21 and > 14 drinks per week in men and women, respectively), cholestatic diseases or rhabdomyolysis]. The outcome was assessed with the modified Rankin scale (mRS) score at discharge and in-hospital mortality. Adverse outcome was defined as mRS score at discharge ⩾ 2. Dependency at discharge was defined as mRS score between 2 to 5.

RESULTS: NAFLD was present in 7.7% of the study population. Patients with NAFLD had lower serum high-density lipoprotein cholesterol and higher triglyceride levels than patients without NAFLD ($P < 0.05$ for both comparisons). Demographic data, the prevalence of other cardiovascular risk factors and the prevalence of established CVD did not differ between the two groups. At admission, the NIHSS score did not differ between patients with and without NAFLD (6.3 ± 6.4 and 8.8 ± 9.6, respectively; P = NS). At discharge, the mRS score did not differ between the two groups (1.9 ± 2.2 and 2.6 ± 2.2 in patients with and without NAFLD, respectively; P = NS). Rates of dependency at discharge were also similar in patients with and without NAFLD (36.8% and 55.0%, respectively; P = NS) as were the rates of adverse outcome (42.9% and 58.6%, respectively; P = NS). In-hospital mortality rates also did not differ between the 2 groups (8.0% and 7.0% in patients with and without NAFLD, respectively; P = NS).

CONCLUSION: The presence of NAFLD in patients admitted for acute ischemic stroke does not appear to be associated with more severe stroke or with worse in-hospital outcome.

Key words: Nonalcoholic fatty liver disease; Stroke; Outcome; Aminotransferases; γ-glutamyl transpeptidase; Cardiovascular disease; Type 2 diabetes mellitus; Obesity; Cardiovascular risk

Core tip: This is the first study that assessed the prevalence of nonalcoholic fatty liver disease (NAFLD) in patients admitted with acute ischemic stroke and the association between NAFLD and stroke severity and in-hospital outcome. NAFLD was present in 7.7% of the patients and was not associated with stroke severity or with in-hospital outcome (dependency at discharge and in-hospital mortality).

Tziomalos K, Giampatzis V, Bouziana SD, Spanou M, Papadopoulou M, Pavlidis A, Kostaki S, Bozikas A, Savopoulos C, Hatzitolios AI. Association between nonalcoholic fatty liver disease and acute ischemic stroke severity and outcome. *World J Hepatol* 2013; 5(11): 621-626 Available from: URL: http://www.wjgnet.com/1948-5182/full/v5/i11/621.htm DOI: http://dx.doi.org/10.4254/wjh.v5.i11.621

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is defined as the presence of hepatic steatosis, proved histologically or by imaging, in the absence of increased alcohol consumption, pharmacotherapy or inherited disorders that can lead to secondary fat accumulation in the liver[1]. NAFLD is the commonest cause of elevated aminotransferases and ranges from isolated fatty deposition in the liver (steatosis) to liver steatosis with inflammation and fibrosis (nonalcoholic steatohepatitis, NASH)[1,2]. The prevalence of NAFLD in the general population ranges between 34%-46% whereas NASH is observed in approximately 12% of the general population[3-5].

Isolated hepatic steatosis and particularly NASH can progress to cirrhosis and are associated with increased incidence of hepatocellular cancer[1,5-8]. Moreover, patients with NAFLD appear to have increased cardiovascular risk compared with the general population[9,10]. Indeed, cardiovascular disease (CVD) is the leading cause of death in patients with NAFLD[9-12]. The frequent coexistence of NAFLD with established cardiovascular risk factors including obesity, type 2 diabetes mellitus (T2DM) and metabolic syndrome may in part explain the increased cardiovascular risk of these patients[9,10,13]. However, some studies indicated that NAFLD is independently associated with higher cardiovascular morbidity and mortality[11,12].

Ischemic stroke is the fourth leading cause of mortality in the developed world[14]. Studies in the general population suggested that patients with NAFLD have increased risk for stroke[9,10]. A recent case-control study in 103 patients with ischemic stroke and 200 controls also showed that elevated aminotransferase levels is associated with increased risk for ischemic stroke[15]. However, there is a paucity of data regarding the association between NAFLD and the severity and outcome of acute ischemic stroke. Accordingly, the aim of the present study was to determine the prevalence of NAFLD in patients admitted with acute ischemic stroke and to evaluate the association of NAFLD with stroke severity and in-hospital outcome.

MATERIALS AND METHODS

We prospectively studied all patients who were admitted in our Department with acute ischemic stroke between September 2010 and August 2012 (n = 415; 39.5% males, mean age 78.8 ± 6.6 years).

At admission, demographic data (age, sex), history of cardiovascular risk factors (hypertension, atrial fibrillation, smoking, alcohol consumption, family history of premature CVD, chronic kidney disease), history of concomitant CVD (coronary heart disease (CHD), previous stroke, congestive heart failure) and pharmacological treatment were recorded. Anthropometric parameters (weight, height, waist and hip circumference, waist to hip ratio) and systolic and diastolic blood pressure were also measured. The severity of stroke was assessed with the National Institutes of Health Stroke Scale (NIHSS) score at admission.

Routine laboratory investigations were performed the first day after admission after overnight fasting and included serum levels of glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), creatinine, uric acid, alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transpeptidase (γ-GT), alkaline phosphatase (ALP), bilirubin (total, direct and indirect) and creatine kinase. Low-density lipoprotein cholesterol (LDL-C) levels were calculated using Friedewald's formula[16]. Glomerular filtration rate (GFR) was estimated using the Modification of Diet in Renal Disease equation[17]. Chronic kidney disease was defined as estimated GFR < 60 mL/min per 1.73 m^2.

Nonalcoholic fatty liver disease was defined as serum ALT and/or AST levels above the upper limit of normal in the absence of other causes of elevated aminotransferases levels (chronic hepatitis B or C, drug toxicity, increased alcohol consumption (> 21 and > 14 drinks per week in men and women, respectively), cholestatic diseases or rhabdomyolysis)[1]. Liver ultrasonography was performed in all patients with elevated aminotransferases levels.

All patients underwent brain computed tomography at admission and a second brain computed tomography was performed if clinically indicated.

The outcome was assessed with the modified Rankin scale (mRS) score at discharge and in-hospital mortality. Adverse outcome was defined as mRS score at discharge ⩾ 2. Dependency at discharge was defined as mRS score between 2 to 5. The length of hospitalization was also recorded.

The study was approved by the Ethics Committee of the Medical School of the Aristotle University of Thessaloniki.

Statistical analysis

All data were analyzed with the statistical package SPSS

Table 1 Clinical characteristics of patients with nonalcoholic fatty liver disease and of those without nonalcoholic fatty liver disease

	Patients with NAFLD (n = 32)	Patients without NAFLD (n = 383)	P value
Age (yr)	77.4 ± 7.7	78.8 ± 6.4	NS
Males (%)	40	37.1	NS
Systolic blood pressure (mmHg)	150 ± 24	145 ± 24	NS
Diastolic blood pressure (mmHg)	84 ± 18	80 ± 13	NS
Hypertension	80%	82.5%	NS
Smoking (current/past)	17.4%/17.4%	10.9%/22.9%	NS
Package-years	17 ± 30	11 ± 31	NS
Type 2 diabetes mellitus	24%	31.8%	NS
Type 2 diabetes mellitus duration (yr)	3.9 ± 9.9	3.1 ± 6.7	NS
Atrial fibrillation	29.2%	38.9%	NS
Alcohol consumption (units/wk)	1.2 ± 2.5	2.3 ± 12.4	NS
Waist (cm)	107 ± 13	104 ± 12	NS
Waist/hip	1.03 ± 0.05	0.97 ± 0.07	NS
Body mass index (kg/m^2)	26.9 ± 3.5	27.3 ± 5.0	NS
Overweight/obese	47.1 %/17.6 %	40.7 %/24.3 %	NS
Family history of cardiovascular disease	31.8%	14.7%	NS
Coronary heart disease	37.5%	27.6%	NS
Previous ischemic stroke	21.7%	43.5%	NS
Chronic kidney disease	31.6%	35.1%	NS
Chronic heart failure	23.8%	23.2%	NS

NS: Not significant; NAFLD: Nonalcoholic fatty liver disease.

(version 17.0; SPSS, Chicago, IL, United States). Data are presented as percentages for categorical variables and as mean and standard deviation for continuous variables. Differences in categorical and continuous variables between groups were assessed with the χ^2 test and the independent samples t-test, respectively. Multiple logistic regression analysis was performed to identify independent predictors of dependency at discharge and of in-hospital mortality including factors with $P < 0.20$ by descriptive analysis. In all cases, a two-tailed $P < 0.05$ was considered significant.

RESULTS

NAFLD was present in 7.7% of the study population. Ultrasonography showed increased liver echogenicity in all these patients. Clinical characteristics of patients with NAFLD and patients without NAFLD are shown in Table 1. Demographic data, the prevalence of cardiovascular risk factors and the prevalence of established CVD did not differ between the two groups. Anthropometric characteristics and blood pressure at admission were also comparable in patients with and without NAFLD.

Laboratory characteristics of patients with NAFLD and patients without NAFLD are shown in Table 2. Patients with NAFLD had lower serum HDL-C and higher TG levels than patients without NAFLD ($P < 0.05$ for both comparisons). Serum LDL-C, glucose and uric acid levels and the estimated GFR did not differ between the two groups. Patients with NAFLD had higher serum ALT and AST levels than patients without NAFLD ($P < 0.001$ for both comparisons). Serum γ-GT levels were also higher in the former ($P < 0.05$). In contrast, serum ALP and bilirubin levels were comparable in the 2 groups.

At admission, the NIHSS score did not differ between patients with and without NAFLD (6.3 ± 6.4 and 8.8 ± 9.6, respectively; P = NS). The outcome of the 2 groups is shown in Table 3. The duration of hospitalization was comparable in patients with and without NAFLD (8.0 ± 5.1 and 6.7 ± 4.2 d, respectively; P = NS). The mRS score at discharge also did not differ between the two groups (1.9 ± 2.2 and 2.6 ± 2.2 in patients with and without NAFLD, respectively; P = NS). Rates of dependency at discharge were also similar in patients with and without NAFLD (36.8% and 55.0%, respectively; P = NS) as were the rates of adverse outcome (42.9% and 58.6%, respectively; P = NS). In-hospital mortality rates also did not differ between the 2 groups (8.0% and 7.0% in patients with and without NAFLD, respectively; P = NS).

In multiple logistic regression analysis, independent predictors of dependency at discharge were age (RR = 1.16, 95%CI: 1.05-1.28, $P < 0.005$), history of stroke (RR = 3.66, 95%CI: 1.25-10.71, $P < 0.05$) and the NIHSS score at admission (RR = 1.61, 95%CI: 1.34-1.92, $P < 0.001$). Independent predictors of in-hospital mortality were diastolic blood pressure at admission (RR = 1.06, 95%CI: 1.01-1.11, $P < 0.05$) and the NIHSS score at admission (RR = 1.17, 95%CI: 1.10-1.23, $P < 0.001$).

Among the 32 patients with NAFLD, 24 did not drink alcohol at all. When these 24 patients were compared with the rest of the study population (n = 391), similar results were obtained.

DISCUSSION

The present study suggests that NAFLD might not be

Table 2 Laboratory characteristics of patients with nonalcoholic fatty liver disease and of those without nonalcoholic fatty liver disease

	Patients with NAFLD (n = 32)	Patients without NAFLD (n = 383)	P value
Glucose (mg/dL)	125 ± 64	113 ± 46	NS
LDL-C (mg/dL)	112 ± 42	112 ± 40	NS
HDL-C (mg/dL)	39 ± 12	47 ± 15	<0.05
Triglycerides (mg/dL)	144 ± 65	119 ± 51	<0.05
Uric acid (mg/dL)	5.6 ± 1.8	5.7 ± 1.8	NS
eGFR (mL/min/1.73 m^2)	65 ± 19	69 ± 23	NS
Alanine aminotransferase (U/L)	35 ± 21	17 ± 13	<0.001
Aspartate aminotransferase (U/L)	56 ± 23	23 ± 17	<0.001
γ-glutamyl transpeptidase (U/L)	38 ± 29	24 ± 31	<0.05
Alkaline phosphatase (U/L)	71 ± 24	71 ± 32	NS
Total bilirubin (mg/dL)	0.83 ± 0.46	0.77 ± 0.48	NS
Direct bilirubin (mg/dL)	0.30 ± 0.19	0.28 ± 0.28	NS
Creatine kinase (U/L)	91 ± 40	119 ± 197	NS

LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; eGFR: Estimated glomerular filtration rate; NS: Not significant; NAFLD: Nonalcoholic fatty liver disease.

Table 3 Severity of stroke and outcome of patients of patients with nonalcoholic fatty liver disease and of those without nonalcoholic fatty liver disease

	Patients with NAFLD (n = 32)	Patients without NAFLD (n = 383)	P value
National Institutes of Health Stroke Scale score at admission	6.3 ± 6.4	8.8 ± 9.6	NS
Duration of hospitalization (d)	8.0 ± 5.1	6.7 ± 4.2	NS
Modified Rankin scale score at discharge	1.9 ± 2.2	2.6 ± 2.2	NS
Dependency at discharge	36.8%	55%	NS
Adverse outcome	42.9%	58.6%	NS
In-hospital mortality	8%	7%	NS

NS: Not significant; NAFLD: Nonalcoholic fatty liver disease.

associated with more severe stroke and that NAFLD does not appear to predict a worse in-hospital outcome in patients admitted with acute ischemic stroke.

The prevalence of NAFLD in patients with acute ischemic stroke in our study was 7.7%. To the best of our knowledge, there are no data regarding the prevalence of NAFLD or NASH in this population. The prevalence of NAFLD in the general population ranges between 34%-46%[3-5]. Since T2DM and obesity are associated with increased risk for both NAFLD and stroke, it might have been expected to find a higher prevalence of NAFLD in patients with acute stroke than in the general population. However, the diagnosis of NAFLD in the general population is primarily based on identification of hepatic steatosis with imaging[3,5]. In contrast, in the present study we defined NAFLD as the presence of elevated aminotransferases levels in the absence of other causes of chronic liver disease. It has been reported that less than one third of patients with NAFLD have elevated aminotransferases levels[3,5,18]. Therefore, the different definition of NAFLD might have contributed to the lower prevalence of NAFLD in our study.

We did not find any correlation between the presence of NAFLD and the severity of stroke at admission as assessed with the NIHSS. This is the first study that evaluated the association. A possible explanation for this finding is that patients with NAFLD and those without NAFLD had similar prevalence of CVD risk factors, except for the lower serum HDL-C and higher TG levels in the former. However, the association between these lipids and stroke severity has been inconsistent in epidemiological studies[19]. On the other hand, it is unclear whether NAFLD independently increases CVD risk or the higher incidence of cardiovascular events in this population is due to the increased prevalence of established CVD risk factors, particularly T2DM and obesity[9,10,20]. Some studies in the general population and in patients with T2DM suggested that NAFLD increases the risk for CVD even after adjusting for other CVD risk factors[15,21,22]. Moreover, NAFLD is independently associated with increased carotid intima-media thickness, a well-established marker of subclinical atherosclerosis[23-25]. Our results suggest that NAFLD might not be independently associated with greater stroke severity but larger studies are needed to resolve this clinically important and controversial issue.

