Primary liver cancer is one of the most common cancers in the world. Intrahepatic cholangiocarcinoma (ICC) is the second most frequent primary liver cancer after hepatocellular carcinoma (HCC), which accounts for more than 80% of these cases. Combined hepatocellular cholangiocarcinoma (CHC) is also occasionally found, but is relatively rare with a frequency of ~1% of the total primary liver cancers (Bhagat et al., 2006). Hepatocellular carcinoma is strongly associated with chronic viral hepatitis B and C and liver cirrhosis. In addition, other forms of chronic inflammation of the liver including alcoholic liver disease, hemochromatosis and congenital and acquired metabolic diseases also contribute to the development of HCC (El-Serag and Rudolph, 2007). On the other hand, the etiology of ICC remains unclear (Shaib et al., 2007; Welzel et al., 2007; Blechacz and Gores, 2008b). Though several risk factors, including hepatolithiasis, inflammatory bile duct disease (sclerosing cholangitis), liver fluke infection and anatomical abnormalities associated with biliary tract inflammation have been described, most occur in absence of known etiological factors (Ben-Menachem, 2007; Blechacz and Gores, 2008b).

The findings of CHC, particularly in patients of viral hepatitis B or C, indicate a common origin and/or shared pathogenic mechanisms for both HCC and ICC (Bhagat et al., 2006). Though several environmental carcinogenesis models of cholangiocarcinoma including infestation with liver flukes, especially the species Opisthorchis viverrini and Clonorchis sinensis, have been established in animals, neither the etiological correlation nor any preclinical models associated viral hepatitis with ICC are available (Blechacz and Gores, 2008a).

Herein, we present our retrospective case–control studies on the association of viral hepatitis B and C with ICC. At the time of HCC diagnosis, HCC patients with viral hepatitis B is ~10 years younger than those with viral hepatitis C, we re-evaluated the association of viral hepatitis-related ICC with viral hepatitis-HCC. We compared the incidence and age distribution of ICC to that of HCC in patients with chronic viral hepatitis B or C, thereby contributing to our understanding of the pathogenesis of ICC and indirectly to that of HCC.

**MATERIALS AND METHODS**

**Study subjects**

The institutional ethics committee approved all protocols applied in this study. We selected consecutive cases of diagnosed ICC among inpatients who were admitted between 1991 and 2005 to our hospital, the Chang Gung Memorial Hospital at the Lin-Ko
Liver cirrhosis

The criteria for the diagnosis of cirrhosis were as follows: clinical manifestations of chronic hepatitis with portal hypertension and/or hepatic decompensation, laboratory tests and hepatic ultrasonography. Characteristic signs included portal hypertension (varices, thrombocytopenia or splenomegaly) and/or signs of hepatic decompensation (jaundice, prolonged prothrombin time and ascites).

Laboratory tests

Blood samples were tested for the surface antigens of hepatitis B virus (HBsAg) and antibodies against hepatitis C virus (anti-HCV) using enzyme-linked immunosorbent assays (Abbott Laboratories, North Chicago, IL, USA).

Statistical methods

Independence tests were performed using unpaired t-test for continuous variables and χ²-square or Fisher’s exact test for categorical variables. We calculated the OR (odds ratio), RR (relative risk) and PPV (positive predictive value) of ICC.

We used univariate logistic regression to determine the variables that predisposed to ICC, and then employed multivariate logistic regression without intercept to select the most important variable.

For each factor in the different populations, we also used logistic regression to estimate the OR and 95% CI (confidence interval) of the population-attributable risk, which was calculated using the OR of that factor and its prevalence in the control group.

RESULTS

Patient characteristics

We enrolled 160 consecutive patients of ICC and 160 age-matched individuals as controls to search the risk factors for ICC (Table 1 and Figure 1).

