

Hepatitis C-associated hepatocellular carcinomas in non-cirrhotic livers

Matthew M Yeh¹, Hubert Darius-J Daniel² and Michael Torbenson²

¹Department of Pathology, University of Washington School of Medicine, Seattle, WA, USA and

²Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Chronic hepatitis C viral infection can lead to cirrhosis and hepatocellular carcinoma. It is generally believed that hepatitis C infection is not oncogenic *per se*, but that the presence of cirrhosis determines the increased risk for hepatocellular carcinoma. However, a search of surgical pathology files from two large tertiary care centers for the years 2001–2008 identified a total of 18 hepatocellular carcinomas in non-cirrhotic livers with chronic hepatitis C infection. In six cases the background livers showed bridging fibrosis, while the remainder showed lower stages of fibrosis. Cases were negative for clinical and serological evidence of hepatitis B co-infection, and occult hepatitis B test was negative by PCR of formalin-fixed, paraffin embedded tissues. The tumors were also negative for *TP53*, exon 7, codon 249 mutations, a characteristic mutation strongly linked to aflatoxin exposure. The hepatocellular carcinomas had typical histology with no enrichment for unusual growth patterns or histological features. Among all resected hepatocellular carcinomas in non-cirrhotic livers over this time period, the prevalence of 16% with HCV infection was significantly greater than that expected by chance. In conclusion, these results demonstrate that hepatocellular carcinomas can arise in livers chronically infected with hepatitis C but without cirrhosis. These findings raise the possibility that in some cases hepatitis C infection and inflammation can be directly oncogenic. It is also possible that established cirrhosis may have regressed in some cases. Regardless of the mechanism, these findings highlight an important and previously under-recognized risk for hepatocellular carcinoma in HCV-infected individuals who do not have cirrhosis.

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Hepatocellular carcinoma is one of the most common malignant neoplasms in the world and has increased in numbers in the United States over the past two decades.¹ Although many of the specific etiologies that lead to hepatocellular carcinoma are well known, the underlying molecular mechanisms leading to hepatocarcinogenesis remain incompletely defined. Nevertheless, cirrhosis from any cause remains one of the strongest known risk factors for hepatocellular carcinoma. Despite this fact, hepatocellular carcinoma may develop in non-cirrhotic livers in some individuals. In particular, hepatitis B virus (HBV) infection has been identified as a cause of hepatocellular carcinoma in the non-cirrhotic liver and HBV can be directly oncogenic, leading to hepatocellular carcinoma through integration of the

viral genome into the host genome.² In addition, the X protein of HBV is a potent transactivator that can interact with p53, interfering with its tumor-suppressor activity.³ In part because of these properties of HBV, liver cirrhosis in HBV-infected patients is not a prerequisite for hepatocarcinogenesis. In contrast, it is generally believed that the presence of cirrhosis determines the increased risk for hepatocellular carcinoma in individuals with chronic hepatitis C viral (HCV) infection.^{4–6} To date there is very little data on HCC arising in HCV infected, but non-cirrhotic, livers. The aims of this study are to characterize the clinical, pathologic and molecular features of hepatocellular carcinomas arising in non-cirrhotic livers of HCV-infected individuals.

Correspondence: Dr MM Yeh, MD, PhD, Department of Pathology, University of Washington School of Medicine, 1959 NE Pacific Street, NE140D, Box 356100, Seattle, WA 98195, USA.

E-mail: myeh@u.washington.edu

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Materials and methods

Case Selection

The surgical pathology files at two tertiary medical centers from January 1, 2001, to July 1, 2008, were

searched for HCV-related hepatocellular carcinomas that arose in non-cirrhotic livers and were surgically resected with intent to cure. Fibrolamellar carcinomas were excluded. Medical records were examined for HCV and HBV histories, including serological and virology tests. Due to the multi-center and retrospective nature of this study, the testing platforms differed among the patients. Chronic hepatitis C infection was defined as presence of hepatitis C viral RNA in the blood as detected by quantitative or qualitative PCR assays. In some referral cases, actual laboratory results were not available and clinical notes reporting HCV positivity were accepted for study inclusion. Serum testing for α -fetoprotein (AFP), when available, was recorded as the highest value available within the 6 months prior to surgical resection.

