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The association between *Clonorchis sinensis* seropositivity and hepatocellular carcinoma in an endemic area: a study in Guangxi, China

Qing-Li Yang[1], Xi-Wei Lu[2], Zhong-Liao Fang[3], Yu-Qiu Gao[1], Yi-Ning He[1], Yan Huang[1], Yue Dai[1], Ming-Yong Liang[4], Carlos H. F. Chan[5*] and Zhi-Hua Jiang[3*]

Abstract

Background Chronic infection with *Clonorchis sinensis* (*C.sinensis*) has been associated swith the development of intrahepatic cholangiocarcinoma (ICC); however, the relationship between *C.sinensis* and hepatocellular carcinoma (HCC) remains uncertain.

Methods This study examined 120 patients with liver cancer in the clonorchiasis endemic area of Hengzhou, Guangxi, China. The type of cancer, the differentiation grade according to Edmondson Steiner's classification, and the pathological characteristics of HCC were determined through postoperative tissue biopsy. *C.sinensis* infection was detected by measuring serum specific IgG antibody, and hepatitis B virus (HBV) infection was determined by detecting serum HBsAg and HBV DNA in HCC tissues. The *C.sinensis* infection rates in control groups were drawn from the local general population based on previous surveys. The association between *C.sinensis* infection and HCC was analyzed by comparing the differences in *C.sinensis* infection rates between the two groups.

Results Of the patients evaluated, 98 (81.7%) had HCC, 21 (17.5%) had ICC, and 1 (0.8%) had comorbidity of HCC/ICC. Among the HCC patients, 24 (24.5%) were solely infected with HBV, 71 (72.4%) were *C. sinensis* seropositive, and 3 (3.1%) showed no evidence of infection. *C. sinensis* seropositive rates in HCC patients are much higher than in general outpatient and non-liver cancer inpatients ($\chi^2 = 141.92$, $p < 0.001$), as well as in the local residents ($\chi^2 = 82.61/21.38$, $p < 0.001$). There were no significant differences in the pathological type, differentiation grade, and lesion composition between the tumor associated with *C.sinensis*/HBV mono- and co-infection ($p > 0.05$). Among the patients with *C.sinensis*-related HCC, 8 (8.2%) were solely *C.sinensis* seropositive, while 63 (64.3%) were co-infected with HBV. Infection with *C. sinensis* and HBV has a significant impact on the pathological types of liver cancer ($\chi^2 = 22.86$, $p < 0.001$).

Conclusions These findings indicate that HCC still accounts for the majority of liver cancer in this region. In addition to being most commonly related with HBV infection, HCC may also be related to *C. sinensis* infection. Co-infection of *C. sinensis* and HBV may enhance the development of HCC in this area.

*Correspondence:
Carlos H. F. Chan
carloshfchan@gmail.com
Zhi-Hua Jiang
gxcdcjzh@163.com

Full list of author information is available at the end of the article

Clinical trial Not applicable.

Keywords *Clonorchis sinensis*, Hepatitis B virus, Hepatocellular carcinoma, Pathogeny

Background

The adult worms of *Clonorchis sinensis* (*C.sinensis*) reside within the intrahepatic bile ducts of humans and animals, leading to the development of clonorchiasis and intrahepatic cholangiocarcinoma (ICC) [1, 2]. Clonorchiasis is predominant in East Asia [3], with over 15 million individuals estimated to be infected globally [4]. Guangxi, China, is recognized as a major region affected by clonorchiasis [5, 6]. According to the 2015 surveillance of major human parasitic diseases, the weighted infection rate of *C.sinensis* in Guangxi was 6.7%, with approximately 2.92 million people affected, ranking it the highest in China [7]. Additionally, Guangxi is also known as a high prevalence area for hepatitis B. Hepatitis B virus (HBV) infection is the primary cause of hepatocellular carcinoma (HCC) in this region [8, 9]. In recent decades, there have been individual HCC cases have been reported to be infected with *C.sinensis*, which suggests that *C.sinensis* infection not only triggers ICC but may also be associated with the occurrence of HCC. Between 2014 and 2015, we identified a total of 10 cases of *C.sinensis*-related HCC in the clonorchiasis epidemic zone of Hengzhou City, Guangxi, including six cases of *C.sinensis* mono-infection and four cases of co-infection with HBV [10]. To further establish the association between *C.sinensis* infection and HCC, a larger group survey of liver cancer patients in Hengzhou, Guangxi was subsequently conducted.

Methods

Study design

The retrospective case-control study was conducted to investigate the association between *C.sinensis* infection and HCC. The study population consisted of liver cancer patients and control groups drawn from the local general population, which include general outpatient and non-cancer hospitalized patients, as well as local residents. Liver cancer patients were assessed for different types of infections, *C.sinensis*/HBV mono- or co-infections, while the local general population were evaluated solely for the prevalence of *C.sinensis* infections based on previous surveys [6, 11, 12].