Of the 160 ICC patients, 60 were seropositive for HBsAg (37.5%); 21 were seropositive for anti-HCV (13.1%), including seven cases of seropositivity for both HBsAg and anti-HCV; 41 had intrahepatic-duct stones (IHD stones) (25.6%); and 21 had cirrhosis (25.6%) (Figure 1A). In the control group, 22 were seropositive for HBsAg (13.8%); 10 were seropositive for anti-HCV (6.3%) and none had IHD stone or cirrhosis (Figure 1B). In addition, patients with gallstone were seen in both the ICC group (18 patients, 11.3%) and the control group (21 patients, 13.8%) (Table 1), whereas patients with IHD stones (41 patients, 25.6%), cirrhosis (41 patients, 25.6%), and biliary parasite (C. sinensis) (1 patients) were only found in the ICC group (Table 1).

Univariate analysis of the risk factors for ICC

As shown in Table 1, the sero-positive rates for HBsAg and anti-HCV in the ICC group were significantly higher than those in the control group (HBsAg: 37.5 vs 13.8%, P<0.001; and anti-HCV: 13.1 vs 6.3%, P=0.038). In addition, the prevalence of IHD stones and cirrhosis were also higher in ICC patients than in the controls (IHD stones: 25.6 vs 0%, P<0.001; and cirrhosis: 25.6 vs 0%).


P < 0.001). However, the difference in the prevalence of gallbladder stones or biliary parasite infection between the ICC and control groups was not statistically significant (gallbladder stones: 11.3% vs 13.8%, P = 0.499; and biliary parasite infection: 0.6% vs 0%, P = 0.317, respectively). It is worthy to mention that liver flukes, especially clonorchiasis and opisthorchiasis, which are prevalent in East and South Asia, have been proved to be the important causes of ICC. In our series, only one case of ICC had liver fluke infection. Low incidence of liver fluke infestation in our ICC patients could be explained by low prevalence of clonorchiasis in the past 13 years in Taiwan (<0.01% since 1995), though it was once very prevalent (about 1.5% in 1969; 0.2% in 1993 (Epidemiology Report, Division of Epidemiology, Department of Health, Taiwan, 1993; 9(5))).

To evaluate the contribution of each risk factor to ICC development, the ORs were analysed (Table 2). Patients with viral hepatitis B showed a 3.8-fold increase in the risk for ICC (95% CI: 2.17 – 6.53, P < 0.001), and patients with viral hepatitis C showed a 2.3-fold increase in the risk for ICC (95% CI: 1:03 – 4.98, P = 0.038). The PPV for ICC in the patients of viral hepatitis B and C were 18.13 × 10−5 and 10.92 × 10−5, respectively, which were also ~ three-fold higher than that in our general population (the prevalence of ICC in our population = 2.48 × 10−10). As there were no cases of cirrhosis or IHD stones in the control group, neither the OR nor the PPV for ICC among patients of viral hepatitis B and/or C. Univariate logistic regression analysis of the effects of cirrhosis on these patients with chronic viral hepatitis showed that cirrhosis increased the risks for ICC by 2.5-, 3.2- and 12.6-fold in patients who were seropositive for HBsAg and/or anti-HCV.

### Multiple logistic regression analysis

To determine the independent factor(s) in the development of ICC, multiple logistic regression analysis was conducted. As shown in Table 3, seropositivity for HBsAg, seropositivity for anti-HCV, and the presence of IHD stones were independent factors associated with ICC. Again, the OR’s for seropositivity for HBsAg and seropositivity for anti-HCV were 4.985 (95% CI: 2.775 – 8.945, P < 0.001) and 2.709 (95% CI: 1.162 – 6.318, P = 0.021), respectively.

| Risk factors | Additional factors | No. | P-value | Odds ratio | 95% CI |
|--------------|-------------------|-----|---------|------------|-------|
| HBsAg | With Cirrhosis | 38 | 0.015* | 2.468 | 1.196 – 5.095 |
| Without | 22 | | | |
| Anti-HCV | With Cirrhosis | 11 | 0.017* | 3.167 | 1.231 – 8.148 |
| Without | 10 | | | |
| HBsAg + Anti-HCV | With Cirrhosis | 3 | 0.002* | 12.625 | 2.533 – 62.923 |
| Without | 4 | | | |