In the general population, most hepatocellular carcinomas arise in cirrhotic livers. However, approximately 15–20% of hepatocellular carcinoma are reported to arise in non-cirrhotic livers.⁷ In addition, 1.6% of the total US population have antibodies to HCV,⁸ with the highest prevalence within the 40- to 49-year age group, which has a prevalence of 4.3%. Thus, some co-occurrence of HCV infection and hepatocellular carcinoma in non-cirrhotic livers is expected to occur by chance. To investigate this possibility further, the total number of cases of all hepatocellular carcinomas arising in non-cirrhotic liver was determined to see if the prevalence of HCV-related hepatocellular carcinomas in non-cirrhotic livers was greater than that expected by chance. The prevalence of HCV-related hepatocellular carcinomas was compared with that of the overall US population (1.6%) and, as the most conservative approach, was also compared with the segment of the US population with the highest prevalence (4.3%).

Molecular Analysis

All identified cases had available paraffin-embedded tissues for analysis. To exclude hepatitis B infection, including occult hepatitis B, genomic DNA was extracted from paraffin blocks of the resected tumors and non-tumorous tissues. Sections of 10 μ m thickness were microdissected and digested with proteinase-K, 1 mg/ml at 56 °C for 16 h and PCR was performed with primers directed against a region of the HBV-surface gene. To evaluate for *CTNNB1* (β -catenin) mutations, the same DNA extracts were amplified with primers directed against exon 3 of the β -catenin gene as previously described.⁹ To exclude aflatoxin exposure, exon 7 of the *TP53* gene was amplified and sequenced using primers as previously reported.¹⁰ Aflatoxin exposure induces a characteristic G-to-T transversion at codon 249. The amplicons were purified and directly sequenced in the forward and reverse directions.

Histological Analysis

The pathological features were reviewed by two pathologists (MM Yeh and M Torbenson). Routine H&E stains and trichrome stains were examined. The background inflammation and fibrosis in the non-neoplastic liver was graded and staged using the modified Histologic Activity Index, or 'Ishak' system, with inflammation graded on a scale of 0–18 and fibrosis on a scale of 0–6. Pericellular fibrosis was also separately noted when present. Steatosis was scored as 0 (0–4% steatosis), 1 (5–30% steatosis), 2 (31–60% steatosis) and 3 (greater than 60% steatosis). The presence of steatohepatitis was also noted when appropriate. The tumor differentiation was graded as per the Edmonson–Steiner (ES) grading system¹¹ on a scale of 0–4. Tumors were staged according to the TNM staging system. In addition, the tumor growth pattern was evaluated as per the WHO classification schema.¹²

Results

Clinical and Laboratory Findings

During the study period, there were total 51 HCC in non-cirrhotic livers from Johns Hopkins. Underlying liver disease included unknown ($n=32$), chronic HCV ($n=9$), chronic HBV ($n=5$) infection, and associated with hepatic adenomas ($n=5$). There were total 63 HCC in non-cirrhotic livers from the University of Washington Medical Center and underlying liver diseases included unknown ($n=45$), chronic HCV ($n=9$) and chronic HBV infection ($n=9$).

The study then focused on the 18 hepatocellular carcinomas that arose in non-cirrhotic livers of individuals with chronic HCV infection (Table 1). The average age at resection was 58 ± 6.8 (mean \pm s.d.) years. There were 14 men and four women, including nine Whites, five Blacks, two Asians and two Egyptians. Only a single individual had additional identifiable causes of liver disease (iron overload and significant alcohol use).

In the 11 patients with available genotype information (Table 1), no enrichment for unusual HCV genotypes was observed: genotype 1A ($n=3$), 1B ($n=3$), 2B ($n=2$), 3A ($n=1$) and 4 ($n=2$). Hepatitis C viral loads averaged 6.4 log IU/ml in the 11 cases with available information. Among the 16 cases with available AFP information, only three had serum AFP levels greater than 30 ng/ml: 5188 ng/ml, 2329 ng/ml and 2303 ng/ml.