Another important finding of the present study is that the presence of NAFLD in patients with acute

ischemic stroke does not appear to be associated with worse functional outcome at discharge. An earlier study suggested that NAFLD is associated with increased incidence of CHD but it does not predict the clinical outcome of patients with established CHD[26]. In patients with acute ischemic stroke, T2DM appears to be associated with worse functional outcome[27,28] whereas obesity is not[29]. Nevertheless, neither T2DM nor obesity was more frequent in patients with NAFLD in our study. There are no studies that evaluated the association between NAFLD and functional outcome in patients with acute ischemic stroke. Our findings suggest that this relationship might be week or absent but this remains to be confirmed or rejected in larger studies.

We did not find a difference in in-hospital mortality rates between patients with NAFLD and those without. In contrast, previous studies in the general population reported higher mortality rates in patients with NAFLD, with CVD being the leading cause of death[11,12]. There are no studies that evaluated the association between NAFLD and short- or long-term mortality in patients with acute ischemic stroke. However, a recent smaller study from India (n = 116 patients with acute ischemic stroke) reported that elevated ALT was associated with higher risk of death at 1 mo[30]. In the general population, some studies also found higher CVD mortality rates in patients with raised ALT[31] but others did not[32,33]. In the present study, mortality rates were higher in patients with NAFLD than in those without but this difference did not reach significance. It is possible that the small number of patients with NAFLD limited the statistical power of our study resulting in a type Ⅱ statistical error.

Apart from aminotransferases, serum γ-GT levels are also frequently elevated in patients with NAFLD. However, γ-GT is not a specific marker of NAFLD since it is also elevated in other hepatic (*e.g.*, cholestasis, alcohol- and drug-induced hepatitis) as well as extrahepatic diseases[1,8]. Some studies in the general population reported that γ-GT is an independent predictor of CVD events, including stroke[8,32-34]. However, when NAFLD was defined as the presence of elevated aminotransferases and/or γ-GT in the present study, again there was no association between NAFLD and stroke severity or outcome (data not shown).

In conclusion, the present study suggests that the presence of NAFLD in patients admitted for acute ischemic stroke might not be associated with more severe stroke or with worse in-hospital outcome. However, given the small number of patients with NAFLD in the present report and the lack of other studies evaluating this association, larger studies are needed to further evaluate the predictive value of NAFLD in this high-risk population.

COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) is the commonest cause of elevated aminotransferases and ranges from isolated fatty deposition in the liver (steatosis) to liver steatosis with inflammation and fibrosis nonalcoholic steatohepatitis (NASH). The prevalence of NAFLD in the general population ranges between 34%-46% whereas NASH is observed in approximately 12% of the general population.

Innovations and breakthroughs

This is the first study that assessed the prevalence of NAFLD in patients admitted with acute ischemic stroke and the association between NAFLD and stroke severity and in-hospital outcome.

Applications

The presence of NAFLD in patients admitted for acute ischemic stroke does not appear to be associated with more severe stroke or with worse in-hospital outcome.

Peer review

This is an interesting article, it is well written.

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BRIEF ARTICLE

Increased bone mineral density in patients with non-alcoholic steatohepatitis

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Abstract

AIM: To determine the relationship between non-alcoholic steatohepatitis (NASH) and bone mineral density (BMD).

METHODS: A total of 38 patients (25 males) with a diagnosis of histologically proven NASH and 42 healthy controls (24 males) were enrolled in the study. Demographic features, clinical findings, complete blood count and routine biochemical analysis, as well as adrenal, thyroid and gonadal functions, were recorded. Additionally, intact parathormone, 25-OH-vitamin-D3, tumor necrosis factor-α, interleukin-6, interleukin-1, insulin-like growth factor-1 and insulin-like growth factor binding protein-3 levels were measured in both groups. Furthermore, lumbar spine and femoral neck BMD of both groups were measured by the dual-energy X-ray absorptiometry (DXA) method.

RESULTS: The mean age was 41 ± 12 years in the NASH group and 43 ± 11 years in the control group. Among demographic features, waist circumference was significantly larger in the NASH group compared to the control group ($P < 0.019$). Among laboratory parameters, serum triglyceride ($P < 0.008$), alanine transaminase ($P < 0.0001$), aspartate transaminase ($P < 0.001$), alkaline phosphatase ($P < 0.016$), gamma glutamyl transferase ($P < 0.0001$), ferritin ($P < 0.001$) and 25-OH-vitamin-D3 levels ($P < 0.0001$) were significantly higher in the NASH group compared to the control group. Lumbar BMD was significantly higher in the NASH group compared to the control group (1.057 ± 0.119 g/cm^2 *vs* 0.941 ± 0.133 g/cm^2; $P < 0.001$, respectively). In the NASH group, there was no significant relationship between BMD and fibrosis stage in liver biopsy.

CONCLUSION: NASH increases BMD and may be related to an elevated serum 25-OH-vitamin D3 level.

Key words: Non-alcoholic steatohepatitis; Hepatic osteodystrophy; Bone mineral density

Core tip: Identifying the relationship between non-alcoholic steatohepatitis and bone mineral density (BMD) and its underlying mechanism is important. We found that patients with biopsy-proven non-alcoholic steatohepatitis (NASH) had higher lumbar BMD and serum 25-OH-vitamin-D3 levels compared to healthy controls. We did not find a significant relationship between serum levels of thyroid hormones, sex hor-

mones, parathormone and cytokines, such as tumor necrosis factor-α, interleukin-1 (IL-1), IL-6, insulin-like growth factor-1, IGFBP-3 levels and BMD. An elevated serum 25-OH-vitamin D3 level may be the principle responsible factor in the increased bone mineral density in patients with NASH.

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INTRODUCTION

The histological spectrum of non-alcoholic fatty liver disease (NAFLD) spans from generally benign, bland steatosis to steatosis with evidence of hepatocellular inflammation and damage (non-alcoholic steatohepatitis or NASH), which may be complicated by progressive fibrosis and cirrhosis[1,2]. Insulin resistance, oxidative stress and an inflammatory cascade are believed to play integral roles in the pathogenesis and progression of NAFLD[3]. Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) induce insulin resistance by inhibiting the activation of insulin receptor substrate[4]. Low serum insulin-like growth factor-1 (IGF-1) levels are associated with a determinant of the metabolic syndrome, including insulin resistance, serum leptin levels, waist-to-hip ratio and type 2 diabetes mellitus[5]. In the circulating blood, most of the IGF-1 binds serum insulin-like growth factor binding protein-3 (IGFBP-3), which therefore lowers the bioavailability of IGF-1[6].

Hepatic osteodystrophy is a bone disease of multifactorial origin associated with chronic liver disease[7]. Osteoporosis accounts for the majority of cases, whereas osteomalacia is rare in the absence of advanced liver disease and severe malabsorption. The reported prevalence of osteoporosis among patients with chronic liver disease ranges from 20% to 100%, depending on patient selection and diagnostic criteria[8]. The pathogenesis is considered as multifactorial and remains unclear in some aspects[9]. Histologically, hepatic osteodystrophy is similar to postmenopausal and aging-related bone loss in that trabecular bone is more rapidly and severely affected than cortical bone[8]. Decreased bone mineral density (BMD) has been reported in patients with primary biliary cirrhosis, primary sclerosing cholangitis, chronic active hepatitis and patients with alcoholic liver disease[9]. Recently, decreased BMD in obese children with NASH[10,11] and in female patients with ultrasoud-proven NASH[12] have been reported.

The aims of our study were to determine: (1) BMD in patients with biopsy proven NASH; (2) the relationship between BMD and clinical parameters; and (3) the relationship between BMD and laboratory parameters.

MATERIALS AND METHODS

Patient population

A total of 38 patients with a diagnosis of NASH were prospectively and consecutively enrolled in the study. The diagnosis of NASH was unequivocally established in all patients based on the following criteria: (1) persistently raised ALT level (> 1.5 times the upper normal limit) for more than 6 mo; (2) a liver biopsy showing the presence of steatosis (> 5%), as well as lobular and/or portal inflammation, with or without Mallory bodies, fibrosis or cirrhosis; and (3) appropriate exclusion of other liver disease, such as alcoholic liver disease, viral hepatitis, autoimmune hepatitis, drug or toxin induced liver disease, primary biliary cirrhosis, biliary obstruction, space occupying lesions in the liver, hemochromatosis, Wilson's disease and α-1 antitrypsin-deficiency-associated liver disease.

All patients had a history of no alcohol consumption, confirmed by family members who were in close contact with the patients. No patient had a history of gastrointestinal surgery or ingestion of drugs known to produce hepatic steatosis in the previous 6 mo. None of the patients had been treated with drugs for the treatment of NASH before liver biopsy. Clinical symptoms and physical examination findings were recorded in all patients. The presence and absence of a space occupying lesion was verified by ultrasonography. At the time of the study, none of the patients showed clinical, biochemical or histological evidence of cirrhosis. In all cases, liver biopsies were performed as part of the evaluation of abnormal liver biochemistry.

Control group

Fifty healthy subjects without risk factors for laboratory and ultrasonographic evidence of liver disease were included in the study. All cases were selected from people who applied to our hospital for a routine check-up and who had no complaints. No cases had a history of alcohol intake and drug use. In all cases, complete physical examination and abdominal ultrasonography were performed. Cases with steatosis on ultrasonography and elevated liver enzymes were excluded. Of the 50 cases originally included in the study, 5 were excluded due to the presence of steatosis on ultrasonography and 3 were excluded from the study because of elevated liver enzymes. Liver biopsy was not performed in the control group.

Clinical and laboratory measurements

Body mass index (BMI) was calculated using the following formula: weight (kg)/height (m^2). We defined obesity as a BMI greater than 30. Subjects fasted overnight before blood samples were obtained. The index of insulin resistance was calculated using the fasting value of plasma glucose and the serum level, according to the homeostasis model assessment (HOMA) index as [(insulin) × (glucose)]/22.5. Abdominal ultrasonographic exami-

Table 1 Demographic features of patients in the non-alcoholic steatohepatitis and in the control group

Parameter	NASH group mean ± SD (range)	Control group mean ± SD (range)	*P* value
n (Female/Male)	13/25	18/24	0.428
Age (yr)	41 ± 12 (18-69)	42.8 ± 10 (24-65)	0.499
Smoking *n* (%)	10 (26)	9 (26)	0.810
BMI (kg/m²)	29 ± 4 (20-39)	27 ± 5 (21-39)	0.338
Waist (cm)	98 ± 9 (77-118)	93 ± 9 (76-113)	0.019

NASH: Non-alcoholic steatohepatitis; BMI: Body mass index.

nations were performed using 7.5 MHz probe (Toshiba SSH-140 A machine).

In all subjects, complete blood count, glucose, urea, creatinine, aspartate transaminase (AST), alanine transaminase (ALT), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), total bilirubin, albumin, cholesterol, triglyceride, hepatitis B surface antigen, antibody to hepatitis B surface antigen, antibody to hepatitis C virus, antinuclear antibody, anti-smooth muscle antibody, anti-mitochondrial antibody, ferritin, ceruloplasmin, α-1 antitrypsin, insulin, intact parathormone (iPTH), 25-OH-vitamin D3, follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), testosterone, free T4 (FT4), thyroid stimulating hormone (TSH) and dehydroepiandrostenedione sulfate (DHEAS) were determined by standard laboratory techniques. Because of seasonal variations in serum vitamin D levels[13], the control group and NASH group were studied in the same season. Measurement of IGF-1, IGFBP-3, TNF-α, IL-1 and IL-6 were performed by chemiluminescent immunometric assay on an automated system (Immulite 1000, Diagnostic Products Corp., Los Angeles, CA, United States).

Liver histology

A liver biopsy was performed in all patients and stained with hematoxylin-eosin, Masson's trichrome and rhodanine. Each liver biopsy was evaluated by an experienced pathologist to determine the severity of liver injury, steatosis, inflammatory cell accumulation, necroinflammatory activity and fibrosis. The degree of fibrosis was assessed using a 5 grade scale: 0 = none, normal connective tissue; 1 = mild, foci of pericellular fibrosis in zone 3; 2 = moderate, perivenular or pericellular fibrosis confined to zone 3 and 2 regions, with or without portal/periportal fibrosis; 3 = severe, bridging or septal fibrosis; and 4 = cirrhosis. The level of fatty infiltration was assessed and graded on a scale of 1 to 3: 1 = mild (5%-33% of hepatocytes affected); 2 = moderate (33%-66%); 3 = severe (> 66%). Lobular inflammation was assessed semi-quantitatively following the criteria of Brunt *et al*[14]. The presence or absence of Mallory bodies was recorded in all liver biopsies.

BMD measurement

BMD was measured by the dual-energy X-ray absorptiometry (DXA) method using Hologic machines (Hologic Discovery QDR 4500A, Waltham United States). Bone mass was expressed in absolute values (g/cm^2), T-score (number of standard deviations compared with a young (30-year-old) adult sex-matched reference population) and Z-score (number of standard deviations compared with an age and sex-matched reference population). As defined by World Health Organization, a T-score between -1 and -2.5 indicates osteopenia, whereas a T-score less than -2.5 indicates osteoporosis (World Health Organization. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. Technical report no. 843. Geneva, Switzerland: World Health Organization, 1994). A Z-score less than -2 indicates a value in the lowest 2.5th percentile of the reference range, a value associated with a considerably larger increase in the risk of fracture. The BMD of the lumbar spine at L_1-L_4 and BMD of the left femoral neck were measured in all patients in the NASH and the control group.

Statistical analysis

Results are expressed as mean ± SD and number of patients with a condition. Statistical analyses were carried out by using the statistical packages for SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, United States). Mean and SD were calculated for continuous variables. Differences between the groups according to non-numerical values were tested by the χ^2 test and Fisher exact tests. The normal distribution of numerical values was analyzed by the Kolmogorov-smirnov test. Normal distributed values were analyzed by the Student's *t*-test and non-normal distributed values were analyzed by the Mann-Whitney *U* test. Two-sided *P* values were considered statistically significant at $P \leqslant 0.05$. The study was approved by the Institutional Review Board and all patients gave informed consent for participation in this research.

RESULTS

NASH group

Table 1 shows the demographic features of both groups. There were 25 (60%) males and 13 (40%) females in the NASH group. In the clinical history, 22 (58%) patients had fatigue, 27 (71%) dyspepsia and 6 (16%) patients had right upper quadrant pain. Physical examination revealed obesity in 10 (26%), hypertension in 7 (18%) and hepatomegaly in 7 (18%) patients. None of the patients had space occupying lesions on the ultrasonographic examination.