### Synergistic effects of cirrhosis on the development of ICC in patients who were seropositive for HBsAg or anti-HCV

Cirrhosis is always associated with chronic inflammation either because of chronic B and/or chronic hepatitis C or because of bile duct inflammation, and ~90% of HCC cases were detected in cirrhosis patients. Therefore, it is interesting to examine whether cirrhosis played a synergistic role in ICC development in patients with viral hepatitis B and/or C. Univariate logistic regression analysis of the effects of cirrhosis on these patients with chronic viral hepatitis showed that cirrhosis increased the risks for ICC by 2.5-, 3.2- and 12.6-fold in patients who were seropositive for HBsAg or anti-HCV.
The incidence of HCC, ICC and combined HCC with ICC in our population

Sharing common disease profiles for both viral hepatitis-associated ICC and HCC including viral-hepatitis etiology, synergic effects of cirrhosis and age distributions suggests the involvement of similar long-term disease process, and even a common cell origin for carcinogenesis. It is, therefore, intriguing to speculate that the relative incidences for HCC and ICC and the CHC might be related to the ratio of hepatocytes and cholangiocytes, both of which were differentiated from the hepatic progenitor cells (oval cells). We then calculated the relative incidences for HCC, ICC and CHC in our population. We retrieved the annual incidence of HCC, ICC and CHC from our national cancer registration system (the Taiwan Cancer Registration System, 2004 – 2006) (http://crs.cph.ntu.edu.tw). The new diagnosed cases of HCC, ICC and CHC in our population during the period 2004 – 2006 were 26543 cases, 1949 cases and 114 cases, respectively. The case/number ratio of HCC:ICC:CHC was 233:17:1, which was consistent with the number ratio of hepatocytes to cholangiocytes in the liver.

**DISCUSSION**

Though association of viral hepatitis B and C with ICC has been documented (Wiwanitkit, 2005; Perumal et al, 2006; Shaib et al, 2007; Hsing et al, 2008; Zhou et al, 2008; El-Serag et al, 2009), the role of viral hepatitis B and C in the development of ICC in viral hepatitis endemic areas remains to be addressed. Herein, we not only show a strong etiological association of viral hepatitis B and C with ICC, but also provide circumstantial evidence to support the hypothesis of involvement of a common carcinogenesis process for both viral hepatitis-associated ICC and HCC. First, viral hepatitis B and C were independent risk factors and the major cause for ICC development. Our findings are consistent with that reported by El-Serag et al (2009) recently. Through a population based cohort study, El-Serag et al (2009) showed a strong association of HCC and ICC with HCV infection, but the association was not observed for extrahepatic cholangiocarcinoma. Second, as generally observed in HCC, cirrhosis—a pathological result of long-term chronic inflammation in the liver—exerted synergistic effects on ICC development in patients of viral hepatitis B and C. Third, both viral hepatitis-associated ICC and HCC had similar age profiles and difference in age distributions between patients with chronic hepatitis B and patients with chronic hepatitis C. Fourth, the ratio of disease incidences for HCC:ICC:CHC was consistent with the ratio of hepatocyte number to cholangiocyte number in the liver. These findings strongly indicated the involvement of similar long-term disease processes in the development of viral hepatitis-associated ICC and viral hepatitis-associated HCC.

It is known that chronic infection, for example, because of viral hepatitis B or C virus infection, plays a primary role not only in trans-activating cellular proto-oncogenes and/or disrupting tumour suppressor genes, but also in causing chronic inflammation. Sustained cell proliferation in a micro-environment rich in inflammatory cells, cytokines/chemokines, growth factors and DNA-damaging agents (such as reactive oxygen and nitrogen species because of long-term inflammation) will lead to permanent genetic alterations and subsequent neoplastic transformation of the proliferating cells (Komori et al, 2008). For example, interleukin-6, an inflammatory cytokine, promotes human cholangiocarcinoma cells grown in vivo by inhibiting apoptosis through the activation of miRNAs including miR let-7a and miR370, thereby modulating the activation of STAT-3 pathways (Meng et al, 2007, 2008; Smirnova et al, 2007). It has also been shown that the sustained upregulation of the transcription factor nuclear factor kappaB (NF-κB) in the liver cells, through the paracrine action of tumour necrosis factor-α secreted from the