Next analysis was performed to investigate whether the frequency of HCV infection in non-cirrhotic livers with hepatocellular carcinoma was higher than the baseline frequency in the general population (Figure 1). Both the individual and the combined prevalence of 16% (95% confidence interval, 8–22%) were found to be higher than that expected by chance, (all $P < 0.01$, χ^2 -test). Repeat

Table 1 Clinical and laboratory findings

Individual	Gender	Age	Race	HCV genotype	HCV viral load	AFP (ng/ml)	Presentation	Outcome
1	M	66	Egyptian	4A	NA	NA	Found on ultrasound screen	NED at 17 months; lost to follow-up
2 ^a	F	66	Black	NA	NA	5	Pruritis	Death at 20 months after surgery
3	M	58	Black	1A	NA	6	RUQ pain	NED at 23 months
4	M	45	White	1A	6.29 log	14	NA	Death at 23 months after surgery
5	F	62	Black	1B	6.81 log	17	Found on ultrasound screen for follow-up of liver cysts and mildly elevated AFP	Death at 26 months after surgery
6	M	56	White	1A	6.86 log 9 months after surgery	21	Found at the time of liver biopsy by radiology, biopsy for staging and grading of HCV	NED at 32 months
7	M	49	White	1B	6.27 log 13 months after surgery	5188	Elevated AFP	Suspicious lung nodule at 23 months
8	F	55	Black	3A	6.66 log 28 months after surgery	11	Found on imaging screen	NED at 11 months
9	M	49	White	NA	6.13 log	2329	Found on w/u for colon met	Death at 2 months after surgery
10 ^a	M	67	Asian	NA	NA	2303	Elevated AFP	Lung metastasis at 30 months
11 ^a	M	58	White	NA	NA	7.9	Found on imaging screen (CT)	Recurrence at 5 months
12	M	57	White	2B	6.24 log	27.1	Found on ultrasound screen	Multiple residual HCC at 5 months
13	F	59	Egyptian	4	6.92 log	14.1	RUQ pain and elevated LFT	Recurrent HCC at 20 months
14	M	68	Asian	1B	5.27 log	28	RUQ pain, weight loss	Recurrent HCC, death at 24 months after surgery
15	M	52	White	2B	1.426e IU/ml	<5	Found on imaging screen (CT)	Recurrent HCC, death at 48 months after surgery
16 ^a	M	67	White	NA	NA	NA	Found on imaging (CT) for chest pain	NED at 3 months
17	M	57	White	NA	1.900e IU/ ml	22.8	Found on ultrasound screen	NED at 9 months
18 ^a	M	60	Black	NA	NA	12	RUQ pain	NED at 9 months

Abbreviations: AFP, α -fetoprotein; CT, computed tomography; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NA, not available; NED, no evidence of disease; RUQ, right upper quadrant; w/u, workup; met, metastasis.

^aNo HCV RNA testing was available for these individuals and diagnosis of chronic HCV is based on the clinical diagnosis in the medical records.

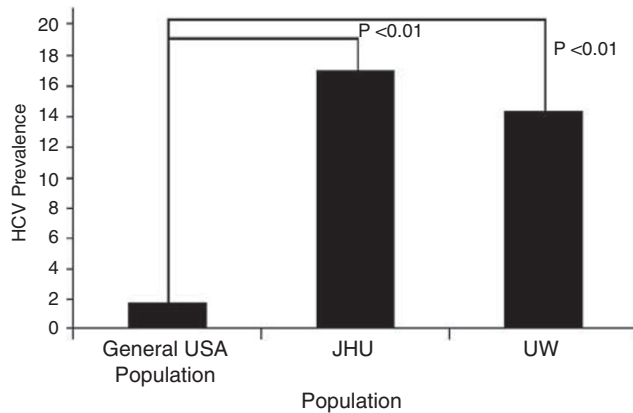


Figure 1 The combined prevalence (16%) of HCV in the non-cirrhotic livers with hepatocellular carcinoma at two institutes (17% for JHU (Johns Hopkins University) and 14% for UW (University of Washington)) is significantly higher than the prevalence of HCV antibodies in the general US population (1.6%).

analysis using the peak prevalence of anti-HCV antibodies in the US of 4.3% for the 40- to 49-years time interval remained significantly different than the combined prevalence of 15% ($P = 0.004$, χ^2 -test).