Complete blood count was within normal range in all patients. Table 2 shows the biochemical findings of both groups. There were elevations in serum ALT levels in all (100%), AST in 34 (89%), alkaline phosphatase in 4 (11%), GGT in 21 (55%), total bilirubin in 5 (13%), total cholesterol in 12 (32%), triglyceride in 22 (58%) and ferritin in 15 (39%) patients. Serum urea, creatinine, calcium and phosphorus levels were within normal limits in all patients. Two patients had slightly decreased serum

Table 2 Biochemical findings of the non-alcoholic steatohepatitis and the control group

Parameter	NASH group mean ± SD (range)	Control group mean ± SD (range)	*P* value
Urea (mg/dL)	28 ± 7 (13-52)	29 ± 8 (14-51)	0.631
Creatinine (mg/dL)	0.7 ± 0.1 (0.6-1.1)	0.8 ± 0.1 (0.5-1.1)	0.631
T. cholesterol (mg/dL)	191 ± 42 (104-314)	187 ± 44 (98-296)	0.668
Triglyceride (mg/dL)	208 ± 210 (67-1420)	136 ± 67 (33-298)	0.008
Calcium (mg/dL)	9.5 ± 0.4 (8.6-10.3)	9.4 ± 0.54 (8.6-10.6)	0.239
Phosphorus (mg/dL)	3.4 ± 0.4 (2.1-4.3)	3.6 ± 0.6(2.3-5.7)	0.288
ALT (U/L)	114 ± 115 (51-775)	20 ± 8 (8-36)	< 0.0001
AST (U/L)	55 ± 31 (28-190)	20 ± 7 (2-35)	< 0.0001
ALP (U/L)	93 ± 44 (43-266)	72 ± 18 (44-123)	0.016
GGT (U/L)	121 ± 214 (23-1135)	29 ± 15 (8-65)	< 0.0001
T bilirubin (mg/dL)	0.8 ± 0.6 (0.2-3.2)	0.6 ± 0.3 (0.2-1.5)	0.165
Albumin (g/dL)	4.2 ± 0.5 (2.3-5.1)	4.2 ± 0.3 (3.5-5.1)	0.214
Ferritin (ng/mL)	208 ± 193 (22-901)	75 ± 82 (3-470)	< 0.001

NASH: Non-alcoholic steatohepatitis; AST: Aspartate transaminase; ALT: Alanine transaminase; GGT: Gamma glutamyl transferase; ALP: Alkaline phosphatase.

Table 3 Serum hormone and cytokine results of the non-alcoholic steatohepatitis and the control group

Parameter	NASH group mean ± SD (range)	Control group mean ± SD (range)	*P* value
HOMA	5.7 ± 5.25 (1.1-23.59)	5.6 ± 10.1 (0.7-59.9)	0.192
Cortisol (μg/dL)	12.6 ± 5.8 (1.6-27)	11.6 ± 5.8 (2.9-28)	0.375
TSH (uIU/mL)	1.7 ± 1.09 (0.2-5.3)	1.36 ± 1 (0.01-4)	0.104
FT4 (ng/dL)	1.22 ± 0.19 (0.9-1.8)	1.22 ± 0.22 (0.8-107)	0.999
FSH (mIU/mL)	21 ± 41 (1.6-299)	19 ± 37 (0.3-177)	0.729
LH (mIU/mL)	10.4 ± 10.8 (1.2-44)	11 ± 15 (0.1-64)	0.974
E2 (pg/mL)	39.1 ± 72 (3.8-435)	41 ± 47 (2-199)	0.404
DHEAS (ug/dL)	190 ± 109 (25-501)	146 ± 90 (33-366)	0.071
iPTH (pg/mL)	46.3 ± 20 (18-130)	50 ± 16 (19-105)	0.086
25-OH-vit-D3 (ug/L)	17 ± 6 (5.1-39)	13 ± 7 (3.8-36)	< 0.0001
Testosterone (ng/mL)	2.5 ± 1.9 (0.03-5.8)	1.7 ± 1.9 (0.02-5.4)	0.075
IGF-1 (ng/mL)	141 ± 94 (30-527)	144 ± 47 (67-292)	0.099
TNF-α (pg/mL)	11.6 ± 6.7 (4-31.7)	14 ± 7 (2-4.9)	0.062
IL-6 (pg/mL)	2.2 ± 0.6 (2-4.9)	3 ± 2.4 (2-12)	0.8
IL-1 (pg/mL)	8.2 ± 16.5 (5-107)	5.3 ± 2 (5-17)	0.291
IGFBP-3 (ug/mL)	4.1 ± 1.68 (1.1-8.6)	4.2 ± 0.8 (2.6-6.02)	0.909

NASH: Non-alcoholic steatohepatitis; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; IGF-1: Insulin-like growth factor-1; IGFBP-3: Insulin-like growth factor binding protein-3; HOMA: Homeostasis model assessment; TSH: Thyroid stimulating hormone; LH: Luteinizing hormone; E2: Estradiol; DHEAS: Dehydroepiandrostenedione sulfate; iPTH: Intact parathormone; FT4: Free T4; FSH: Follicle-stimulating hormone.

albumin levels.

Table 3 shows hormone and cytokine levels of both groups. There were elevations in the serum cortisol level in 3 (8%) patients, TSH in 1 (3%) and iPTH level in 4 (11%) patients. Serum FSH, LH, DHEAS and testosterone levels were within normal range in all patients. We found decreased serum 25-OH-vitamin D3 levels in 4 (11%) patients and decreased serum IGF-1 levels in 16 (42%) patients. There were elevations in the serum TNF-α level in 16 (42%), IL-1 in 2 (5%) and IGFBP-3 in 16 (42%) patients. Serum IL-6 level was within normal range in all patients.

Table 4 shows histopathological analysis of the liver section obtained from patients in the NASH group. Most of the patients (71%) had moderate or severe steatosis. There was mild or moderate fibrosis (grade I or Ⅱ) in 21 (55%) patients. Twelve patients (32%) had no fibrosis and none of the patients had cirrhosis in the liver biopsy.

Table 5 shows the bone mineral density of both groups. Five male patients (11%) had osteoporosis, 13 (34%) patients (9 males and 2 premenopausal and 2 postmenopausal females) had osteopenia and 20 (57%) patients had normal bone mineral density.

Control group

There were 18 (42.8%) females and 24 (57%) males in the control group. Physical examination revealed hypertension in 10 (23.8%) and obesity in 13 (30.9%) patients.

Complete blood count, serum urea, creatinine, ALT, AST, alkaline phosphatase and albumin levels were within normal range in all patients. There were elevations in serum levels of total cholesterol level in 13 (30.9%), triglyceride in 7 (16.6%), calcium in 3 (7.1%), phosphorus

Table 4 Histopathological findings according to Brunt classification of liver biopsy obtained from the non-alcoholic steatohepatitis group *n* (%)

Parameters	Grade 0	Grade 1	Grade 2	Grade 3
Steatosis	0 (0)	11 (29)	15 (39)	12 (32)
Ballooning	1 (3)	11 (29)	26 (68)	-
Lobular inflammation	0 (0)	21 (55)	16 (42)	1 (3)
Portal inflammation	8 (21)	24 (63)	6 (16)	0 (0)
Fibrosis	12 (32)	18 (47)	3 (8)	5 (13)

in 1 (2.3%), GGT in 3 (7.1%), total bilirubin in 1 (2.3%) and ferritin in 3 (7.1%) patients.

There were elevations in the serum levels of cortisol in 4 (9.5 11%), THS in 1 (2.3%) and iPTH in 6 (14.2%) patients. Decreased serum 25-OH-vitamin D3 levels were found in 14 (33.3%) patients. Serum FSH, LH, E2, testosterone, DHEAS and FT4 levels were within normal range in all patients.

There were elevations in the serum levels of TNF-α in 23 (54.7%), IL-1 in 1 (2.3%), IL-6 in 1 (2.3%) and IGFBP-3 in 15 (35.7%) patients. Decreased serum IGF-1 levels were found in only 13 (30.9%) patients.

There was osteoporosis in 11 (26.1%) (5 males, 3 premenopausal and 3 postmenopausal females) patients. There was osteopenia in 13 (30.9%) (7 males, 5 premenopausal and 1 postmenopausal females) patients. Bone mineral density was within normal range in 11 (26.1%) patients.

Comparison of the NASH and the control group

There were no significant differences between the NASH group and control group regarding age, gender, BMI and the incidence of smoking and hypertension. Waist circumference was significantly larger in the NASH group compared to the control group (98 ± 9 cm *vs* 93 ± 9 cm, respectively; $P = 0.019$). There were no significant differences between the NASH group and the control group regarding complete blood count, HOMA score, serum urea, creatinine, total cholesterol, calcium, phosphorus, total bilirubin, albumin, cortisol, TSH, FT4, FSH, LH, E2, DHEAS, iPTH, IGF-1, TNF-α, IL-1, IL-6 and IGFBP-3 levels.

Serum triglyceride (208 ± 210 *vs* 136 ± 67; $P = 0.008$), ALT (114 ± 115 *vs* 20 ± 8; $P < 0.0001$), AST (55 ± 31 *vs* 20 ± 7; $P < 0.001$), ALP (93 ± 44 *vs* 72 ± 18; $P = 0.016$), GGT (121 ± 214 *vs* 29 ± 15; $P < 0.0001$), ferritin (208 ± 193 *vs* 75 ± 82; $P < 0.001$) and 25-OH-vitamin D3 levels (17 ± 6 *vs* 13 ± 7; $P < 0.0001$) were significantly higher in the NASH group compared to the control group.

Lumbar BMD was significantly higher in the NASH group compared to the control group (1.057 ± 0.119 g/cm^2 *vs* 0.941 ± 0.133 g/cm^2, $P = 0.001$). Lumbar T-score and Z-score were also significantly higher in the NASH group compared to the control group [(-0.77) ± (1.25) *vs* (-1.58) ± (1.36); $P = 0.009$ and (-0.44) ± (1.38) *vs* (-1.18) ± (1.28); $P = 0.019$; respectively]. There was no significant difference between the NASH group and control group regarding femoral BMD, femoral T-score and Z-score.

DISCUSSION

Hepatic osteodystrophy is a bone disease of multifactorial origin associated with chronic liver disease. Both osteoporosis and osteopenia are part of this condition. Histologically, hepatic osteodystrophy is similar to postmenopausal and aging-related bone loss in that trabecular bone is more rapidly and severely affected than cortical bone[8]. On the basis of BMD measurements, the reported prevalence of low BMD ranges from 13 to 70%[8,15]. Cholestatic liver disease has a higher incidence of hepatic osteodystrophy than non cholestatic liver disease, but BMD loss is present in cirrhosis of all etiologies. The mean T-score evaluated by DXA in the lumbar spine has been found to be -2.22 in primary biliary cirrhosis, -1.93 in primary sclerosing cholangitis, -1.23 in chronic active hepatitis and -0.86 in alcoholic cirrhosis[9]. Decreased BMD in obese children with NASH[10,11] and in female patients with ultrasound-proven steatosis and elevated ALT[12] have been reported. Our findings were not compatible with previous studies. We found that NASH has a promoting effect on lumbar BMD compared to healthy controls (-1.057 ± 0.119 g/cm^2 *vs* 0.941 ± 0.133 g/cm^2, $P = 0.001$ respectively). Our study population consisted of an adult population with both genders and most patients with NASH had low fibrosis scores on liver biopsy. We also found that patients with NASH had a higher level of serum 25-OH-vitamin-D3 compared to healthy controls. NASH has an insignificant promoting effect on femoral BMD compared to healthy controls. This different promoting effect on lumbar and femoral bone may be related to the structure of those bones.

Potential inciting factors that either directly or indirectly alter the bone mass include IGF-1 deficiency, hyperbilirubinemia, hypogonadism (estrogen and testosterone deficiency), alcoholism, excess tissue iron deposition, subnormal vitamin D levels, vitamin D receptor genotype, osteoprotegerin deficiency, vitamin K deficiency, immunosuppressive therapy preceding and following liver transplantation[9,10], together with less exercise and muscle activity compared to healthy persons[16]. Vitamin D is a key regulator of bone metabolism and several studies in adults have shown that vitamin D increases bone mineral density[17] and prevents osteoporotic fractures[18]. Among NAFLD, a significant correlation between decreased serum 25-hydroxyvitamin D concentration and histological severity of hepatic steatosis, necroinflammation and fibrosis have been reported previously[19,20]. There are inverse correlations between serum 25-OH-vitamin D3 levels and all adiposity measurement, including BMI percentage, waist circumference, total fat mass, percentage of body fat and subcutaneous abdominal adipose tissue[13,21]. There are seasonal variations in serum vitamin D levels and its level is higher in summer than winter[13]. There were no

Table 5 Dual-energy X-ray absorptiometry results of patients in the non-alcoholic steatohepatitis and the control group

Parameter	NASH group mean ± SD (range)	Control group mean ± SD (range)	*P* value
Lumbar BMD	1.057 ± 0.119	0.941 ± 0.133	0.001
Lumbar T-score	-0.77 ± 1.25 (-2.9-2.6)	-1.58 ± 1.36 (-3.7-2.2)	0.009
Lumbar Z-score	-0.44 ± 1.38 (-3.3-2.9)	-1.18 ± 1.28 (-2.9-2.5)	0.019
Femoral BMD	1.004 ± 0.118	0.972 ± 0.130	0.305
Femoral T-score	0.32 ± 0.87 (-2-1.8)	0.062 ± 1.02 (-1.7-2.1)	0.242
Femoral Z-score	0.65 ± 0.92 (-1.5-2.8)	0.35 ± 0.94 (-1.6-2)	0.178

NASH: Non-alcoholic steatohepatitis; BMD: Bone mineral density.

known factors that can cause hepatic osteodystrophy in our patients with NASH. In this study, there was an elevated serum 25-OH-vitamin-D3 level in the NASH group compared to the control group (17.3 ± 6.1 *vs* 14.07 ± 10.8; $P < 0.0001$, respectively). It may be the main responsible factor for the elevated BMD of patients with NASH. All our patients in the NASH group and control group were included in the study at the same season. Both serum 25-OH-vitamin D3 levels and mean waist circumferences were significantly higher in the NASH group compared to the control group. Therefore, our results were not compatible with previously reported literature. We suggest that the relationship between serum 25-OH-vitamin D3 levels and adiposity parameters, BMD and histopathological changes in NASH should be investigated in a multicenter, multiregional, prospective study.