**Table 5** Comparison of age at diagnosis amongst different groups of ICC patients

| Factors       | Case no. | Age: Mean ± s.d. | P-value |
|---------------|----------|------------------|---------|
| HBsAg         |          |                  |         |
| +             | 60       | 56.4 ± 11.1      | 0.000*  |
| –             | 100      | 64.6 ± 11.3      |         |
| Anti-HCV      |          |                  |         |
| +             | 21       | 65.6 ± 9.17      | 0.096   |
| –             | 139      | 60.9 ± 12.3      |         |
| IHD stone     |          |                  |         |
| +             | 59       | 64.9 ± 11.8      | 0.005*  |
| –             | 101      | 59.5 ± 11.7      |         |
| Cirrhosis     |          |                  |         |
| +             | 41       | 59.8 ± 10.8      | 0.306   |
| –             | 119      | 62.1 ± 12.3      |         |
| All cases     | 160      | 61.5 ± 12.0      |         |

Anti-HCV = anti-hepatitis C virus antibody; HBsAg = hepatitis B surface antigen. 
+/–: seropositivity/seronegativity. * P<0.05 was considered significant (two-sample t-test).
neighbouring endothelia and inflammatory cells, may lead to the tumour development (Maeda et al, 2005; Choi et al, 2006; Sakurai et al, 2006; Luedde et al, 2007), given the activation of mitogenic and anti-apoptotic genes through NF-κB pathways. In spite, the hypothesis of sharing common disease process for the carcinogenesis of viral associated ICC and HCC remains to be verified using cellular and animal models.

It has been known that men are about two–four times more likely to develop HCC than women (Bosch et al, 2004). Recent studies by using a chemical carcinogen (diethylnitrosamine) model of HCC in mice showed that the gender disparity in HCC was owing to suppression of interleukin-6 production in Kupffer cells by oestrogen (Naugler et al, 2007). Our studies showed a more profound degree of gender disparity in HCC (male/female = 2.91, the National Database, 2006) than in ICC (male/female = 1.31, based on the National Database, 2006). In addition, the gender disparity in both HCC and ICC was even more prominent in patients with chronic hepatitis B (male/female = 5.04 in HCC or 2.16 in ICC) than in patients with chronic hepatitis C (male/female = 2.35 in HCC or 1.29 in ICC), suggesting that additional viral etiology and environmental factors also contribute to the gender disparity in both HCC and ICC development.

What are the target cells of chronic inflammation that induce similar carcinogenic processes for ICC and HCC in chronic viral hepatitis? Considering that tumour formation is a multi-step process involving the accumulation of mutations over years or even decades before the occurrence of neoplastic transformation, the target cells should be able to proliferate as well as survive in the liver for a long period. Some hepatocytes and bile duct cells (cholangiocytes) can temporally proliferate, and act as the target cells for carcinogenesis. The targeting of the proliferating cholangiocytes in the liver could explain why chronic inflammation of bile ducts, such as that caused by sclerosing cholangitis, hepatic fluke infection or hepatic lithiasis, is only associated with cholangiocarcinoma but not HCC. However, these proliferating cholangiocytes might not be the primary target cells for HCC development in patients with viral hepatitis B and C, unless transdifferentiation from the cholangiocyte to the hepatocyte lineage occurs. Similarly, proliferating hepatocytes can be the target cells for HCC. However, the requirement of a transitional differentiation process from the hepatocyte to the cholangiocyte lineage makes it less likely that viral hepatitis-associated ICC arises from the proliferating hepatocytes. More likely is that viral hepatitis-associated HCC and ICC are derived from the same cells of origin that have the potential to develop into both the hepatocyte and cholangiocyte lineages, and have long been exposed to the milieu related to genome injury and consequently, tumour transformation (Alison, 2006; Libbrecht, 2006; Nomoto et al, 2006; Roskams, 2006; Zhang et al, 2008). Indeed, it is now generally believed that cancer can originate from stem cells or progenitor cells rather than from differentiated cells as these are the only cells that persist in the tissue for a sufficient length of time to acquire the requisite number of genetic changes for neoplastic transformation (Alison and Lovell, 2005).