Pathologic Findings in Background Livers

Sections of the background non-neoplastic livers were available in all 18 resection specimens. The typical histological features of chronic hepatitis C were found in all the cases, including mild-to-moderate portal inflammatory infiltrates composed predominantly of mature-appearing lymphocytes, mixed with occasional plasma cells, eosinophils and Kupffer cells (Figure 2a). Lymphoid aggregates eccentrically located in the portal tracts were common. Scattered foci of inflammatory infiltrates were present in the hepatic lobules (Figure 2b), composed predominantly of lymphocytes and Kupffer cells. On trichrome stains, eight cases showed moderate portal fibrosis (Figure 2c), six cases showed bridging fibrosis (Figure 2d) and the remaining livers showed no significant or only minimal fibrosis. None of the cases were histologically cirrhotic.

Tumor Characteristics

As shown in Table 2, the average tumor size was 5.9 cm and the tumors were unifocal in nine cases and multifocal in nine cases. Pathological staging of hepatocellular carcinoma¹³ was T1 in six cases, T2 in eight cases and T3 in four cases. The histology of the tumors (not available for review in one case due to complete tumor necrosis) showed the typical morphology of hepatocellular carcinoma (Figures 2e and f). The observed growth patterns were typical of hepatocellular carcinoma and included a pseudo-glandular pattern in six cases, solid pattern in nine

cases and a mixed pattern in two cases. The hepatocellular carcinomas were classified as Edmonson–Steiner grade-1 in two cases, grade-2 in five cases, grade-3 in seven cases and grade-4 in three cases, respectively.

Molecular Results

All cases were negative for HBV DNA in the liver tissues. No mutations were seen in exon 3 of β -catenin or in exon 7 of *TP53*.

Discussion

In the US, approximately 50% of hepatocellular carcinomas are caused by chronic HCV infection.¹⁴ The pathogenesis of hepatocellular carcinoma in chronic HCV infection is generally accepted as chronic inflammation and injury, which leads to fibrosis with eventual progression to cirrhosis and subsequent development of hepatocellular carcinoma.¹⁵ Cirrhosis has generally been considered a prerequisite for HCV-infected livers to develop hepatocellular carcinoma. However, in this study we document the presence of hepatocellular carcinoma arising in non-cirrhotic livers infected with chronic HCV. This represents an important advance in our understanding of the natural history of HCV infection. The combined prevalence of HCV-positive cases among all resections for hepatocellular carcinoma in non-cirrhotic livers was 16%, significantly higher than the general prevalence of HCV-infected individuals in the US population.

Chronic HBV infection is an important risk factor for hepatocellular carcinoma, and it is well known that cirrhosis is not a prerequisite for the development of hepatocellular carcinoma, especially in Asian and African patients.¹⁵ Cancers can arise in non-cirrhotic livers largely because HBV DNA is capable of integrating into the host genome and can subsequently cause malignant transformation of hepatocytes.² Furthermore, the HBV X protein has many possible pro-oncogenic properties, including disrupted p53 tumor-suppressor activity,³ and this can occur early in the process of tumorigenesis.¹⁶ In addition, in occult HBV infection, defined as the presence of viral DNA in blood or liver tissues in persons who are HBsAg-negative,⁹ HBV virus can continue to replicate at low levels,¹⁷ which could cause persistent inflammation and injury, which may contribute to the formation of hepatocellular carcinoma. In the current study, co-infection with HBV does not appear to have played a role as all cases for HBV DNA in the liver tissue were negative as shown by PCR.