Dehydroepiandrosterone (DHEA) and its sulfate derivative (DHEAS) are the most abundant circulating C_{19} steroids in humans and are produced primarily from the adrenal glands. The actions of DHEA in humans are thought to be mediated primarily through conversion to sex hormones[22]. DHEA is the precursor for 30%-50% of circulating androgens in older men[23] and more than 70% in older women[24]. It has been postulated that the decline in DHEA with aging contributes to physiological changes that are dependent on sex hormones, such as the loss of bone and muscle mass. It has been reported that DHEA replacement therapy for 1 year improved hip bone mineral density in older adults and spine bone mineral density in older women[25]. Charlton *et al*[26] reported that more advanced NAFLD is strongly associated with low circulating DHEAS. In our study, most of the patients with NASH had early grade (less than grade Ⅱ) liver fibrosis, verified by biopsy. Therefore, early stages of NASH may not have any negative effect on serum DHEAS levels. A high serum parathormone level has been associated with increased bone remodeling, excessive bone loss and increased fracture risk[27]. The inverse correlation between serum parathormone levels and BMD in all skeletal sites has been reported previously[28]. Although there was no statistically significant difference between the two groups, elevated serum DHEAS (190 ± 109 *vs* 146 ± 90; $P = 0.071$, respectively) and decreased serum iPTH levels (46.3 ± 20 *vs* 50 ± 16, $P = 0.086$, respectively) may have an additional promoting effect on the elevated bone mineral density in our patients with steatohepatitis. The concomitant elevation in serum 25-OH-vitamin D3 and DHEAS levels and decline in serum iPTH levels in the NASH group compared to the control group can explain pathophysiologically elevated BMD in our patients with NASH.

There is increasing evidence that several cytokines regulate metabolism, inflammatory response, cell death, regeneration and fibrosis in normal and injured liver tissue[29]. Obesity is characterized by a broad inflammatory response and many inflammatory mediators exhibit patterns of expression and/or impact insulin action that correlates with the progression of metabolic syndrome[30]. The tissue expression and circulating cytokines, such as TNF-α, IL-1, IL-6, adiponectin and interferon-γ, have been associated with obesity-related insulin resistance[31]. IGF-1 is a multipotent anabolic hormone with beneficial effects on glucose homeostasis by its action as an insulin sensitizing mediator. Hepatocytes are the main source of circulating IGF-1 whose secretion is stimulated by growth hormone[32]. Garcia-Galiano *et al*[33] reported glucose > 110 mg/dL, IL-6 > 4.81 pg/mL, IGF-1 < 130 ng/mL, HOMA > 4.5 and IGF-1 < 110 ng/mL as independent predictors of hepatic steatosis and NASH, respectively. In a population based study, positive correlation between hyperechogenic liver pattern and low serum IGF-1 and low serum IGF-1/IGFBP-3 ratios have been reported[34]. The relationship between low serum IGF-1 and fibrosis stage in patients with NAFLD has also been reported[35]. In our study, we did not find a significant relationship between serum TNF-α, IL-1, IL-6, IGF-1, IGFBP-3 and NASH. The prospective collection of all data, the presence of a control group and the histopathological diagnosis of NASH may enhance the validity of our study.

In conclusion, we found that NASH has a promoting effect on bone mineral density. This increase was not related to serum cytokine levels, including TNF-α, IL-1, IL-6, IGF-1 and IGFBP-3. Elevated serum 25-OH-vitamin D3 levels may be the main responsible factor for increased bone mineral density in NASH. Elevated serum DHEAS and decreased serum iPTH levels (but the significance level was not achieved) may have an additional promoting effect on bone mineral density in NASH.

COMMENTS

Background

Non-alcoholic steatohepatitis (NASH) may be complicated by progressive fibrosis and cirrhosis. Hepatic osteodystrophy is a bone disease of multifactorial origin associated with chronic liver disease and histologically similar to postmenopausal or aging-related bone loss. The pathogenesis is considered multifactorial and remains unclear in some aspects. The relationship between NASH and bone mineral density (BMD) is important topic.

Research frontiers

Potential inciting factors, such as the stage of liver disease, insulin-like growth factor 1 (IGF-1) levels, sex hormones, vitamin D, parathormone and circulating cytokine levels, that may influence BMD in NASH may give a new frontier to fight two prevalent conditions like NASH and osteoporosis.

Innovations and breakthroughs

Cholestatic liver disease has higher incidence of hepatic osteodystrophy than non cholestatic liver disease, but BMD loss is present in all etiologies of cirrhosis. There are a few publications regarding decreased BMD and its underlying mechanism in patients with NASH. In this study, the authors showed that patients with liver biopsy-proven NASH had a significantly higher lumbar BMD and 25-OH-vitamin-D3 level than the healthy control group. There was no significant relationship between BMD and fibrosis stage in liver biopsy of patients with NASH. This increase in lumbar BMD was also not related to serum TNF-α, interleukin (IL)-1, IL-6, IGF-1 and IGFBP-3. Elevated serum 25-OH-vitamin D3 level may be the main responsible factor for increased bone mineral density in NASH. The results of this study were not compatible with previously reported studies.

Applications

Elevated serum 25-OH-vitamin-D3 level may have a protective effect on BMD in patients with NASH. Therefore, supplemental supportive administration of 25-OH-vitamin-D3 to prevent bone loss in patients with NASH may not be required until the development of cirrhosis.

Terminology

NASH is steatosis with evidence of hepatocellular inflammation and damage of liver and it may progress to advanced fibrosis, cirrhosis and hepatocellular cancer. Hepatic osteodystrophy is a bone disease of multifactorial origin associated with chronic liver disease.

Peer review

Interestingly, the authors report that NASH patients have a higher lumbar BMD and 25-OH-vitamin D3 level than healthy controls.

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BRIEF ARTICLE

IL-28B polymorphisms and treatment response in hepatitis C virus patients with persistently normal alanine aminotransferase

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Abstract

AIM: To examine the association between the interleukin 28B (*IL-28B*) genotype and treatment response in hepatitis C virus (HCV)-infected patients with persistently normal alanine aminotransferase (PNALT).

METHODS: We compared the treatment response of HCV-infected patients with PNALT to that of patients with non-PNALT. Between February 2010 and April 2013, 278 patients infected with HCV were enrolled in this study. All of the patients were treated with peginterferon-alpha 2a or 2b plus ribavirin. In addition, 180 μg of peginterferon alpha-2a or 1.5 μg/kg peginterferon alpha-2b per week plus weight-based ribavirin (600-1000 mg/d) were typically administered for 24 wk to HCV genotype 2-infected patients or for 48-72 wk to HCV genotype 1-infected patients. In all of the patients, the *IL-28B* rs8099917 genotype was determined using a TaqMan single-nucleotide polymorphism assay. HCV RNA was measured using the COBAS TaqMan HCV test.

RESULTS: Female patients were dominant in the PNALT group ($P < 0.0001$). Among 72 HCV genotype 1-infected patients with PNALT, the early virologic response (EVR) rates ($P < 0.01$) and the sustained virologic response (SVR) rates ($P < 0.01$) were higher in patients with the IL-28B TT genotype than in those with the *IL-28B* TG/GG genotype. In HCV genotype 1-infected patients with PNALT, multivariate logistic-regression analysis showed that SVR was independently predicted by the *IL-28B* rs8099917 TT type ($P < 0.05$) and having an EVR ($P < 0.01$). The *IL-28B* rs8099917 TT genotype strongly correlated with treatment response in HCV genotype 1-infected Asian patients with PNALT.

CONCLUSION: The *IL-28B* genotype may be useful for selecting HCV genotype 1-infected patients with PNALT who should receive interferon-based treatment.

Key words: Hepatitis C virus; Interleukin 28B; Persistent normal alanine aminotransferase levels; Standard of care; Treatment response

Core tip: Whether the interleukin 28B (*IL-28B*) genotype affects the treatment response to peginterferon plus ribavirin in hepatitis C virus (HCV)-infected patients with persistently normal alanine aminotransfer-

ase (PNALT) is unclear. We examined the association between the *IL-28B* genotype and treatment response in HCV-infected patients with PNALT. Opinions about the appropriate treatment method for HCV-infected patients with PNALT differ. In the present study, we found that *IL-28B* rs8099917 TT was associated with SVR in HCV genotype 1-infected Asian patients with PNALT. The determination of *IL-28B* genotype is important for the successful treatment of HCV genotype 1-infected patients with PNALT.

Miyamura T, Kanda T, Nakamura M, Jiang X, Wu S, Nakamoto S, Mikami S, Takada N, Imazeki F, Yokosuka O. *IL-28B* polymorphisms and treatment response in hepatitis C virus patients with persistently normal alanine aminotransferase. *World J Hepatol* 2013; 5(11): 635-641 Available from: URL: http://www.wjgnet.com/1948-5182/full/v5/i11/635.htm DOI: http://dx.doi.org/10.4254/wjh.v5.i11.635

INTRODUCTION

Hepatitis C virus (HCV) is a causative agent of acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC)[1-3]. Peginterferon-alpha 2a or 2b plus ribavirin treatment leads to sustained virologic response (SVR) rates of approximately 50% and 80% in patients infected with HCV genotype 1 and genotype 2 or 3, respectively[4-6]. The standard of care has been peginterferon plus ribavirin until the recent approval of combination therapies, including telaprevir and boceprevir. Until the development of an interferon-free regimen, peginterferon alpha plus ribavirin will play a critical role in the eradication of this virus.

Persistently normal alanine aminotransferase (PNALT) is present in 25%-40% of patients with chronic HCV infection[7,8]. Although an elevated alanine aminotransferase (ALT) level suggests progressive liver damage in chronic HCV infection, normal ALT levels do not always exclude significant liver damage. Zeuzem *et al*[9] reported that the SVR rates in patients with PNALT were similar to those in patients with abnormal ALT. However, opinions about the appropriate treatment method for HCV-infected patients with PNALT differ[7-11].

Genome-wide association studies have revealed a strong relationship between single-nucleotide polymorphisms (SNPs) near interleukin 28B (IL-28B) on chromosome 19 and the virologic response to peginterferon plus ribavirin treatment in patients worldwide who are infected with HCV genotype 1[12-14] as well as an association with the natural clearance of this virus[15,16]. IL-28B has antiviral properties and can interact with human interferon responses[17-21]. Associations between IL-28B variants and HCC development[22] and recurrence[23] have recently been reported. Moreover, an association between the *IL-28B* rs12979860 CC genotype and higher ALT levels has also been described[24]. It is possible that the *IL-28B* genotype is associated with inflammatory activity in the liver and the progression of hepatic fibrosis.

In clinical practice, it is difficult to make the decision to treat HCV-infected patients with PNALT. In the present study, we investigated whether *IL-28B* rs8099917 genetic variations were useful for the prediction of treatment response in HCV-infected patients with PNALT.

MATERIALS AND METHODS

Ethics

This work was conducted in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Written informed consent was obtained from each patient participating in this study. The study was approved by the ethics committee of Chiba University, Japan (permission number 282 and 1462), and conformed to the tenets of the Declaration of Helsinki.

Patients

Between February 2010 and April 2013, 278 patients infected with HCV were enrolled in this study. All patients were treated with peginterferon-alpha 2a or 2b plus ribavirin at Chiba University Medical School Hospital, Kikkoman General Hospital, or Toho University, Sakura Medical Center. The patients were eligible if they met the following inclusion criteria: (1) infection with HCV; (2) age $\geqslant$ 20 years; (3) no absolute contraindications for peginterferon plus ribavirin therapy such as pregnancy, severe heart disease, abnormal hemoglobinemia, chronic renal failure, mental disorders, severe liver failure, or autoimmune diseases; (4) absence of HIV infection; (5) no currently active drug abuse; and (6) no drug allergy to interferon or nucleos(t)ide analogues. Some of these patients had previously been included in other studies[19,25,26].

Among these 278 HCV RNA-positive patients, 178 had ALT elevation (each value exceeding the higher limit of the normal range was considered abnormal)[7], and 100 exhibited normal ALT levels at least 3 times during a 24-mo period (considered as PNALT patients).

Treatment regimens

In the present study, 180 μg of peginterferon alpha-2a or 1.5 μg/kg of peginterferon alpha-2b per week plus weight based ribavirin (600-1000 mg/d) were typically administered for 24 wk to HCV genotype 2-patients or for 48-72 wk to HCV genotype 1-patients.

Serum HCV RNA, HCV genotype, ALT, other liver function, and hematological tests

HCV RNA was measured using the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). The linear dynamic range of this assay was 1.2 to 7.8 log IU/mL. HCV genotypes were determined using the antibody serotyping method of Tsukiyama-Kohara *et al*[27], and Tanaka *et al*[28]. Serum ALT measurement and other liver function tests were performed according to standard methods. The normal range of serum ALT was considered 8-42 IU/L.

IL-28B SNP genotyping

SNP rs8099917 was examined in plasma by allelic discrimi-

Table 1 Patient baseline and demographic characteristics, and treatment response in the present study

	Total	PNALT	Abnormal ALT	*P* value
Number of patients	278	100	178	
Age (yr)	55.6 ± 11.4	56.2 ± 10.9	55.3 ± 11.6	0.526
Gender (male/female)	136/142	31/69	105/73	< 0.0001
AST (IU/L)	56.6 ± 44.7	29.8 ± 12.1	71.3 ± 49.1	< 0.0001
ALT (IU/L)	70.5 ± 64.3	27.6 ± 7.2	94.6 ± 69.4	< 0.0001
γ-GT (IU/L)	50.7 ± 57.3	26.2 ± 22.1	64.1 ± 65.7	< 0.0001
WBC (/mm^3)	5190 ± 1500	5060 ± 1490	5260 ± 1510	0.28
Hemoglobin (g/dL)	14.4 ± 7.1	13.5 ± 1.2	15.0 ± 8.9	0.094
Platelets (× 10^4/mm^3)	17.1 ± 5.6	17.7 ± 5.9	16.8 ± 5.5	0.20
Previous treatment (-/+)	211/67	75/25	136/42	0.90
IL-28B SNP(Maj/Min)	189/89	70/30	119/59	0.68
VR/Null response	203/65	84/16	129/49	0.042
RVR (+/-)	32/200	12/70	20/130	0.93
EVR (+/-)	116/118	46/36	70/82	0.18
SVR (+/-)	143/135	56/44	87/91	0.30

Data are expressed as the mean ± SD. *P* values are for comparisons between the persistent normal alanine aminotransferase (PNALT)- and abnormal alanine aminotransferase (ALT)-groups by Student's *t*-test or χ^2 test. AST: Aspartate aminotransferase; γ-GT: Gamma-glutamyl transpeptidase; WBC: White blood cell count; IL-28B: Interleukin 28B; SNP: single-nucleotide polymorphisms; Maj: Major genotype; Min: Minor genotype; VR: Virologic response; RVR: Rapid virologic response; EVR: Early virologic response; SVR: Sustained virologic response.

nation using TaqMan minor groove binding (MGB) probes as described previously[26]. Briefly, we used DNA Extract All Reagents Kit (Applied Biosystems Inc., Foster City, CA, United States) to prepare the DNA sample from fresh plasma. Probes for the TaqMan MGB assay were manufactured by Applied Biosystems. Thermal cycling was performed in an ABI Step One Real-Time PCR system (Applied Biosystems) according to the manufacturer's protocol. Activation of TaqMan GTXpress Master Mix (Applied Biosystems) and the initial denaturation cycle were at 95 ℃ for 20 s, followed by 40 cycles at 95 ℃ for 3 s and 60 ℃ for 20 s. We analyzed SNP rs8099917 TT as the major genotype and TG/GG as the minor genotype in the present study.