Site of case investigation, consent to participate and ethics approval

The study included a population of 120 patients diagnosed with liver cancer who underwent surgical treatment at People's Hospital of Hengzhou (PHH) between August 2020 and May 2023. All cases were confirmed to originate from the local population. The investigation and specimen collection procedures were reviewed and approved by the Guangxi Ethics Review Committee (approval No. GXIRB 2020-0022), and all the participants provided written informed consent prior to their participation.

Serological testing

Prior to the surgery, blood samples were obtained from each patient and the serum was separated and stored at -80 °C. The qualitative detection of the IgG antibody against *C.sinensis* and hepatitis B virus surface antigen (HBsAg) in serum samples was conducted using the enzyme-linked immunosorbent assay (ELISA) method. A liver fluke IgG antibody detection kit (Shenzhen Huakang Biomedical Engineering Co., Ltd., Shenzhen; National Medical Device Approval No. 20173401117) was used to detect the *C.sinensis* IgG antibody, while a diagnostic kit for HBsAg (Shanghai Rongsheng Biopharmaceutical Co., Ltd., Shanghai; National Medical Products Administration Approval No. S10950045) was used to detect HBsAg. For the detection of *C.sinensis* IgG antibody, the following steps were performed: The serum was diluted 1:10 with dilution buffer and added to a microplate coated with *C.sinensis* antigen. The mixture was incubated at 37 °C for 1 h, followed by washing the plate with washing buffer 5 times for 1 min each. After drying, 50 µL of enzyme conjugate was added and incubated at 37 °C for 30 min. The plate was washed again 5 times for 1 min each. The double antibody sandwich method was used for the detection of HBsAg. 50 µL of the serum to be tested was added to a microplate coated with anti-HBs and incubated at 37 °C for 1 h. Then, 50 µL of enzyme conjugate was added and incubated at 37 °C for 30 min. The plate was washed 5 times with washing buffer for 10 s each. Finally, the specific substrates were added for color development and the reaction was terminated. All experiments included blank, positive, and negative controls. All OD_{450} values were measured using the Bio Tek Epoch microplate reader. The results were determined based on the cut-off value (CO), a $CO \geq 0.07 +$ negative control OD_{450} mean (or 0.05) is regarded positive for *C. sinensis* IgG, while a $CO \geq 2.1 \times$ negative control OD_{450} mean (or 0.05) is considered positive for HBsAg.

Detection of HBV DNA

Liver cancer tissues were collected during the operation and stored at -80°C. The cancer tissue samples weighing between 30 and 50 mg were homogenized in a heating block at 56°C for 6 hours. During this process, intermittent grinding was performed using 1 mL of lysis buffer containing 100 mM Tris, 50 mM EDTA, 2% SDS, and

500 g/ml proteinase K at a pH of 8.0. Subsequently, Tris-saturated phenol and chloroform-isopentanol at a ratio of 24:1 (v/v) were used to extract and purify the entire DNA content. The DNA was then precipitated using ethanol and subsequently dissolved in 200 μL of 8 mM NaOH solution (pH 8.0). The DNA concentration was measured using spectrophotometry at a wavelength of 260 nm using the NanoDrop One device (Thermo Scientific). For the quantification of HBV DNA, primers based on the HBV *PreC/C* gene were designed using Primer-BLAST (available at nih.gov). The primer sequences were as follows: forward 5'- ACTTTTTCACCTCTGCCTAA-3' and reverse 5'- AGCTCCAAATTCTTTATA-3'. These primers were synthesized by Sangon Biotech Co., Ltd. in Shanghai, China. Real-time quantitative PCR (qPCR) was used to detect HBV DNA with the TB Green® Premix Ex Taq qPCR Mix. Specifically, 20 μL of the TB Green® Premix Ex Taq qPCR Mix (2 ×), 0.8 μL of each primer (10 μM), and 1 μL of the DNA template were combined with MilliQ water to achieve a final volume of 40 μL. The amplification and data acquisition steps were carried out using an Analytik Jena qTOWER^3/G touch Real-Time PCR system with V4.0 software. The cycling parameters included a pre-denaturation step at 95 °C for 30 s, followed by 50 cycles of amplification at 95 °C for 5 s, and annealing and extension at 60 °C for 30 s. Subsequently, a melt curve analysis ranging from 65 to 95 °C was performed to determine the melting temperature (Tm) for the specific DNA product populations that were identified. The HBV DNA copy number was standardized using 100 ng of tissue DNA.