HCV is an RNA virus that does not integrate into the host genome and the mechanism of HCV-associated hepatocellular carcinoma arising in non-cirrhotic livers is not clear. Several HCV-gene products, including the core, NS3, NS4B and NS5A,

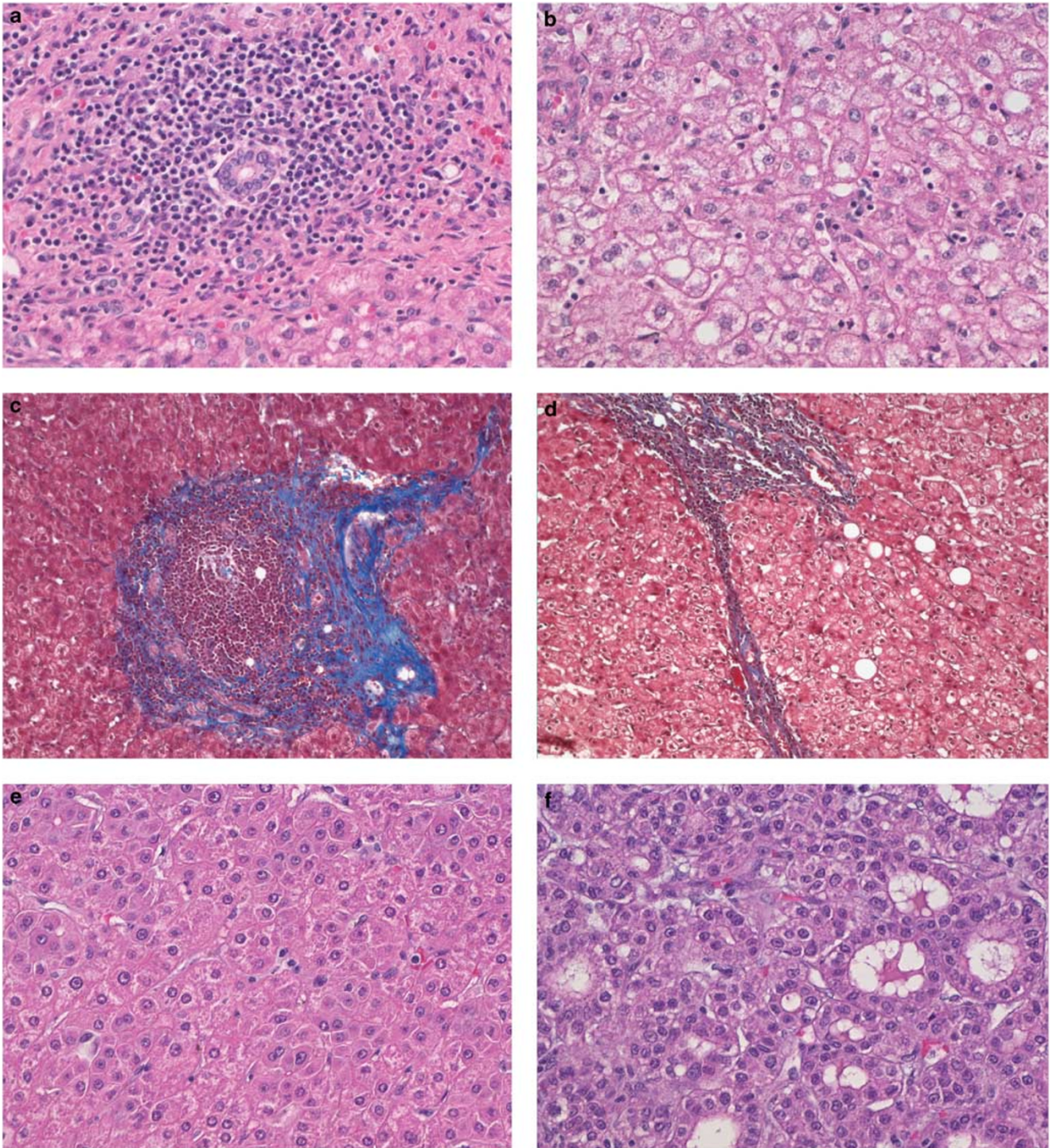


Figure 2 The cases showed the typical histology of chronic hepatitis C, with mild-to-moderate portal inflammatory infiltrates (**a**, $\times 250$ original magnification), composed predominantly of mature-appearing lymphocytes mixed with occasional plasma cells, eosinophils and Kupffer cells. Lymphoid aggregates eccentrically located in the portal tracts were common. Scattered foci of inflammatory infiltrates were present in the hepatic lobules, composed predominantly of lymphocytes and Kupffer cells (**b**, $\times 250$). Masson trichrome stains showed periportal fibrosis (**c**, $\times 40$) or bridging fibrosis (**d**, $\times 40$). Hepatocellular carcinoma showed the classical histology with thickened trabecular (**e**, $\times 100$) and/or pseudoglandular formation (**f**, $\times 100$).

are capable of malignant transformation of hepatocytes in cell model systems,¹⁸ and in some individuals this could potentially be sufficient to cause hepatocellular carcinoma. The expression of HCV

proteins has also been shown to alter several potentially oncogenic pathways via cell signaling, transcriptional modulation, apoptosis, transformation and translational regulation, and through

Table 2 Histological findings in the resected livers