Definition of treatment response

SVR was defined as undetectable serum HCV RNA at 24 wk after the end of treatment. Patients who had undetectable HCV RNA within the initial 4 wk of treatment were considered to have had a rapid virologic response (RVR). Patients with undetectable HCV RNA within the initial 12 wk were considered to have had a complete early virologic response (cEVR) (described as EVR in this study).

Statistical analysis

The results are expressed as the mean ± SD. Student's *t*-test or the χ^2 test was used to determine statistical significance. Variables with $P < 0.05$ in univariate analyses were retained for multivariate logistic regression analysis. For all tests, two-sided *P*-values were calculated, and the results were considered statistically significant at $P < 0.05$. The statistical analysis was performed using the Excel Statistics program for Windows, version 7 (SSRI, Tokyo, Japan).

RESULTS

Patient characteristics

The baseline characteristics are shown in Table 1. Of the 278 total patients, 100 (36.0%) and 178 (64.0%) were in the PNALT and abnormal ALT groups, respectively. Female patients were dominant in the PNALT group ($P < 0.0001$), whereas male patients were dominant in the abnormal ALT group ($P < 0.0001$). The AST, ALT, and γ-GT levels in the PNALT group were lower compared with those in the abnormal ALT group ($P < 0.0001$) (Table 1). In the PNALT group, 15 patients had relapsed after treatment, and 10 patients were null responders. In the abnormal ALT group, 23 patients had relapsed after treatment, and 19 patients were null responders. Of the 278 patients, 215 (77.3%), 60 (21.5%), and 3 (1.0%) were classified into HCV genotypes 1, 2, and unknown, respectively. HCV genotype 1 patients in PNALT and abnormal ALT groups were 72 (72.0%) and 143 (80.3%), respectively. The proportions of *IL-28B* genotypes did not differ between the PNALT and abnormal ALT groups (Table 1).

Virologic response

Of the 278 total patients, 211 (75.8%) and 67 (24.1%) were treatment naïve and retreated, respectively. SVR was obtained in 143 (51.4%) of the 278 patients. Within treatment groups, SVR was achieved in 120 (56.8%) of 211 treatment-naive and 23 (34.3%) of 67 retreated patients, respectively. The age of SVR patients (53.1 ± 12.8 years) was lower than that of non-SVR patients (58.3 ± 8.9 years) ($P = 0.00011$).

We next compared the virologic responses (VR) between the PNALT and abnormal ALT groups, and the proportions of treatment-naïve and retreated patients did not differ between these 2 groups (Table 1 and Figure 1). Additionally, the proportion of each *IL-28B* SNP rs8099917 did not differ between the 2 groups. Of interest, significantly fewer null responders were included in the PNALT group than in the abnormal ALT group ($P = 0.042$). However, the proportions of patients with an RVR, EVR, or SVR did not differ between the 2 groups (Table 1).

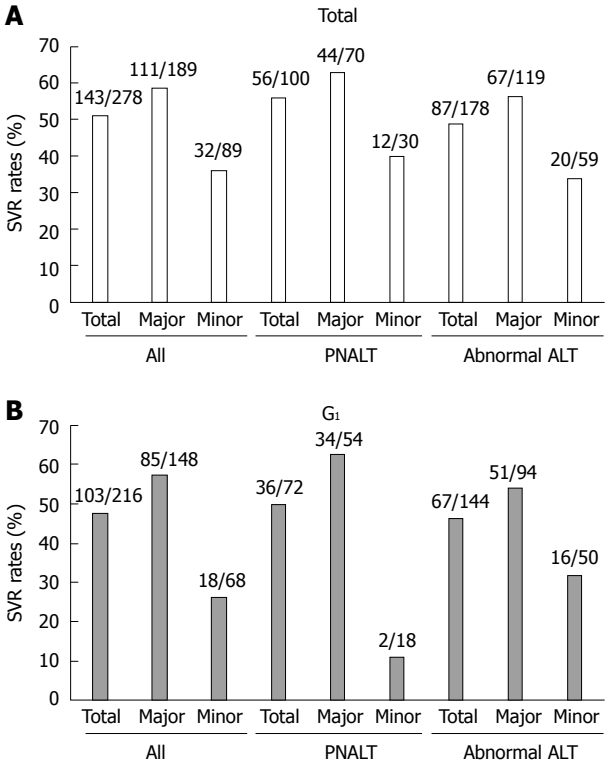

Figure 1 Sustained virologic response rates according to interleukin 28B single-nucleotide polymorphism rs8099917, and alanine aminotransferase levels. A: All hepatitis C virus (HCV)-infected patients; B: HCV genotype 1-infected patients. SVR: Sustained virologic response; PNALT: Persistent normal alanine aminotransferase; ALT: Alanine aminotransferase.

Patient characteristics and VR according to the IL-28B SNP

Among the 100 PNALT patients, 70 and 30 patients had the *IL-28B* rs8099917 major and minor genotypes, respectively (Table 2). In this PNALT group, patients with the *IL-28B* rs8099917 major genotype were older than those with the *IL-28B* rs8099917 minor genotype. The γ-GT levels in the *IL-28B* rs8099917 major group were lower compared with those in the *IL-28B* rs8099917 minor group. However, we observed lower hemoglobin levels in the *IL-28B* rs8099917 major group compared with those in the *IL-28B* rs8099917 minor group. In the PNALT group, other than RVRs, virologic responses were better in the *IL-28B* rs8099917 major group (Table 2).

Among the 178 abnormal ALT patients, 119 and 59 had the *IL-28B* rs8099917 major and minor genotypes, respectively (Table 2). The γ-GT levels in the *IL-28B* rs8099917 major group were lower than those in the *IL-28B* rs8099917 minor group. In the abnormal ALT group, other than RVRs, virologic responses were better in the IL-28B major group (Table 2).

HCV genotype 1-infected patients with PNALT and the IL-28B SNP

Among 72 PNALT patients infected with HCV genotype 1, 54 and 18 had the *IL-28B* rs8099917 major and minor genotypes, respectively (Table 3). Among the patients with the *IL-28B* rs8099917 major genotype, 8 had relapsed after treatment, and 4 were null responders. In patients with the *IL-28B* rs8099917 minor genotype, 1 had relapsed after treatment, and 5 were null responders. The ALT and γ-GT levels in the *IL-28B* rs8099917 major group were lower than those in the *IL-28B* rs8099917 minor group (Table 3). In HCV genotype 1-infected patients with PNALT, virologic responses were better in the IL-28B major group (Table 3). However, among the HCV genotype 2-infected patients with PNALT, virologic responses did not differ between the IL-28B major and minor groups although the number of HCV genotype 2-infected patients was smaller in the present study (data not shown). Among these patients, an SVR occurred in 71.4%, 62.5%, and 83.3% of all, IL-28B major, and IL-28B minor patients, respectively.

Predictors of SVR in HCV genotype 1-infected patients with PNALT

To clarify the predictors of SVR, we compared pretreatment and treatment factors between SVR and non-SVR-HCV genotype 1-infected patients with PNALT (Table 3). In HCV genotype 1-infected patients with PNALT, univariate analysis showed that AST, γ-GT, *IL-28B* SNP rs8099917, virologic response, and having an EVR contributed to the achievement of SVR. Factors significantly associated with SVR by univariate analysis were included in a multivariate logistic regression analysis. In HCV genotype 1-infected patients with PNALT, SVR was independently predicted by the *IL-28B* rs8099917 major genotype and having an EVR (Table 4).

DISCUSSION

The main finding of the present study evaluating *IL-28B* SNP rs8099917 was that this genotype may be a useful predictors of SVR following treatment with peginterferon-alpha plus ribavirin in HCV genotype 1-infected patients with PNALT. This finding is in line with previous reports indicating that *IL-28B* SNPs rs1297986 and rs8099917 could predict hepatitis C treatment-induced viral clearance[12-15]. Importantly, the present study results indicated that *IL-28B* SNP rs8099917 and EVR are useful surrogate markers of SVR even in HCV genotype 1-infected patients with PNALT.

Nunnari *et al*[7] reported that the frequency of *IL-28B* SNP rs12979860 did not differ between the hyper-ALT and PNALT groups. Furthermore, the natural history of HCV carriers with PNALT is most likely not always benign and could reflect a more severe evolution of liver disease[29]. Controversies exist regarding the appropriate treatment method for HCV-infected patients with PNALT[29]. The most recent guidelines recommended that HCV-infected PNALT-patients with moderate or severe fibrosis should be treated[8,10]. Tanaka *et al*[14] has shown that the *IL-28B* SNP rs8099917 TT genotype strongly correlates with treatment response in HCV genotype 1-infected Asian patients. It has also been reported that

Table 2 Baseline characteristics of hepatitis C virus-infected patients, according to interleukin 28B single-nucleotide polymorphism rs8099917

IL-28B rs8099917	PNALT group (n = 100)			Abnormal ALT group (n = 178)		
	Major	Minor	*P* value	Major	Minor	*P* value
Number of patients	70	30		119	59	
Age (yr)	57.7 ± 10.8	52.6 ± 10.5	0.031	55.3 ± 11.3	55.4 ± 12.3	0.95
Gender (male/female)	21/49	10/20	0.92	68/51	37/22	0.58
AST (IU/L)	29.3 ± 13.1	30.9 ± 9.7	0.58	69.4 ± 51.4	75.4 ± 44.1	0.47
ALT (IU/L)	26.8 ± 7.2	29.5 ± 7.0	0.086	92.1 ± 70.4	99.5 ± 67.8	0.5
γ-GT (IU/L)	21.0 ± 11.5	38.4 ± 33.8	0.00069	54.0 ± 41.3	85.1 ± 95.7	0.0056
WBC (/mm^3)	5130 ± 1440	4900 ± 1640	0.52	5280 ± 1,680	5230 ± 1070	0.84
Hemoglobin (g/dL)	13.3 ± 1.0	13.9 ± 1.5	0.02	15.3 ± 10.8	14.4 ± 1.1	0.52
Platelets (× 10^4/mm^3)	17.2 ± 5.7	18.8 ± 6.3	0.21	17.0 ± 5.3	16.4 ± 5.8	0.49
Previous treatment (-/+)	54/16	21/9	0.61	93/26	43/16	0.55
VR/Null response	63/7	21/9	0.027	98/21	31/23	0.000059
RVR (+/-)	9/50	3/20	0.92	17/84	3/46	0.12
EVR (+/-)	38/21	8/15	0.029	59/44	11/38	0.00011
SVR (+/-)	44/26	12/18	0.058	67/52	20/39	0.0079

Data are expressed as the mean ± SD. *P* values are for comparisons between the major genotype group and minor genotype group among the persistent normal alanine aminotransferase (PNALT) group or among abnormal alanine aminotransferase (ALT) group by Student's *t*-test or the χ^2 test. AST: Aspartate aminotransferase; γ-GT: Gamma-glutamyl transpeptidase; WBC: White blood cell count; IL-28B: Interleukin 28B; Maj: Major genotype; Min: Minor genotype; VR: Virologic response; RVR: Rapid virologic response; EVR: Early virologic response; SVR: Sustained virologic response.

Table 3 Hepatitis C virus genotype 1-infected patient with persistent normal alanine aminotransferase and interleukin 28B single-nucleotide polymorphism

IL-28B rs8099917	Major	Minor	*P* value	SVR	Non-SVR	*P* value
Number of patients	54	18		36	36	
Age (yr)	58.4 ± 10.9	54.6 ± 11.0	0.20	56.6 ± 13.4	58.4 ± 8.0	0.49
Gender (male/female)	17/37	7/11	0.77	14/22	10/26	0.45
AST (IU/L)	30.3 ± 14.5	34.5 ± 10.3	0.29	13.2 ± 1.1	32.0 ± 6.3	< 0.00010
ALT (IU/L)	27.5 ± 6.7	31.7 ± 5.6	0.019	27.3 ± 7.4	29.8 ± 5.7	0.11
γ -GT (IU/L)	21.7 ± 11.9	47.3 ± 39.3	0.00024	19.7 ± 10.6	39.2 ± 32.7	0.0011
WBC (/mm^3)	5020 ± 1410	4570 ± 1350	0.27	5000 ± 1350	4780 ± 1470	0.51
Hemoglobin (g/dL)	13.3 ± 0.9	13.7 ± 1.5	0.17	13.2 ± 1.1	13.5 ± 1.1	0.25
Platelets (× 10^4/mm^3)	16.6 ± 6.0	17.0 ± 5.6	0.80	17.1 ± 6.0	16.2 ± 5.8	0.51
Previous Treatment (-/+)	42/12	12/6	0.52	29/7	25/11	0.41
VR/Null response	46/8	9/9	0.0064	36/0	19/17	< 0.00010
RVR (+/-)	6/38	0/16	0.28	6/27	0/27	0.057
EVR (+/-)	25/19	1/15	0.0013	23/10	3/24	< 0.00010
SVR (+/-)	34/20	2/16	0.00040			
IL-28B SNP rs8099917 (Maj/Min)				34/2	20/16	0.00040

Data are expressed as the mean ± SD. *P* values are for comparisons between the major genotype group and minor genotype group or between sustained virologic response (SVR) and non-SVR groups by Student's *t*-test or the χ^2 test. AST: Aspartate aminotransferase; γ-GT: Gamma-glutamyl transpeptidase; WBC: White blood cell count; IL-28B: Interleukin 28B; Maj: Major genotype; Min: Minor genotype; VR: Virologic response; RVR: Rapid virologic response; EVR: Early virologic response; SVR: Sustained virologic response.

Table 4 Factors associated with sustained virologic response among hepatitis C virus genotype 1-infected patients with persistent normal alanine aminotransferase by multivariate analysis

Factor	Category	Odds ratio	95%CI	*P* value
IL-28B rs8099917	Major/Minor	7.11	1.305-38.799	0.023
EVR	(+/-)	13.28	3.242-54.399	0.0003

IL-28B: Interleukin 28B; Maj: Major genotype; Min: Minor genotype; EVR: Early virologic response.

linkage disequilibrium between the two *IL-28B* SNPs, rs8099917 and rs12979860, is strong in Japanese HCV patients[30]. In the present study, *IL-28B* SNP rs8099917 but not *IL-28B* SNP rs12979860 was evaluated.

Peginterferon-alpha plus ribavirin treatment led to an SVR rate of approximately 50% in patients infected with HCV genotype 1[4]. The efficacy and safety of peginterferon and ribavirin combination therapy in patients with HCV and PNALT are similar to those in patients with abnormal ALT[9]. Dual peginterferon/ribavirin therapy is no longer the standard therapy for chronic HCV infection. Combination therapy with telaprevir or boceprevir led to higher SVR rates in patients infected with HCV genotype 1[31]; however, severe adverse events are often observed with the use of these drugs[32,33]. In daily clinical practice, it must be decided

whether patients with HCV and PNALT should receive treatment based the balance between disease progression and treatment efficacy. Until new interferon-sparing regimens are introduced[31], our findings suggest that *IL-28B* SNP rs8099917 could be helpful in selecting patients with HCV and PNALT who should receive treatment.