Postoperative tissue biopsy

The postoperative liver tissue specimens were examined by pathology at the local hospital, PHH, and our laboratory, the Guangxi Key Laboratory of Translational Medicine for Treating High-Incidence Infectious Diseases with Integrative Medicine (LHIID), respectively. Additional tissue samples were fixed with a 4% paraformaldehyde solution and stored at room temperature. The fixed specimens underwent paraffin embedding, sectioning, and staining with hematoxylin and eosin (H&E). The Zeiss Apotome.2 imaging system was used to observe the pathological types of liver cancer (ICC/HCC). Additionally, the differentiation of HCC was assessed using the grading system I ~ IV based on Edmondson Steiner's criteria [13, 14]. Furthermore, the presence of inflammatory cell infiltration, fibrosis, and necrosis in the tissue sections was documented.

Table 1 Pathological examination of liver in patients with liver cancer

Institution	HCC (%)	ICC (%)	HCC + ICC (%)	Total (%)
PHH	94 (81.7)	21 (18.3)	0	115 (100)
LHIID	91 (84.3)	16 (14.8)	1 (0.9)	108 (100)

PHH People's Hospital of Hengzhou, *LHIID* Guangxi Key Laboratory of Translational Medicine for Treating High-Incidence Infectious Diseases with Integrative Medicine, *HCC* hepatocellular carcinoma, *ICC* intrahepatic cholangiocarcinoma

Statistical analysis

Descriptive statistics of frequencies and rate/ratio (%) were used for categorical variables. Pearson's chi-square (χ^2) test was used to compare and test significance in observed differences. These included comparing the *C.sinensis* seropositive rates between different populations, assessing the impact and association of *C.sinensis* and HBV infection on different types of liver cancer, and comparing the pathological type, differentiation degree, and ratio of pathological constituents in HCC following *C.sinensis*, HBV mono-infection, and HBV co-infection, respectively. The statistical analysis was conducted using IBM SPSS Statistics 19.0 software, and a $p < 0.05$ was considered statistically significant.

Results

The state and relationship between HBV and *C. sinensis* infection in patients with liver cancer

A comprehensive investigation was conducted of a study participants of 120 patients suffering from liver cancer, ranging in age from 33 to 76 years. Of these patients, 104 were male and 16 were female. The postoperative liver tissue specimens of 115 cases were examined by pathology at a local hospital, PHH, while 108 cases were examined in our laboratory, LHIID. Among the cases, 103 underwent simultaneous pathological examination by both institutions, resulting in a high accordance rate of 98.1% (101/103). Additionally, 17 cases were examined exclusively by one institution. In cases where there were inconsistent pathological results, preference was given to the results obtained from our laboratory (Table 1).

In terms of the specific diagnoses, of all the patients with liver cancer, 98 cases were diagnosed as HCC, 21 cases as ICC, and 1 case as a comorbidity of HCC/ICC. These diagnoses accounted for 81.7%, 17.5%, and 0.8% of the cases, respectively. Serum specific IgG antibodies were detected in order to determine past or current *Clonorchis sinensis* infection in patients with liver cancer [15]. Among the HCC patients, 71 cases (72.5%) tested positive for the *C.sinensis* IgG antibody. Of these, 60 cases were positive for both HBsAg and HBV DNA, with a viral load ranging from 6 to 23,270,015 copies per 100 ng of DNA. Three cases were positive for HBV DNA but not for HBsAg, with a viral load ranging from 1 to 124 copies per 100 ng of DNA. On the other hand, 11 cases were negative for both HBsAg and HBV DNA. In this investigation, HBV infection was defined as the presence of HBsAg, HBV DNA, or both. Notably, HCC tissues and serum from 8 patients with *C.sinensis* seropositive did

not yield any detectable HBV DNA (Table 2). The infection rate of *C. sinensis* in HCC patients is significantly higher than that in general outpatients and non-liver cancer inpatients (22.1% [3172/14363]) [11], as well as in local residents surveyed twice at different years (28.8% [503/1748] [12] and 48.6% [1109/2282] [6]) (Table 3). All patients diagnosed with ICC tested positive for *C.sinensis* IgG antibody.

There is a certain degree of association between the types of *C.sinensis*/HBV infection and the different types of liver cancer occurrence ($\chi^2 = 22.86$, $p = 0.000$). The occurrence of ICC is solely associated with *C.sinensis* infection, whereas the occurrence of HCC is not only commonly associated with HBV, but also with *C.sinensis* infection. HBV mono-infection is only associated with HCC, while *C.sinensis* mono-infection and co-infection with HBV are both associated with ICC and HCC. *C.sinensis* mono-infection may have a minor relationship with HCC, however co-infection with HBV may increase the association.