The speculation that both HBV- and HCV-associated HCC and ICC are derived from liver progenitor cells (oval cells) is further supported by the occurrence of CHC, in which both the histological components of HCC and ICC are found simultaneously. Recently, it has been suggested that CHCs may arise from hepatic progenitor cells that retain their potential to differentiate into the hepatocytic and biliary lineages (Libbrecht, 2006; Zhang et al, 2008).

Hepatic progenitor cells (oval cells), residing in the canal of Herring in the liver, are multipotent progenitor cells that can give rise to both hepatocytes and cholangiocytes. They represent the replicating cellular compartment after liver injury. The degree of reactivation of hepatic progenitor cells has been associated with the degree of inflammatory activity in viral hepatitis and cirrhosis (Roskams et al, 2003; Roskams, 2006). Recently, cells with stemness properties have been identified in human HCC, further supporting the speculation that hepatic progenitor cells are involved in the carcinogenesis of HCC as well as ICC (Barajas et al, 2007; Ma et al, 2007; Yamashita et al, 2007; Zen et al, 2007; Qiao et al, 2008; Yang et al, 2008; Zhang et al, 2008).

The reason why the incidence of ICC in patients of viral hepatitis B or C is substantially lower than that of HCC if both arise from the same hepatic progenitor cells with a common pathogenic process remains unknown. Our explanation is that the incidence of HCC vs ICC is determined by the ratio of the number of the progenitor cells differentiating into the two lineages hepatocytes vs cholangiocytes. Indeed, hepatocytes represent approximately 80–90% of the liver cells, whereas bile duct cells account approximately 5–7% of the liver cells. If this is the case, the incidence of HCC:ICC:CHC should be approximately 225:15:1 (p:q:pq, where p and q are the incidences for HCC and ICC, or the relative numbers of hepatocytes and cholangiocytes in the liver, respectively). Consistent with this estimation, the incidence of HCC:ICC:CHC in our population was 233:17:1 (HCC:ICC:CHC = 26543 cases: 1949 cases: 114 cases (according to the Taiwan Cancer Registration System, 2003–2005) (http://crs.cph.ntu.edu.tw)). Further studies should be undertaken to determine the roles and mechanisms of hepatic progenitor cells in neoplastic transformation into HCC as well as ICC in patients of viral hepatitis B or C.

We conclude that besides other causes of bile duct inflammation, viral hepatitis B and C are the primary independent risk factors for ICC in our country, a viral hepatitis endemic area. Cirrhosis exerts synergistic effects on ICC development in patients of viral hepatitis B or C. Patients with viral hepatitis B developed ICC ~ 9 years earlier than patients with viral hepatitis C did. The incidence of ICC by age distribution in patients with either viral hepatitis B or C was nearly identical to that of HCC in patients with viral hepatitis B or C. The ratio of the incidence of HCC to ICC to CHC might reflect the ratio of the numbers of hepatocytes and cholangiocytes differentiated from the same hepatic progenitor cells. Our findings suggested that both viral hepatitis-associated ICC and HCC originate from the same hepatic progenitor cells through a similar long-term inflammatory carcinogenic process. Our findings also suggest that all risk factors causing chronic inflammation of the liver including chronic viral hepatitis, inflammatory bile duct disease, hepatic lithiasis and hepatic parasite infection could activate a common pathway for the development of liver malignancy. Moreover, suppression of inflammation may reduce cellular dysplasia, thereby preventing malignancy.

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