Baseline plasma interferon-gamma inducible protein-10 (IP-10 or CXCL10) levels are strongly associated with *IL-28B* genotypes[18]. Honda *et al*[17] reported that hepatic interferon-stimulated genes (ISGs) are associated with *IL-28B* genotypes. We have also reported that concomitant assessment of lower-hepatic STAT1-nuclear translocation and *IL-28B* genotypes is useful for the prediction of SVR in HCV-infected patients[19]. Additionally, we have recently demonstrated that IL-28B induces ISGs that are reportedly associated with the progression of HCV-related pathogenesis and antiviral activities against HCV[34]. Further studies will be needed.

In a previous study, the *IL-28B* minor genotype was associated with lower inflammatory activity in the liver[22]. In contrast, the proportion of *IL-28B* genotypes did not differ between patients with PNALT and abnormal ALT in the present study. Further studies will be needed to clarify the association between *IL-28B* SNP rs8099917 and serum ALT levels[35]. In conclusion, *IL-28B* rs8099917 TT was associated with SVR in HCV genotype 1-infected Asian patients with PNALT. This finding sheds new light on the treatment options for HCV genotype 1-infected patients with PNALT.

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COMMENTS

Background

Although the progression of hepatic fibrosis appears to be slow in chronic hepatitis C patients with persistently normal alanine aminotransferase (PNALT), differing opinions about the natural history of hepatitis C virus (HCV) carriers with PNALT exist, suggesting that it is most likely not always benign and that a more severe evolution of liver disease can occur. It is difficult to determine whether chronic hepatitis C patients with PNALT should be treated. Interleukin 28B (*IL-28B*) genotypes have been reported to be predictive of the treatment response to peginterferon plus ribavirin in chronic hepatitis C patients.

Research frontiers

Whether there is an association between the *IL-28B* rs8099917 genotype and treatment response in HCV-infected Asian patients with PNALT is unknown. In this study, the authors demonstrated an association between the *IL-28B* rs8099917 genotype and treatment response in HCV-infected Asian patients with PNALT.

Innovations and breakthroughs

Recent reports have highlighted the importance of the *IL-28B* genotype in the treatment of HCV genotype 1-infected Asian patients with PNALT. This is the first study to report an association between the *IL-28B* rs8099917 genotype and treatment response in HCV-infected Asian patients with PNALT.

Applications

IL-28B rs8099917 appears to be useful for identifying chronic hepatitis C patients with PNALT who will benefit from treatment.

Terminology

IL-28B SNP rs8099917 is located approximately 8 kb upstream of IL-28B, which is in linkage disequilibrium with rs12979860 (located approximately 3 kb upstream of IL-28B). These SNPs are strongly associated with the natural and treatment-induced eradication of HCV.

Peer review

The authors examined the association between the *IL-28B* genotype and treatment response in HCV-infected patients with PNALT. Their study revealed that the proportion of *IL-28B* genotypes did not differ between patients with PNALT and patients with abnormal ALT. The authors also demonstrated an association between the *IL-28B* rs8099917 genotype and treatment response in HCV-infected Asian patients with PNALT. The results are interesting and the *IL-28B* genotype may be very helpful in the treatment of patients with chronic hepatitis C with PNALT.

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BRIEF ARTICLE

Homogeneous phenomenon of the graft when using different genotype characteristic of recipients/donors in living donor liver transplantation

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Abstract

AIM: To investigate the evidence of homogeneous phenomenon on CYP3A5*3 MDR1-3435 and CYP3A4*18 of the liver graft after living donor liver transplantation (LDLT).

METHODS: We identified the proportional change of the CYP3A5*3, MDR1-3435 and CYP3A4*18 from the peripheral blood mononuclear cell of 41 pairs recipient/donor with different genotype polymorphisms and 119 liver graft biopsy samples used with the pyrosequencing technique after LDLT. Polymerase chain reaction/ligase detection reaction assay and restriction fragment length polymorphism was employed for genotyping the CYP3A5*3 and CYP3A4*18 single nucleotide polymorphisms (SNPs). All of the recipients and donors expressed with the similar SNP genotype of CYP3A5*3, MDR1-3435 or CYP3A4*18 were excluded.

RESULTS: The final genetic polymorphisms of the liver graft biopsy samples of CYP3A5*3, MDR1-3435 and CYP3A4*18 was predominated depends on the donor with restriction fragment length polymorphism and seems to be less related to the recipient. The proportional changes of G to A alleles of the 119 samples of CYP3A5*3 (included A > A/G, A/G > A, A/G > G, G > A, G > A/G and A > G), C to T alleles of the 108 samples of MDR1-3435 (included C > C/T, C/T > C, C/T > T, T > C/T and T > C), and T to C alleles of 15 samples of CYP3A4*18 (included T/C > T and T > C/T) were significant different between the recipients and the liver graft biopsy samples ($P < 0.0001$) and less difference when compared with the donors in the pyrosequencing analysis after LDLT.

CONCLUSION: The CYP3A5*3, MDR1-3435 and CYP3A4*18 of the recipient could be modified by the donor so-called homogenous phenomenon when the recipient's blood drained into the liver graft.

Key words: Pyrosequencing; CYP3A5*3; MDR1-3435; CYP3A4*18; Liver biopsy; Living donor liver transplantation

Core tip: The most innovative concept is that pyrosequencing can deeply clarify the proportional change of the A and G alleles in CYP3A5*3, C and T alleles in MDR1-3435, and T and C alleles in CYP3A4*1when the different genotype of single nucleotide polymorphism after living donor liver transplantation. The biogenetic characteristic of the recipient could be modified by a do-

nor you want to change the genetic characteristic. For further confirmation, homogeneous phenomenon was the truly occurred in the cytochrome P450 system when the recipients and donors with different genotype of the single nucleotide polymorphism.

Chiu KW, Nakano T, Chen KD, Hsu LW, Lai CY, Chiu HC, Huang CY, Cheng YF, Goto S, Chen CL. Homogeneous phenomenon of the graft when using different genotype characteristic of recipients/donors in living donor liver transplantation. *World J Hepatol* 2013; 5(11): 642-648 Available from: URL: http://www.wjgnet.com/1948-5182/full/v5/i11/642.htm DOI: http://dx.doi.org/10.4254/wjh.v5.i11.642

INTRODUCTION

The cytochrome P450 is a very important system for the drugs metabolism in the liver. Therefore, the metabolic characteristic of this system is considerable affected when the recipient received a liver graft with different genotype of the drug metabolic isoenzymes after liver transplantation. In our previous study, it was very interesting that the genotypes of the CYP2C19 in the peripheral blood mononuclear cell does not change even the recipient received a liver graft with individually differences genotype of the CYP2C19 after living donor liver transplantation (LDLT)[1]. Nevertheless, the fast drug metabolic characteristic of the liver graft seems to be presenting a more frequency of the abnormal liver function after LDLT[2]. The different genotype of the drug metabolic isoenzyme represented with an uncertain phenomenon when the new liver graft continuously circulated with the original recipient peripheral blood[3]. Recently, we successfully identified the homogeneous phenomenon of the single nucleotide polymorphism (SNP) from the liver graft biopsy specimen using by the pyrosequencing method[4]. Herein, we would like to expose all of the polymorphisms of CYP3A5*3, MDR1-3435 and CYP3A4*18 because of the importance for the immunosuppressive agent metabolism in the liver transplantation setting.

MATERIALS AND METHODS

Based on the liver graft biopsy samples with the different genotype between recipient and donor, 119 liver graft biopsy specimens in 41 cases of CYP3A5*3, 108 liver graft biopsies in 41 cases of MDR1-3435 and 15 liver graft biopsies in 3 cases of 3A4*18 (38 recipients/donors had the similar polymorphism genotype for CYP3A4*18) were enrolled for pyrosequencing analysis prospectively. All of the recipients and donors expressed with the similar SNP genotype of CYP3A5*3, MDR1-3435 or CYP3A4*18 were excluded in this study. We performed 119 liver biopsies in 41 recipients because of the evidence of clinical investigation of abnormal liver function after LDLT as our previous reported[2,5]. They were performed once in 14 recipients, twice in 7, 3 times in 9, 4 times in 1, 5 times in 6, 6 times in 1, 7 times in 2, and 10 times in 1. Base of the haplotypes of the SNP from the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP), there were 10 cases in A > A/G, 3 cases in A/G > A, 12 cases in A/G > G, 2 cases in G > A, 13 cases in G > A/G and 1 case in A > G on the polymorphism genotypes of CYP3A5*3; 14 cases in C > C/T, 16 cases in C/T > C, 3 cases in C/T > T, 7cases in T > C/T and 1case in T > C on the polymorphism genotypes of MDR1-3435; and only 2 cases in T/C > T and 1 case in T > T/C on the polymorphism genotypes of CYP3A4*18 due to 92.7% (38/41) recipients/donors were the similar CYP3A4*18 genotype SNP.

Genomic DNA isolation

Genomic DNA was isolated from the 0.5 mL EDTA-treated whole blood and liver biopsy specimens using the QIAamp DNA mini kit (Qiagen) in accordance with the manufacture's instruction.

*Genotyping of CYP3A5*3, MDR1-3435 and CYP3A4*18*

PCR/ligase detection reaction assay (LDR) and RFLP was employed for genotyping the CYP3A5*3 and CYP3A4*18 SNPs. A PCR assay for the CYP3A5*3 was using forward primer (5'-CATGACTTAGTAGACAGAT GAC-3') and reverse primer (5'-GGTCCAAACAGGGAAGAAATA-3') was performed in a 25 μL of reaction volume; and for genotyping the CYP3A4*18, a PCR assay was using forward primer (5'-CACCCTGATGTCCAGCAGAAA CT-3') and reverse primer (5'-AATAGAAAGCAGATGAACCAGAGCC-3'). The Probe for the CYP3A5*3-A was using (5'-TTTTTTTTTTTTTTTTTTTT TGTGGTCCAAACAGGGAAGAGATAT-3') and for the CYP3A5*3-G was using (5'-TTTTTTTTTTTTTTTTTTTTTTTT-GTGGTCCAAACAGGGAAGAGATAC-3'). The Probe for the CYP3A4*18-G was using (5'-TTTTTTTTTTTTTTTT TTTTTTTT-TACCTCCTCCCTCCTTCTCCATGTAC-3') and for the CYP3A4*18-A (5'-TTTTTTTTTTTTTTTTTTTTTT-TACCTCCTCCCTCCTTCTCC ATGTAT-3'. The PCR conditions consisted of a denaturation step at 95 ℃ for 15 min, followed by 35 cycles of 94 ℃ for 30 s, 65 ℃ for 1 min, and 72 ℃ for 1 min, followed by a final extension step at 72 ℃ for 7 min. The specific amplified fragments were used in an LDR assay to identify the mutations associated with CYP3A5*3 and. CYP3A4*18. The LDR assay was performed as follows: 10 μL of the reaction mix contained 1 μL of 1 × ligase reaction buffer (New England Biolabs, United States), 1 μL of probes (12.5 pmol/μL each), 0.05 μL (2 U) of thermostable Taq DNA ligase (New England Biolabs), and 1 μL of PCR product. The ligation reaction was performed with a GeneAmp PCR System 9600 (Perkin Elmer, United States) as follows: 15 min at 95 ℃, followed by 35 cycles of 30 s at 94 ℃ and 2 min at 60 ℃. PCR-RFLP was

performed to genotype intron 3 (6986 A > G) variant alleles in the CYP 3A5*3 gene and exon 10 (878 T > C) variant alleles in the CYP3A4*18 gene, with slight modifications. PCR products were digested with Ssp I for CYP3A5*3 and with Hpa II for CYP3A4*18. The products were separated by agarose gel electrophoresis and analyzed by an ABI PRISM 377 DNA sequencer[6]. Genotyping was performed using an independent external contractor (Biowing Applied Biotechnology Co. Ltd., China). Genomic DNA was isolated from whole blood using the UltraPure™ Genomic DNA Isolation Kit (Shanghai SBS Genetech Technology Co., China). PCR-RFLP was performed to genotype exon 26 (C3435T) variant alleles in the *MDR1* gene, with slight modifications. A PCR assay was using forward primer (5'-TGCTGGTCCTGAAGTTGATCTGTGAAC-3') and reverse primer (5'-ACATTAGGCAGTGACTCGATGAAGGCA-3'). The PCR conditions consisted of a denaturation step at 95 ℃ for 5 min, followed by 30 cycles of denaturation at 94 ℃ for 30 s, annealing at 54 ℃ to 59 ℃ for 50 s, and elongation at 72 ℃ for 1 min, followed by a final extension at 72 ℃ for 10 min. PCR products were digested with Sau3A I (C3435T) and analyzed by electrophoretic separation on agarose gels, followed by direct visualization over an ultraviolet transilluminator after ethidium bromide staining[7,8].

*Pyrosequencing for CYP3A5*3, MDR1-3435 and CYP3A4*18 genotyping*

DNA amplification: One of the known primers of CYP3A5*3, MDR1-3435 and CYP3A4*18 was used for amplification of DNA for PCR analysis was biotinylated, respectively. Primers for pyrosequencing of CYP3A5*3, MDR1-3435 and CYP3A4*18 were designed with PyroMark Assay Design Software 2.0. In the PCR assay (PyroMark PCR Kit-Qiagen), we used a forward primer and a reverse primer that was biotinylated at the 5'- end of the CYP3A5*3, MDR1-3435 and CYP3A4*18 respectively. The assay was performed in a 25-μL reaction volume. The PCR conditions consisted of initial denaturation at 95 ℃ for 15 min, followed by 45 cycles of denaturation at 94 ℃ for 30 s, annealing at 60 ℃ for 30 s, extension at 72 ℃ for 30 s, and a final extension step at 72 ℃ for 10 min. The PCR products were separated on 2% agarose gels.

Pyrosequencing analysis: Biotinylated PCR products were immobilized on streptavidin-coated Sepharose beads (Streptavidin Sepharose High Performance, GE Healthcare). All of the streptavidin-coated Sepharose beads (2 μL per sample) were mixed with binding buffer (40 μL per sample) in a tube. High-purity water was then added to a total volume of 80 μL per well, including the PCR product (20 μL). This immobilization mix was incubated for 10 min at 25 ℃ with continuous mixing (1400 rpm) on a shaking device, and the sequencing primer was then diluted to 0.3 μmol/Lin annealing buffer. Next, 25 μL of the solution was transferred to each well of a PyroMark Q24 Plate. After immobilization, the liquid was removed by aspirating the beads with a Vacuum Prep Tool and the beads were treated for approximately 5 s with 75% ethanol, 5 s with denaturation buffer, and 5 s with washing buffer. The PyroMark Q24 Plate containing the samples was heated at 80 ℃ for 2 min using a PyroMark Q24 Plate Holder and a heating block. The plate was then removed from the plate holder and the samples were allowed to cool to room temperature (15-25 ℃) for at least 5 min, and the reagents, including enzyme and substrate mixtures, and nucleotides were added to the cartridge (PyroMark Q24, Qiagen)[4,9]. The samples were analyzed using a PyroMark Q24 system (Qiagen) according to standard protocols. The order of nucleotide dispensation was chosen based on suggestions provided by the PyroMark Assay Design Software 2.0 (Figure 1A).