Table 2 Liver cancer patients infected with *C. sinensis* and HBV*

Infection	HCC (%)	ICC (%)	HCC+ICC (%)	Total (%)
C.sinensis	8 (8.1)	10 (47.6)	0	18 (15.0)
HBV	24 (24.5)	0	0	24 (20.0)
C.sinensis + HBV	63 (64.3)	11 (52.4)	1 (100)	75 (62.5)
UN	3 (3.1)	0	0	3 (2.5)
Total (%)	98 (100)	21 (100)	1 (100)	120 (100)

* Patients with HBV were detected by the presence of serum HBsAg and/or HBV DNA in hepatic tissues. *HBV* hepatitis B virus, *HCC* hepatocellular carcinoma, *ICC* intrahepatic cholangiocarcinoma, *UN* un-infection

Table 3 HCC risk of patients infected with *C. sinensis*

Compare with	C. sinensis infection (%)	OR	95% CI	χ^2	*p*
Control group 1[a]	22.1	9.28	5.95~14.48	141.92	0
Control group 2[b]	28.8	6.51	4.13~10.26	82.61	0
Control group 3[c]	48.6	2.78	1.77~4.37	21.38	0

a, b, c: Refer to Chen YX, 2011 [11], Yang YC, et al., 2007 [12], and Xin H, et al., 2016 [6], respectively. *HCC* hepatocellular carcinoma, *OR* odds ratio, *CI* confidence interval

Histopathological analysis of HCC associated with *C.sinensis* and HBV infection

A total of 88 cases of HCC associated with *C.sinensis* and HBV infection were subjected to pathological analysis in our laboratory. Pathological examination revealed that the morphology of the cancer cells in 54 patients resembled normal hepatocytes, exhibiting solid growth, adenoid growth, trabecular growth, and fiberboard growth patterns [16]. These differentiation patterns were classified as hepatocellular carcinoma (HCC) and accounted for 61.4% of the cases. In 28 patients, the cytoplasm of their cancer cells exhibited clear cell hepatocellular carcinoma (CC-HCC) characterized by massive storage of α-glycogen particles [17, 18]; these accounted for 31.8% of the cases. Two cases (2.3%) presented as steatohepatic hepatocellular carcinoma (SH-HCC) with multiple lipid droplets in the cytoplasm [16, 19, 20]. The remaining 4 cases (4.5%) were spindle cell hepatocellular carcinoma (Sp-HCC) consisting mainly of spindle-shaped cells [21]. According to Edmondson Steiner's grading, differentiation grades I ~ IV were observed in 5 (5.7%), 37 (42.0%), 18 (20.5%), and 28 cases (31.8%), respectively. The proportions of inflammatory cell infiltration, fibrosis, and necrosis in HCC tissue sections were 36.8%, 37.9%, and 25.3%, respectively (Fig. 1).

In the case of *C.sinensis* mono-infection, 8 cases of HCC were identified through pathological examination. Among these cases, 4 displayed HCC with trabecular growth, solid growth, and fiberboard growth patterns, 3 were classified as CC-HCC, and 1 was identified as SH-HCC (Fig. 2). The pathological types of HCC with co-infection with *C.sinensis* and HBV were found to be diverse, including the majority of HCC, CC-HCC, and a few cases of SH-HCC and Sp-HCC (Fig. 3). Statistical analysis revealed no significant difference in the proportion of pathological types between *C.sinensis* mono-infection, HBV mono-infection, and co-infection related HCC ($\chi^2 = 5.15$, $p = 0.525$). Similarly, there was no significant difference in the distribution of Edmondson Steiner I ~ IV differentiation among these groups ($\chi^2 = 1.19$, $p = 0.977$). Furthermore, no significant difference was observed in the frequency of inflammatory cell infiltration, fibrosis, and necrosis in HCC tissue sections ($\chi^2 = 0.20$, $p = 0.996$).

Discussion

The primary focus of this study is to examine the association between *C.sinensis* infection and HCC. In a study conducted by Strauss sixty years ago, it was observed that, of five cases of HCC found in patients infected with *C.sinensis*, four were accompanied by Laennec's cirrhosis. However, it should be noted that HBV infection was not excluded in these cases [22]. Nakashima et al. reported two cases of elderly patients with HCC after *C.sinensis* infection forty-five years ago. One patient had a mild infestation with hepatic changes indicative of posthepatitic cirrhosis, while the other had a heavy infestation that exhibited secondary biliary cirrhosis with dilated intrahepatic bile ducts and periductal fibrosis. In both cases, the tumor nodule was solitary, and the cells were differentiated and classified as Grade I of Edmondson-Steiner's scale of anaplasia. HBsAg was determined to be negative in both cases through radioimmunoassay [23]. These early studies suggest a potential relationship between *C.sinensis* infection and the development of HCC. In a more recent investigation conducted by Tan et al. fifteen years ago, it was found that the infection rate