Patient	Tumor size (cm)	Tumor stage ^a	Tumor grade ^b	ES MOD NUC grade ^c	Tumor growth pattern	Inflammation grade in background liver (Ishak scores): interface, lobular, portal	Fibrosis stage in background liver (Ishak scores out of 6)	Steatosis ^d
1	4.7	T2	G2	2	Solid	3,1,3	2	0
2	2.7	T1	G1	1	Solid	1,2,2	3	0
3	4.5	T1	G2	1	Pseudoglandular	2,1,2	2	0
4	3.5	T1	All necrotic	All necrotic	All necrotic	1,1,2	2	0
5	2.8; 1.4	T2	G1	2	Pseudoglandular	1,1,2	1	1
6	3.5	T1	G2	2	Pseudoglandular	1,1,2	2	0
7	4.5; 2.8; 2.0	T2	G2	4	Solid	1,1,2	2, also mild pericellular fibrosis	2 with mild SH
8	3.5; 2.7	T2	G1	3	Solid	2,1,3	3	0
9	15 with numerous adjacent satellite nodules	T3N1	G2	3	Pseudoglandular and solid	1,2,2	3	0
10	4.3	T1	G3	4	Solid	1,1,3	1	1
11	4.2	T2	G2	2	Solid	2,2,3	3	1
12	26 with numerous adjacent satellite nodules	T3	G2	3	Pseudoglandular	2,3,2	2	1
13	4.7;1.1	T1	G2	3	Pseudoglandular	1,1,3	3	2
14	7.0; 2.2	T3	G2	4	Solid	3,2,3	1	0
15	10.8	T2	G2	2	Solid	2,3,2	3	0
16	6.5	T3	G2	3	Solid and pseudoglandular	1,1,3	1	0
17	2; 3.5	T2	G2	3	Solid	2,1,2	2	0
18	5, with numerous adjacent satellite nodules	T2	G2	3	Pseudoglandular	1,1,3	2	0

Abbreviations: ES, Edmonson–Steiner; SH, steatohepatitis.

^aAJCC (American Joint Committee on Cancer) tumor stage.

^bHamilton and Lauri, 2000.¹²

^cNzeako *et al*, 1995.¹¹

^dSteatosis was scored as 0 (0 to 4% steatosis), 1 (5 to 30% steatosis), 2 (31 to 60% steatosis) and 3 (greater than 60% steatosis).

interaction with the translational machinery and post-translational modifications systems.¹⁸ However, the mechanisms of HCV-induced hepatocellular carcinoma in non-cirrhotic livers remains unclear.