Ethics statement

This research was conducted in accordance with the Declaration of Helsinki (2000) of World Medical Association and institutional standards and was granted ethical approval by the institute review board from Chang Gung Memorial hospital (No: 100-2953C). Written informed consent for participation in the study was obtained from participants or from a parent or guardian in case of minor participants. All of the participants had been provided and obtained the written informed consent to participate in this study and the ethics committees had approved all of the consent procedure.

Statistical analysis

Statistical analyses were performed using SPSS software (version 12.0; SPSS, Chicago, IL, United States). Comparisons of parameters of the haplotypes of CYP3A5*3, MDR1-3435 and CYP3A4*18 between the donors and recipients were performed using the Student's *t*-test with 2SD. *P* values less than 0.05 were considered statistically significant.

RESULTS

We performed 119 liver graft biopsies as part of clinical investigations after LDLT. For the SNP of CYP3A5*3, A allele was the wide type and G allele was mutant variant. The detail proportional change of A and G alleles of the CYP3A5*3 in the pyrosequencing between the recipients, donors and liver graft biopsy samples were showed on Table 1. The proportional change of A and G alleles of the CYP3A5*3 SNP in the pyrosequencing with RFLP was significant different between the liver graft biopsy sample and the peripheral blood mononuclear cells of the recipients in A > A/G ($P < 0.0001$), A/G > A ($P < 0.001$), A/G > G ($P < 0.0001$), G > A ($P < 0.0001$), G > A/G ($P < 0.0001$), but less difference to those of the donors (Table 1). For the SNP of MDR1-3435, C allele was the wide type and T was mutant variant. The detail proportional change of C and T alleles of the MDR1-3435 SNP in the pyrosequencing

Table 1 The proportional changes of G and A alleles of the CYP3A5*3 in the pyrosequencing with restriction fragment length polymorphism of peripheral blood mononuclear cells of the recipients and donors, and the liver graft biopsy samples after living donor liver transplantation

CYP3A5*3 intron3 6986A > G A (wide type) SspI[1]	PS	Recipient	Donor	Graft (liver biopsy)		*P* value	
						R *vs* G	D *vs* G
A > A/G, *n* = 10	A	86.23 ± 7.67	24.78 ± 3.90	*n* = 35	35.57 ± 7.52	< 0.0001	>0.05
	G	13.77 ± 7.67	75.23 ± 3.90		64.43 ± 7.52		
A/G > A, *n* = 3	A	21.97 ± 1.32	73.13 ± 19.2	*n* = 5	68.92 ± 11.75	< 0.001	>0.05
	G	78.03 ± 1.32	26.87 ± 19.2		31.08 ± 11.75		
A/G > G, *n* = 12	A	21.73 ± 5.81	6.44 ± 3.38	*n* = 33	9.95 ± 5.68	< 0.0001	>0.05
	G	78.27 ± 5.81	93.56 ± 3.38		90.05 ± 5.68		
G > A, *n* = 2	A	3.06 ± 0.27	94.08 ± 5.87	*n* = 10	47.29 ± 9.44	< 0.0001	< 0.0001
	G	96.94 ± 0.27	5.92 ± 5.87		52.71 ± 9.44		
G > A/G, *n* = 13	A	4.55 ± 1.88	22.97 ± 3.84	*n* = 31	18.75 ± 6.43	< 0.0001	= 0.033
	G	95.45 ± 1.88	77.03 ± 3.84		81.25 ± 6.43		
A > G, *n* = 1	A	84.58	2.26	*n* = 5	11.60 ± 5.26	-	-
	G	15.42	97.74		88.39 ± 5.26		
Total, *n* = 41				*n* = 119			

[1]Restriction enzyme for polymerase chain reaction-restriction fragment length polymorphism. D: Donor; G: Graft; PS: Pyrosequencing; R: Recipient.

Table 2 The proportional changes of C and T alleles of the MDR1-3435 in the pyrosequencing with restriction fragment length polymorphism of peripheral blood mononuclear cells of the recipients and donors, and the liver graft biopsy sample after living donor liver transplantation

MDR1-3435 Exon26 3435C>T C (wide type) Sau3AI[1]	PS	Recipient	Donor	Graft (liver biopsy)		*P* value	
						R *vs* G	D *vs* G
C > C/T, *n* = 14	C	93.10 ± 2.71	46.38 ± 1.94	*n* = 40	56.97 ± 5.17	< 0.0001	< 0.0001
	T	6.90 ± 2.71	53.62 ± 1.94		43.03 ± 5.17		
C/T > C, *n* = 16	C	46.36 ± 1.75	92.94 ± 2.15	*n* = 31	81.96 ± 5.42	< 0.0001	< 0.0001
	T	53.64 ± 1.75	7.06 ± 2.15		18.04 ± 5.42		
C/T > T, *n* = 3	C	46.35 ± 2.77	2.88 ± 1.49	*n* = 5	12.38 ± 6.46	< 0.001	> 0.05
	T	53.65 ± 2.77	97.12 ± 1.49		87.62 ± 6.46		
T > C/T, *n* = 7	C	2.33 ± 0.48	47.52 ± 1.00	*n* = 27	34.38 ± 6.21	< 0.0001	> 0.05
	T	97.67 ± 0.48	52.47 ± 1.00		65.62 ± 6.21		
T > C, *n* = 1	C	4.19	94.19	*n* = 5	63.93 ± 5.25	-	-
	T	95.81	5.81		36.07 ± 5.25		
Total, *n* = 41				*n* = 108			

[1]Restriction enzyme for polymerase chain reaction-restriction fragment length polymorphism. D: Donor; G: Graft; PS: Pyrosequencing; R: Recipient.

between the recipients, donors and liver graft biopsy samples were showed on Table 2. This phenomenon with proportional changes of C and T alleles in the MDR1-3435 was also significant different between the liver graft biopsy samples and the recipients in C > C/T ($P < 0.0001$), C/T > C ($P < 0.0001$), C/T > T ($P < 0.001$) and T > C/T ($P < 0.0001$), and less difference to those of the donors (Table 2). For the SNP of CYP3A4*18, T allele was the wide type and C allele was mutant variant. The detail proportional change of T and C alleles of the CYP3A5*3 SNP in the pyrosequencing between the recipients, donors and liver graft biopsy samples were showed on Table 3. Of the 41 pairs of recipient/donor, there were only 3 pairs (7.3%, 3/41) recipient/donor with different genotype and performed 15 liver graft biopsy samples for further analysis. There was also significant difference of the proportion changes of T and C alleles between the liver graft biopsy samples and the peripheral blood mononuclear cell of the recipient ($P < 0.0001$) but less difference to those of the peripheral blood mononuclear cell of the donor ($P = 0.033$) (Table 3).

DISCUSSION

Anti-rejection agents such as tacrolimus usually target CYP3A5, MDR-1 and CYP3A4, which are the major metabolic isoenzymes of cytochrome P450. In the present study, all of the alleles A and G in CYP3A5*3 SNP, alleles C and T in MDR1-3435 SNP and alleles T and C in CYP3A4*18 SNP of the liver graft biopsy samples were expressed significant proportional change when compared with the recipients after LDLT. The most innovative finding suggested that the characteristic activity

Table 3 The proportional changes of T and C alleles of the CYP3A4*18 in the pyrosequencing with restriction fragment length polymorphism of peripheral blood mononuclear cells of the recipients and donors, and the liver graft biopsy samples after living donor liver transplantation

CYP3A4*18 Exon10 878T>C T (wide type) Hpa II[1]	PS	Recipient	Donor	Graft (liver biopsy)		*P* value	
						R *vs* G	D *vs* G
T/C > T, *n* = 2	T	58.59 ± 3.08	99.02 ± 1.39	*n* = 12	91.86 ± 4.21	< 0.0001	0.033
	C	41.42 ± 3.08	0.99 ± 1.39		8.14 ± 4.21		
T > T/C, *n* = 1	T	100	55.92	*n* = 3	65.23 ± 3.31	-	-
	C	0	44.08		34.77 ± 3.31		
Total, *n* = 3				*n* = 15			

[1]Restriction enzyme for polymerase chain reaction-restriction fragment length polymorphism. D: Donor; G: Graft; PS: Pyrosequencing; R: Recipient.

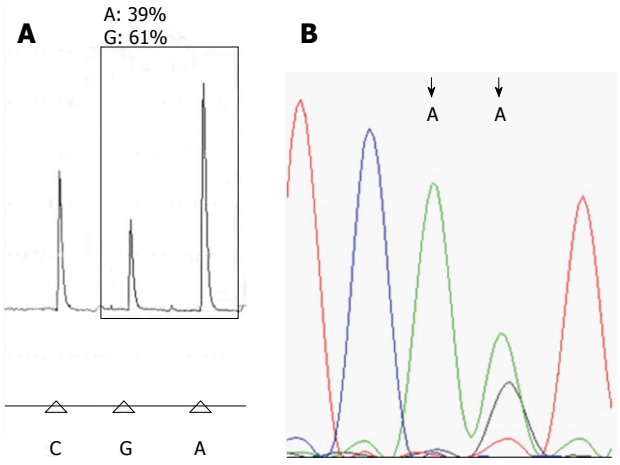

Figure 1 Pyrosequencing and traditional sequence. A: Pyrosequencing: proportional persentage presentation of the alleles; B: Traditional sequence: Single nucleotide polymorphism presentation with the differences wave form.

of the drug metabolism in the cytochrome P450 should be modified by the donor with individual characteristic. In our previous study showed that the CYP2C19 has 3 variant genotypes which have been classified as homozygous extensive metabolizers (HomEM), heterozygous extensive metabolizers (HetEM), and poor metabolizers in characteristic drug metabolism in cytochrome P450. But the donor graft does not affect *CYP2C19* genotype expressed on the peripheral blood in recipient with LDLT[1]. Interestingly, the liver graft still presented the original *CYP2C19* genotype characteristics of the donor when liver graft biopsy for a part of the clinical investigation at that moment. This clinical phenomenon could not be demonstrated used with Western blotting analysis[10] but it represented from the traditional sequencing so-called homogeneous phenomenon[3,4]. In the present study, pyrosequencing goes forward to identify the proportional change only requires a standard biotinylation of one of the PCR primers. It should be easier and more accurate to demonstration the SNP allele when compared with the traditional sequence (Figure 1). The bias of our study was the lack of microdisection of the hepatocyte[11-13] or isolation of the hepatocyte[14,15] from the graft, because we believed that the genetic characteristic of the graft hepatocyte is belongs to the donor originally. The peripheral blood of the recipient continuous goes through into the graft but it may not be contribute the genetic characteristic of the donor graft. During liver graft biopsy process, both of the graft hepatocyte and contaminated peripheral blood from the recipient would be mixed together. Hence, the liver graft biopsy sample is still presented with the original SNP genotype characteristic from the donor but the proportional change of the allele should be contributed with the recipient. It is so-called homogeneous phenomenon and expressed on the polymorphisms of CYP3A5*3, MDR1-3435 and CYP3A4*18. In the early stage within 1 mo after LDLT, this proportional change of the alleles was not stabilized. After a longitudinal followed up liver graft biopsy, the proportional change of the alleles was going to constant more than 1 mo after LDLT[4]. Followed up liver graft biopsy sampling more than 5 times per recipient was 24.4% (10/41) in CYP3A5*3, 17.1% (7/41) in MDR1-3435 and 66.7% (2/3) in CYP3A4*18. It was also suggested that the acute rejection is easily occurred within one month after liver transplantation[2,5] and the anti-rejection agent concentration in the serum was higher in one month later after LDLT[4].

As we know, the effect of MDR1-3435, CYP3A5*3, and CYP3A4*18 SNPs on cyclosporine A pharmacokinetics is very important[16-22]. In our study, the variant polymorphisms of CYP3A5*3 and MDR1-3435 had much differences between the recipients/donors which represented on the liver graft biopsy after LDLT. In contrast, the wide type and mutant variant was only 7.3% (3/41) differences in the polymorphisms of CYP3A4*18 needed liver graft biopsy after LDLT. This results suggested that the metabolic activity of the CYP3A4*18 was relative stabilized at the early stage after LDLT when compared with the CYP3A5*3 and MDRR1-3435, and corresponded that the *CYP3A4*18B* genotype affects cyclosporine A pharmacokinetics during the first month following surgery in Chinese renal transplant recipients. Patients with CYP3A4*18B alleles may require higher doses of cyclosporine A to reach the target levels[23-25].

In conclusion, pyrosequencing technique could be successful establishment of homogeneous phenomenon of the CYP3A5*3, MDR1-3435 and CYP3A4*18. The

genetic polymorphisms characteristic of these isoenzymes of the recipient could be modified by the donor representing a biogenetic change when the peripheral blood drained into the new liver graft.

COMMENTS

Background

Homogenous phenomenon of graft liver *CYP2C19* genotypes including homozygous extensive metabolizers, heterozygous extensive metabolizers, and poor metabolizers after living donor liver transplantation.

Research frontiers

It is very useful and promising information about immunosuppression acted major role in bringing of organ transplantation in these days.

Innovations and breakthroughs

This clinical study is important to deeply clarify the biogenetic characteristic of the cytochrome P450 system when the recipients and donors with different genotype of the single nucleotide polymorphism.

Applications

Pyrosequencing technique could be successful establishment of homogeneous phenomenon of the CYP3A5*3, MDR1-3435 and CYP3A4*18.

Terminology

Homogenous phenomenon: The graft liver from the donor mixed with the recipient circulating blood might be the result of present biogenetic phenomenon of genotype homogenous characteristics.

Peer review

Through pyrosequencing technology, the genotype polymorphisms of CYP3A5*3 MDR1-3435 and CYP3A4*18 were analysised from the peripheral blood mononuclear cell of 41 pairs recipient/donor with different genotype polymorphisms and 119 liver graft biopsy samples. The genetic polymorphisms characteristic of the CYP3A5*3, MDR1-3435 and CYP3A4*18 of the recipient could be modified by the donor representing a biogenetic change so-called homogenous phenomenon when the peripheral blood drained into the new liver graft. This study innovatively found that the liver graft could have the evidence to handle the cytochrome P450 drug metabolizing system in the recipient.