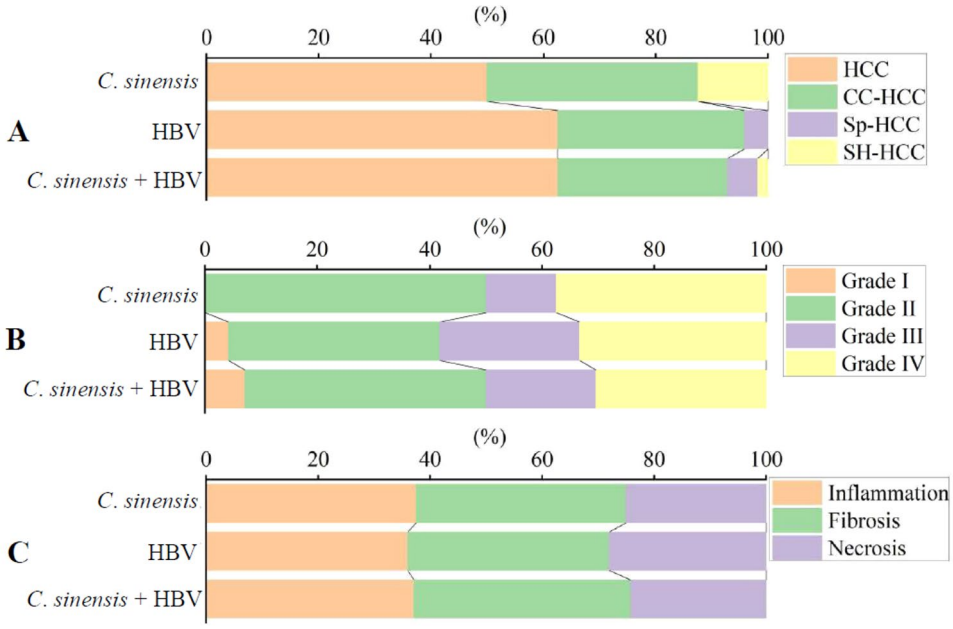

Fig. 1 Analysis of the pathological composition of *C.sinensis* and HBV infection related HCC **A**, Proportion of pathological types (%); **B**, Proportion of Edmondson-Steiner's grade I ~ IV differentiation (%); **C**, Frequency of inflammatory cell infiltration, fibrosis and necrosis in HCC tissue sections (%). *HBV* hepatitis B virus, *HCC* hepatocellular carcinoma

of *C.sinensis* in HCC patients at the First Affiliated Hospital of Guangxi Medical University and Cancer Hospital Affiliated to Guangxi Medical University was 16.4%, while the infection rate in non-HCC patients was only 2.4%. HBsAg was examined using ELISA to determine HBV infection. Based on these findings, researchers suggest that clonorchiasis may be an important risk factor for HCC [24].

In the past decade, we have conducted an ongoing investigation into liver cancer patients in the region of Hengzhou, Guangxi, which is known for its high prevalence of clonorchiasis. The purpose of this study was to clarify the relationship between *C. sinensis* infection and HCC in this particular demographic. We identified six cases of *C.sinensis* infection in a group of 20 liver cancer patients. To rule out the possibility of HBV infection, we conducted tests for HBsAg, which yielded negative results [10]. Over the course of the past three years, we have expanded our research to include a larger sample size of liver cancer patients in Hengzhou City. Our findings revealed eight cases of HCC that were seropositive for *C.sinensis*. Among the liver cancer patients surveyed, we observed that HCC was the predominant type of malignancy, with ICC being less common and is only associated with *C.sinensis* seropositivity.

Previous surveillance revealed that the infection rates of *C.sinensis* in the local residents and outpatient/non-liver cancer inpatients are 28.8% [15] (or 48.6% [6]) and 22.1% [14], respectively. In this investigation, we discovered that 72.5% of HCC patients were infected with *C. sinensis*, which was much higher than the infection rate in the general population in this location. The findings of this survey in a high-clonorchiasis-endemic area revealed a specific connection between *C. sinensis* seropositivity and HCC. Guangxi is also a hepatitis B epidemic area. HBV infection is the leading cause of HCC in this region [8, 9]. In this survey, we determined HBV infection by testing for serum HBsAg and HBV DNA in liver cancer tissues [25]. IgG antibody in identifying *C. sinensis* infection is much valuable [26]. To determine *C. sinensis* infection, we employed a highly specific IgG antibody detection kit that has been nationally approved and confirmed to be consistent with the results of fecal egg testing in our laboratory [27]. The study discovered that the majority of patients with HCC in this location were infected with both HBV and *C. sinensis*, complicating the understanding of the causes and risks associated with HBV-induced HCC [28]. Co-infection with HBV and *C. sinensis*, as well as the resulting HCC, are unavoidable difficulties in this area. Co-infection with HBV and *C. sinensis* increase the risk of HCC development, and it is an inescapable public health hazard in this region.