Besides the possible direct effects exerted by the HCV on the host genome, it is also plausible that hepatocellular carcinoma can develop in non-cirrhotic livers as a result of cycles of inflammation, necrosis and regeneration due to chronic hepatitis C-induced liver cell injury in the liver. Increased cell turnover in this setting of inflammation and oxidative DNA damage may facilitate the accumulation of genetic and epigenetic alterations, which include activation of cellular oncogenes and proliferative signaling pathways, telomerase activation, inactivation of tumor-suppressor genes and over-expression of growth and angiogenic factors.¹⁸

The hepatocellular carcinomas reported in this case series do not represent an unusual subtype of hepatocellular carcinoma: the hepatocellular carcinoma all had the typical clinical and histological characteristics. Sequence analysis found no exon 3 *CTNNB1* (β -catenin) mutations. Earlier studies suggested an association between β -catenin mutations and HCV-associated hepatocellular carcinoma^{19,20} and recent reports have also implicated the HCV core protein in the activation of the Wnt/ β -catenin pathway.²¹ The lack of β -catenin mutations in the 18 patients in this study suggests that alternative pathways exist, which warrant further investigation.

Aflatoxin exposure is known to play an important role in the development of hepatocellular carcinomas.²² In the current study, sequencing of exon 7 of *TP53* showed no mutations in this recognized mutational hot spot for aflatoxin-associated hepatocellular carcinoma,²³ providing evidence that exposure to aflatoxin-B1 did not contribute to the etiology of these carcinomas. Besides HCV infection, only one patient had a significant history of alcohol use and iron overload. The remaining patients did not have any other known risk factors for hepatocellular carcinoma other than chronic HCV infection.

Pathological examination of the background livers in the resection specimens showed that none of the cases had cirrhosis. However, several individuals did have advanced fibrosis with stage-3 or bridging fibrosis. In some of these cases, it is possible that individuals had more advanced fibrosis that has now regressed. Others have also reported cases of hepatocellular carcinoma arising in individuals who were long-term, HCV-sustained virological responders, some of whom did not have cirrhosis.²⁴ Whether HCV-infected patients with bridging fibrosis, but not cirrhosis, should be offered screening for hepatocellular carcinoma is not clear. The point at which the risk of hepatocellular carcinoma starts to climb is unknown and transition to cirrhosis cannot always be accurately determined. However, the European Association for the Study of Liver Disease Single Topic Conference suggested that screening

should be offered to HCV-infected patients with METAVIR stage-3 fibrosis,²⁵ although the cost-efficacy of this recommendation has not been fully assessed.

Dysplastic nodules within the liver are a recognized pre-neoplastic lesion.^{26–29} Most dysplastic nodules are found in the setting of liver cirrhosis, but they may occasionally be seen in non-cirrhotic livers.^{30,31} No dysplastic nodules were identified in the background livers of these cases.

This study has several limitations, the principal one being the retrospective nature of the study. In part because of this, complete virological data in terms of hepatitis C viral loads and HCV genotypes were not available. In addition, as many individuals were primarily surgical referrals, details of prior antiviral therapy were largely not available. It is also recognized that paraffin-embedded tissues are not as sensitive as frozen tissues for excluding occult hepatitis B infection. Despite these limitations, this cohort is important in showing that hepatocellular carcinomas can arise in non-cirrhotic livers in individuals with hepatocellular carcinoma.

In conclusion, this study shows that hepatocellular carcinoma can arise in livers with chronic hepatitis C infection without cirrhosis. These hepatocellular carcinomas had typical clinical and pathological features of hepatocellular carcinoma. There was no evidence of HBV infection and molecular studies provided no evidence for aflatoxin exposure. It is possible that established cirrhosis may have regressed in some cases, but the possibility that HCV infection and inflammation may be directly oncogenic, cannot be excluded. Regardless of the mechanism, these findings highlight an important and previously under-recognized risk for hepatocellular carcinoma in HCV-infected individuals who do not have cirrhosis.

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Disclosure/conflict of interest

The authors declare no conflict of interest

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