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CASE REPORT

Diagnostic challenges of Wilson's disease presenting as acute pancreatitis, cholangitis, and jaundice

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Abstract

Wilson's disease is a rare disorder of copper transport in hepatic cells, and may present as cholestatic liver disease; pancreatitis and cholangitis are rarely associated with Wilsons's disease. Moreover, cases of Wilson's disease presenting as pigmented gallstone pancreatitis have not been reported in the literature. In the present report, we describe a case of a 37-year-old man who was admitted with jaundice and abdominal pain. The patient was diagnosed with acute pancreatitis, cholangitis, and obstructive jaundice caused by pigmented gallstones that were detected during retrograde cholangiopancreatography. However, because of his long-term jaundice and the presence of pigmented gallstones, the patient underwent further evaluation for Wilson's disease, which was subsequently confirmed. This patient's unique presentation exemplifies the overlap in the clinical and laboratory parameters of Wilson's disease and cholestasis, and the difficulties associated with their differentiation. It suggests that Wilson's disease should be considered in patients with pancreatitis, cholangitis, and severe protracted jaundice caused by pigmented gallstones.

Key words: Wilson's disease; Pancreatitis; Cholangitis; Obstructive jaundice; Cholestasis

Core tip: A 37-year-old male patient was diagnosed with acute pancreatitis, cholangitis, and jaundice caused by pigmented gallstones. Due to long-term jaundice and an obscure clinical course, the patient was evaluated for Wilson's disease, which was confirmed using the Wilson's disease score. This patient's unique presentation exemplifies the overlap in the clinical and laboratory parameters of Wilson's disease and cholestasis, and the difficulties associated with their differentiation. This very rare case of acute pancreatitis, as the presenting feature of Wilson's disease, suggests that Wilson's disease should be considered in patients with pancreatitis, cholangitis, and severe protracted jaundice caused by pigmented gallstones.

Nussinson E, Shahbari A, Shibli F, Chervinsky E, Trougouboff P, Markel A. Diagnostic challenges of Wilson's disease presenting as acute pancreatitis, cholangitis, and jaundice. *World J Hepatol* 2013; 5(11): 649-653 Available from: URL: http://www.wjgnet.com/1948-5182/full/v5/i11/649.htm DOI: http://dx.doi.org/10.4254/wjh.v5.i11.649

INTRODUCTION

Wilson's disease is a rare, autosomal recessive disorder of copper transport in hepatic cells, with a reported incidence of 1: 30000[1,2]. The disease may present as a cholestatic liver disease[1,2] or, rarely, as hemolytic anemia[3-8].

Mild pancreatitis, upon presentation, has been described in only one case of Wilson's disease and was attributed to copper deposition in the pancreas[9]. Cholelithiasis, as a result of hemolysis and pigmented gallstone formation, has also been reported in Wilson's disease[10-19]. The atypical clinical presentation of cholangitis in patients with normal findings on bile duct imaging has also been reported[20]. However, pigmented gallstone pancreatitis and cholangitis, with concomitant obstructive jaundice, have not been reported as the presenting features of Wilson's disease.

The diagnosis of Wilson's disease in the setting of obstructive jaundice and cholestatic disease can be challenging because the laboratory and liver biopsy results overlap with those of other cholestatic conditions. In the present report, we describe the case of a patient who presented with acute pancreatitis (caused by pigmented gallstones), which was the first presenting feature of Wilson's disease.

CASE REPORT

A 37-year-old man was admitted to our hospital because of upper abdominal pain, jaundice, fever (39.6 ℃), and diarrhea. He reported no prior exposure to drugs, alcohol, or chemicals. His physical examination revealed bilateral scleral icterus and jaundiced skin as well as abdominal tenderness without hepatosplenomegaly or abdominal masses. Moreover, there were no signs of chronic liver disease, such as spider naevi, clubbing, or caput medusae.

The results of the patient's laboratory examination indicated a hemoglobin level of 14.5 g/dL (normal level: 14-18 g/dL), which decreased to 7.7 g/dL; a leukocyte count of 13.6-17.8 k/μL (normal level: 4.8-10.8 k/μL); a platelet count of 200 k/μL (normal level: 130-400 k/μL); a serum haptoglobin level of 128 mg/dL (normal level: 30-200 mg/dL); a reticulocyte count of 4% (normal level: 0.5-1.5%); reticulocyte production index 1.7 (normal index: 1); a serum amylase level of 2800 U/L (normal level: 28-100 U/L); a serum glutamic-oxaloacetic transaminase level of 196 U/L (normal level: 0-35 U/L); and a glutamate pyruvate transaminase level of 78-150 U/L (normal level: 0-45 U/L). His results also demonstrated a serum alkaline phosphatase level of 312-812 U/L (normal level: 30-120 U/L), a total serum bilirubin level of 15.3-23.6 mg/dL (normal level: 0.3-1.2 mg/dL), a direct bilirubin of 7.2-17.0 mg/dL (normal level: 0-0.3 mg/dL), a serum C-reactive protein level of 170 mg/L (normal level: 0-5 mg/L), an erythrocyte sedimentation rate of 96 mm/h (normal level: 0-15 mm/h), a serum albumin level of 2.36 g/dL (normal level: 3.5-5.2 g/dL), a serum uric acid level of 1.5 mg/dL (normal level: 3.5-7.2 mg/dL), and a serum lactate dehydrogenase level that was within normal limits. The patient's creatinine level at admission was 0.84 mg/dL (normal level: 0.67-1.17 mg/dL), and a blood film indicated anisocytosis and abnormal red blood cells. However, Heinz bodies were not observed.

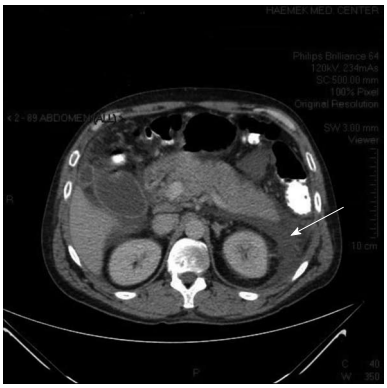

Figure 1 Abdominal computed tomography of the patient showing pancreatic edema and peripancreatic and pararenal fluid (arrow).

Ultrasonography showed a thickened gallbladder wall and several hyperechoic foci, without any acoustic shadows in the gallbladder; the common bile duct was dilated to a diameter of 13.5 mm. Computed tomography (CT) showed a swollen pancreas with peripancreatic and pararenal fluid (Figure 1), a swollen gallbladder, choledochal dilation, and a choledochal stone.

Negative results were noted on blood cultures, and the patient's D-dimer level was 5484 ng/mL (normal level: 0-500 ng/mL). Level of clotting factor Ⅶ was low, at 8% (normal level: 60%-120%) whereas factor V and Ⅷ level were normal, at 61% and 156%, respectively. Tests involving serum antibodies for hepatitis B and C viruses, cytomegalovirus, Epstein-Barr virus, *Coxiella burnetii*, *Brucella*, and *Rickettsia* yielded negative results. Tests for serum anti-smooth muscle and anti-mitochondrial antibodies also yielded negative results.

The patient was intravenously treated with fresh frozen plasma, vitamin K, and antibiotic therapy, consisting of intravenous metronidazole (500 mg, 3 times/d) and intravenous ciproxin (200 mg, 2 times/d). Endoscopic retrograde cholangiopancreatography was performed and numerous pigmented, black stones were removed (Figure 2). However, his bilirubin level remained elevated, and other abnormal liver function test findings were noted. Acute renal failure [creatinine value increased to 4.60 mg/dL (normal level: 0.67-1.17 mg/dL)] and hypotension developed; therefore, treatment with norepinephrine was initiated. Another CT scan indicated the presence of hypoechoic foci in the liver. Due to a fair response and the long-term cholangitis, the antibiotic therapy was temporarily changed to intravenous amikacin (1 g/d) and intravenous ertapenem (1 g/d), and a marked clinical improvement was noted. The presence of pigmented gallstones, anemia, low serum uric acid levels, and protracted jaundice led to the suspicion of Wilson's disease, even though hemolytic episodes had not been observed.

A slit lamp examination did not reveal Kayser–Fleischer rings. However, the patient's ceruloplasmin level was 17 mg/dL (normal level: 20-45 mg/dL), and

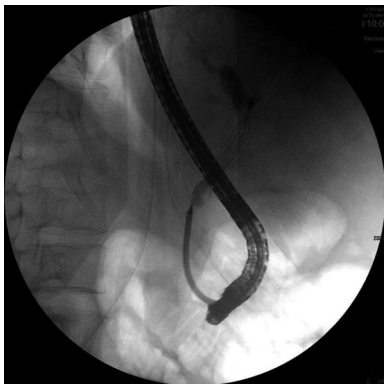

Figure 2 Endoscopic retrograde cholangiopancreatography of the patient showing removal of the stones by using a basket.

his 24-h copper excretion level was 305 μg (normal level: < 60 μg/24 h), which increased to 658 μg after trientine therapy was initiated. A liver biopsy showed chronic intrahepatic cholestasis, interface hepatitis, portal space lymphocytic plasma cells, and granulocyte infiltration (Figure 3); the hepatic copper content in the biopsy specimen was 304 μg/g (normal level: 10-35 μg/g) dry weight. In addition, a molecular genetic analysis of a blood sample revealed a V1140A homozygous mutation in the *ATP7B* gene. Considering these results, a diagnosis of Wilson's disease was established.

Treatment with the chelating agent trientine (250 mg, 3 times/d) was initiated and antibiotic therapy with oral ciprofloxacin (250 mg, 2 times/d) was continued. The patient's condition improved, and there were gradual improvements in the findings of the liver function tests, including normalization of the bilirubin and creatinine levels during the 6-mo follow-up period.

DISCUSSION

Wilson's disease is an inherited disease, caused by one of several possible mutations in the *ATP7B* gene, leading to defective copper excretion into the bile and subsequent accumulation of copper in the liver and central nervous system. The hepatic manifestations of Wilson's disease range from asymptomatic liver enzyme elevations to chronic, active hepatitis, cirrhosis, and acute fulminant hepatic failure[1,2]. Cholelithiasis has also been described in patients with Wilson's disease[10-19], including one study that found cholelithiasis among 25% of Wilson's disease patients[16]. Cholelithiasis is frequently observed in female patients with Wilson's disease, but its incidence is not high among male patients with the disease. Acute cholangitis with bile duct stones has not been described in Wilson's disease, although edema of the gallbladder, mimicking acute cholecystitis and clinical cholangitis, but with normal bile duct imaging findings, has been reported[17,20].

The diagnosis of Wilson's disease in the presence of cholestasis or long-term bile duct obstruction is difficult and challenging. Many characteristic laboratory findings in Wilson's disease overlap with those of other cholestatic

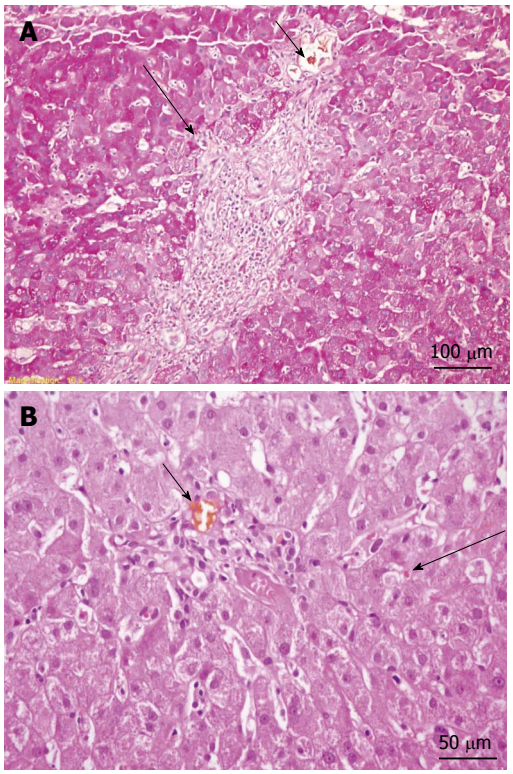

Figure 3 Liver biopsy. A: Periodic acid Schiff-stained section (original magnification, × 200) showing mild interface hepatitis (long arrow) and a bile plug in the portal space bile duct (short arrow); B: Hematoxylin and eosin stain (original magnification, × 400) showing a bile plug in the portal space bile duct (short arrow) and mild intrahepatic cholestasis (long arrow).

conditions. Cholestasis, due to conditions such as chronic active hepatitis, primary biliary cirrhosis, and primary sclerosing cholangitis, results in elevated 24-h urine copper levels. In such cases, the hepatic copper content is usually elevated, but the ceruloplasmin level may be either normal or elevated[21-25]. However, even in cases of severe cholestasis, the 24-h urine copper level does not usually exceed 200 μg/24 h[2]; in the present patient, the copper level was 300 μg/24 h. Liver biopsies in patients with Wilson's disease yield non-specific findings and may indicate chronic, active, hepatitis-like results. The Kayser-Fleischer ring may also be absent in 50% of patients with hepatic Wilson's disease.

The molecular analysis conducted on a blood sample from the present patient showed a homozygous V1140A peptide change (nucleotide change, c.3419 T > C) mutation in the *ATP7B* gene. Over 500 mutations of the *ATP7B* gene have been reported to cause Wilson's disease[2,26], some of which are very rare. These mutations lead to the production of defective copper transporter proteins, resulting in copper accumulation in the tissue. The V1140A mutation is a missense mutation, or sequence polymorphism in codon 3419 T > C of exon 16 on chromosome 13q, which was previously described in a Chinese, Czech-Slovakian, and Thai siblings and in a Yugoslavian cohort with Wilson's disease[27-33].

A single, specific test for diagnosing Wilson's disease does not exist. Therefore, the European Association for

the Study of the Liver practical guidelines established a Wilson's disease scoring system[21]. The diagnostic score is based on six available tests, including the presence of the Kayser-Fleischer ring, the presence of neurologic symptoms, ceruloplasmin values, the presence of hemolytic anemia, liver copper content in the absence of cholestasis, urinary copper levels, and a mutation analysis. A score of greater than or equal to four establishes a diagnosis of Wilson's disease[21,34]; the present patient had a score of six, clearly indicating a diagnosis of Wilson's disease. The low serum uric acid level found in our patient may be characteristic, although not diagnostic, of Wilson's disease and is caused by renal tubular defects[2]. The reversible coagulopathy with anemia observed in the present patient has also been previously described in a patient with Wilson's disease[20]. The pigmented gallstones associated with Wilson's disease are related to hemolytic episodes and might be a presenting feature of the disease[2-8]. In the present patient, the reticulocyte production index was higher than normal whereas the serum haptoglobin level, which is usually decreased in patients with hemolysis, was within normal limits. We hypothesize that this may be due to the presence of acute pancreatitis and cholangitis, considering the fact that haptoglobin is characterized as an acute phase reactant.

In conclusion, Wilson's disease should be considered in patients presenting with pancreatitis, cholangitis, and bile duct obstruction due to pigmented gallstones and who have protracted cholestasis and an obscure disease course.

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Volume with supplement

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Statistical data

Write as mean ± SD or mean ± 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 υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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