This suggests that *C.sinensis* infection on its own may directly induce HCC, while co-infection with HBV may contribute to the higher incidence of HCC. There were no significant differences observed in the pathological type, differentiation degree, or detection frequency of inflammatory cell infiltration, fibrosis, and necrosis in HCC cases with *C.sinensis* infection alone or in co-infection with HBV, when compared to cases with HBV

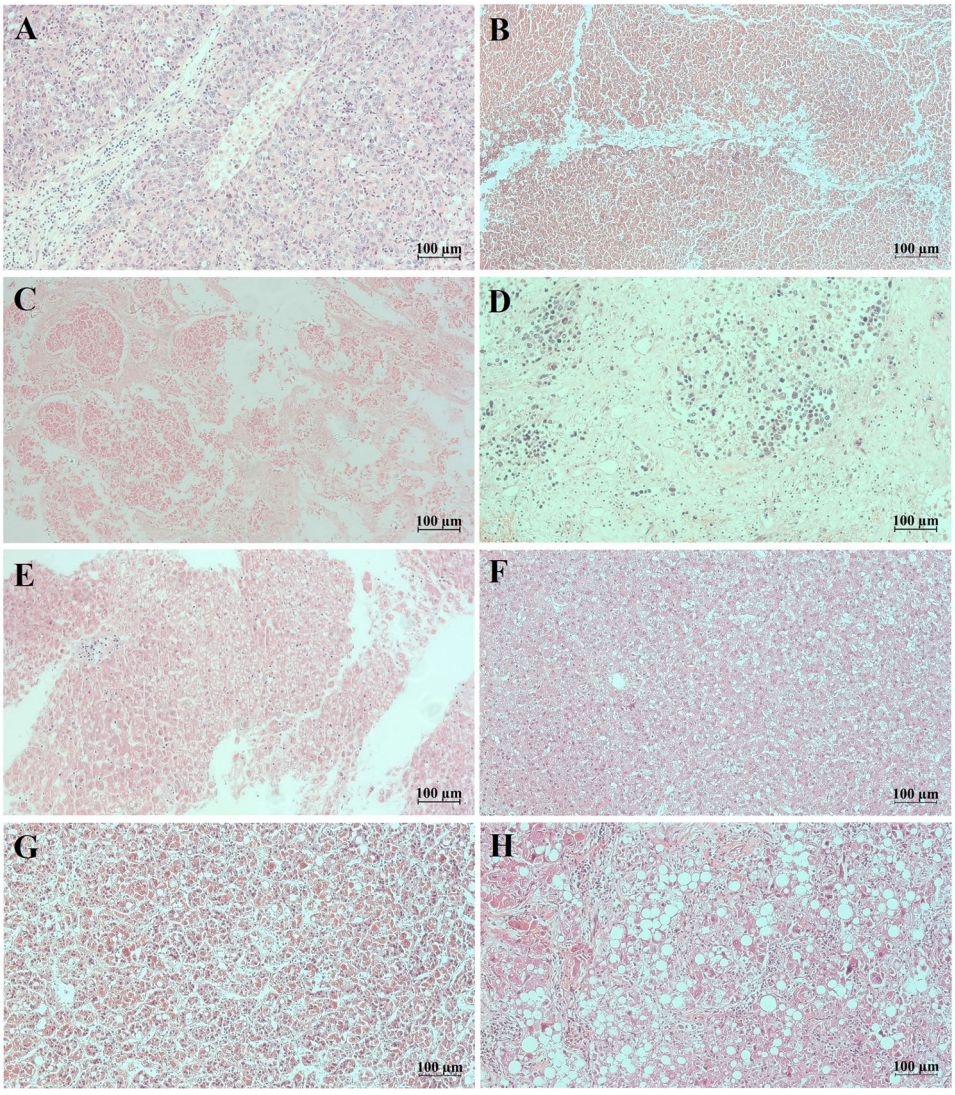

Fig. 2 Histopathology of the liver in HCC patients with *C. sinensis* mono-infection. **A**, **B**, HCC with trabecular growth and solid growth pattern; C, D, HCC with fibrolamellar growth pattern; E ~ G, CC-HCC; H, SH-HCC. *HCC* hepatocellular carcinoma, *CC-HCC* clear cell hepatocellular carcinoma, *SH-HCC* steatohepatic hepatocellular carcinoma; H&E stain, ×100

infection alone. During the investigation, one case of steatohepatic HCC was found in patients seropositive for *C.sinensis*, as well as one case with HBV co-infection. Further confirmation is required to determine the effect and mechanism of *C.sinensis* infection on lipid metabolism in HCC [29, 30].

The mechanisms by which *C.sinensis* infection induces HCC are not yet to be determined. Initial investigations have revealed that the excretory-secretory products (ESPs) of *C.sinensis* can enhance the replication of HBV and elicit a Th2 immune response. It has been observed that patients co-infected with *C.sinensis* and HBV exhibit higher levels of serum HBV DNA than patients infected with HBV alone [31, 32]. Clinical data have demonstrated that patients with co-infection of *C.sinensis* and HBV have significantly elevated levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TB), and hyaluronic acid (HA) compare to those with HBV infection alone [33]. A survey conducted in the high incidence area of clonorchiasis in the Lalin River, Northeast China, reported that out of 854 cases of clonorchiasis, 46 cases were co-infected with HBV (HBsAg positive). However, no significant differences were observed in 16 clinical indicators, including ALT, AST, and TB, between the *C.sinensis* infection, HBV infection, and co-infection groups [34]. In vitro experiments have revealed that co-infection of *C.sinensis* and HBV can promote liver fibrosis and chronic inflammation. Co-stimulation of the hepatic stellate cell line LX-2 with the total proteins from *C.sinensis* adult worms (CsTPs) and HBV positive serum leads to increased transcription of alpha-smooth muscle actin and types I and

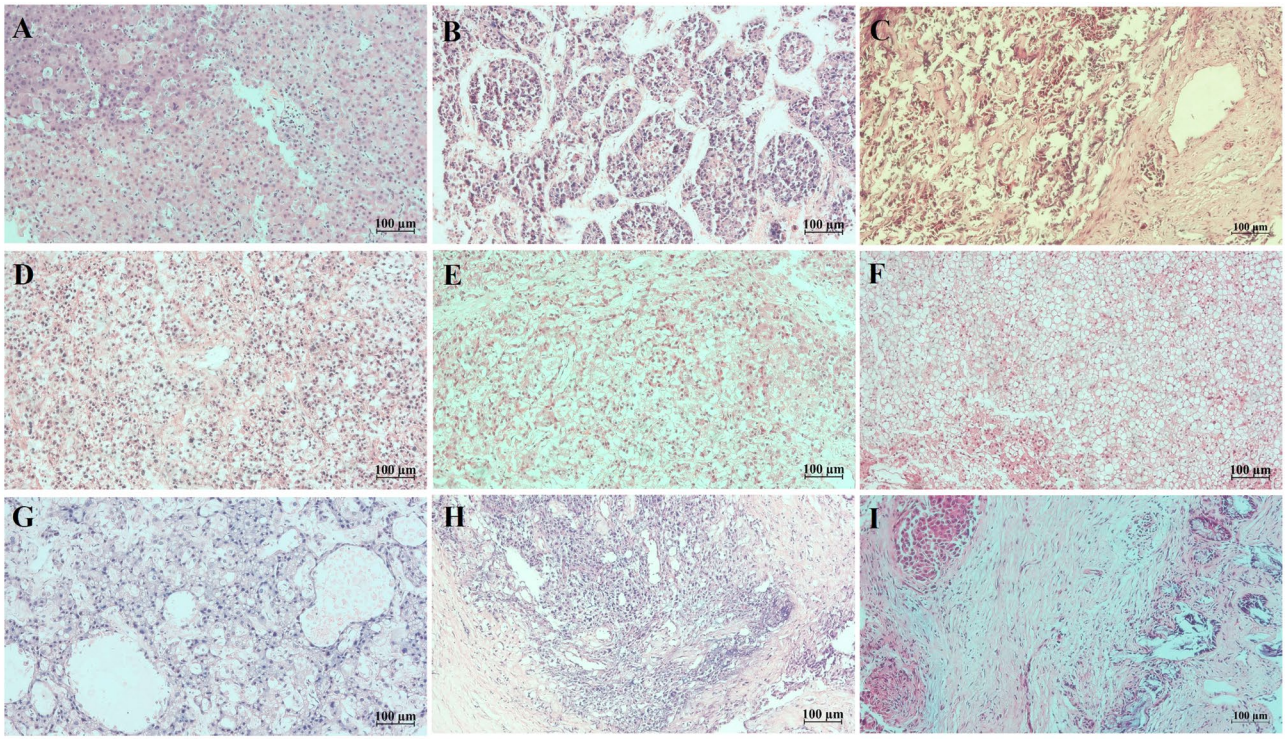

Fig. 3 Typical pathological changes in HCC patients with co-infection of *C. sinensis* and HBV. **A**～**C**, HCC with solid growth, adenoid growth and fibrolamellar patterns; D～F, CC-HCC; G, SH-HCC; H, Sp-HCC; I, HCC/ICC comorbidity. *HBV* hepatitis B virus, *HCC* hepatocellular carcinoma, *CC-HCC* clear cell hepatocellular carcinoma, *SH-HCC* steatohepatic hepatocellular carcinoma, *Sp-HCC* spindle cell hepatocellular carcinoma; H&E stain, ×100

III collagen, as well as pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 [33]. Recent studies have highlighted the ability of parasite extracellular vesicles (EVs) to mediate the host competing endogenous RNA (ceRNA) regulatory network across species through their own non-coding RNA (ncRNA) with regulatory function [35–37]. These EVs have a profound impact on the occurrence of HCC [38, 39]. Specifically, EVs derived from *C.sinensis* (CsEVs) [40] have been found to activate the TLR9 signal, promoting an inflammatory response characterized by the production of IL-6 and TNF-α in mouse biliary epithelial cells (BECs) [41]. The miRNA csi-let-7a-5p, which is enriched in CsEVs, negatively regulates the expression of *SOCS1* and *clec7a* genes in the NF-κB signaling pathway, thereby enhancing the production of TNF-α, IL-6, IL-1β, iNOS, and promoting the differentiation of M1-like macrophages. These processes play a crucial role in the development of bile duct injury and fibrosis following infection [35]. However, the precise role and mechanism of CsEVs in interfering with the miRNA-mediated ceRNA regulatory network in host hepatocytes, and their impact on hepatocellular carcinogenesis, remain unclear.

Clonorchiasis poses considerable public health risks [42], and the HBV is the leading cause of HCC in China [43, 44]. However, chronic alcohol consumption [45], non-alcoholic fatty liver disease (NAFLD) [46] and hepatitis C virus (HCV) infection [47, 48] are not the primary hazards or burdens of HCC in this region. Recently, there have shown that infection with *C. sinensis* can enhance the stemness of HCC and promote its growth by boosting angiogenesis, indicating a bad prognosis and lower patient survival rates following liver resection. However, these research did not investigate the effect of *C. sinensis* infection on the development of HCC [49–52]. This investigation has provided further clarification of the association between *C. sinensis* infection and the occurrence of HCC. In populations with a high incidence of HBV infection and related HCC, the role of *C.sinensis* in inducing HCC may be overshadowed. In China, HBV is the primary risk factor for HCC [43, 44]. However, with the implementation of the "hepatitis B elimination" strategy in 2030 [53, 54], the role of *C.sinensis* in inducing HCC may gradually become more evident. Furthermore, this investigation discovered that three patients with functional cured (FC) hepatitis B [55, 56], who had negative HBsAg but low liver HBV DNA load, were all co-infected with *C.sinensis* and suffering from HCC. Therefore, the potential threat of *C.sinensis* infection cannot be disregarded in the clinical treatment of hepatitis B and the prevention and treatment of HBV-related HCC. In areas with a high prevalence of *C.sinensis*, effective control of human HCC should not only focus on the "hepatitis B elimination" strategy but also actively

implement comprehensive prevention and control measures for clonorchiasis.

It is essential to address the limitations of this study. Firstly, a significant limitation is the study's failure to investigate the seropositivity rate of *C. sinensis* among local residents during the specified period. Additionally, the research was conducted in a high-epidemic area for clonorchiasis, necessitating future validation of the findings in medium- and low-epidemic areas. Secondly, it is important to note that this survey does not adequately identify potential secondary causes of HCC. Furthermore, while the study suggests that *C. sinensis* infection may contribute to the development of SH-HCC, this finding requires confirmation through larger population-based studies.

Conclusions

These results indicate that HCC still dominates the composition of liver cancer in this region, while ICC only accounts for a small number and is all associated with *C.sinensis* seropositivity. The occurrence of HCC was not only commonly associated with HBV, but also with *C.sinensis.C. Sinensis* seropositive individuals are significantly accumulated in HCC patients. HBV mono-infection is only associated with HCC, but *C.sinensis* mono-infection and co-infection with HBV are both associated with ICC and HCC. *C. sinensis* mono-infection may have a minor effect on HCC, however co-infection with HBV may enhance the risk and encourage the development of HCC in this area.

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Author contributions
Q-L Y wrote original draft; Z-H J, X-W L and M-Y L conducted case investigation; Q-L Y, Y-Q G, Y-N H, YH and YD performed the laboratory works, and prepared Figs. 1, 2 and 3; Table 1, and 2; Z-H J, Z-L F and CHFC reviewed and edited the manuscript. All authors reviewed the manuscript.

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Data availability
Data is provided within the manuscript.

Declarations

Ethics approval and consent to participate
The process of this study strictly adhered to the principles of the Helsinki Declaration; the case investigation and specimen collection procedures were reviewed and approved by the Guangxi Ethics Review Committee (approval No. GXIRB 2020-0022). All cases involved in the study agreed to investigation and related detection, and informed consent was obtained.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Guangxi University of Chinese Medicine, Guangxi Key Laboratory of Translational Medicine for Treating High-Incidence Infectious Diseases with Integrative Medicine, Nanning, Nanning, Guangxi 530200, People's Republic of China
[2]People's Hospital of Hengzhou, Nanning, Guangxi 530300, People's Republic of China
[3]Guangxi Zhuang Autonomous Region Center for Disease Prevention and Control, Guangxi Key Laboratory for Viral Hepatitis Prevention and Control, Nanning 530028, Guangxi, People's Republic of China, Nanning, Guangxi, People's Republic of China
[4]Hengzshou Center for Disease Prevention and Control, Nanning, Guangxi 530300, China
[5]Department of Surgery, University of Iowa Health Care, Iowa City IA 52242, USA

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