Helicobacter pylori infection does not promote hepatocellular cancer in a transgenic mouse model of hepatitis C virus pathogenesis

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Abbreviations: hepatitis C virus, HCV; Helicobacter pylori, H. pylori; wild-type, WT; HCV transgenic, HT; gastric histologic activity index, GHAII; altered hepatocellular foci, AHF; large foci of cellular alteration, LFCA; hepatocellular adenoma, HCA; hepatocellular carcinoma, HCC; polymerase chain reaction, PCR; quantitative polymerase chain reaction, qPCR; hematoxylin and eosin, H&E; fluorescent in situ hybridization, FISH; enzyme-linked immunosorbent assay, ELISA; enterohepatic helicobacter species, EHS

Helicobacter pylori (H. pylori) and hepatitis C virus (HCV) infect millions of people and can induce cancer. We investigated if H. pylori infection promoted HCV-associated liver cancer. Helicobacter-free C3BBF1 wild-type (WT) and C3BBF1: Tg(AID1-HCV335358mHVT)) male and female mice were orally inoculated with H. pylori SS1 or sterile media. Mice were euthanized at ~12 mo postinoculation and samples were collected for analyses. There were no significant differences in hepatocellular tumor promotion between WT and HT mice; however, HT female mice developed significantly larger livers with more hepatic steatosis than WT female mice. H. pylori did not colonize the liver nor promote hepatocellular tumors in WT or HT mice. In the stomach, H. pylori induced more corpus lesions in WT and HT female mice than in WT and HT male mice, respectively. The increased corpus pathology in WT and HT female mice was associated with decreased gastric H. pylori colonization, increased gastric and hepatic interferon gamma expression, and increased serum Th1 immune responses against H. pylori. HT male mice appeared to be protected from H. pylori-induced corpus lesions. Furthermore, during gastric H. pylori infection, HT male mice were protected from gastric antral lesions and hepatic steatosis relative to WT male mice and these effects were associated with increased serum TNF-α. Our findings indicate that H. pylori is a gastric pathogen that does not promote hepatocellular cancer and suggest that the HCV transgene is associated with amelioration of specific liver and gastric lesions observed during concurrent H. pylori infection in mice.

Introduction

Helicobacter pylori (H. pylori) and hepatitis C virus (HCV) infect approximately 50% and 2% of the world’s population, respectively.1,2 The World Health Organization’s International Agency for Research on Cancer considers H. pylori and HCV human carcinogens.3 H. pylori infection may induce gastritis leading to gastric adenocarcinoma whereas HCV can induce hepatitis and cirrhosis leading to hepatocellular carcinoma (HCC).4,5 Gastric and liver cancers are ranked in the top ten most common cancers in both men and women worldwide and are two of the most common causes of cancer death.6

During HCV infection, environmental and/or host-associated factors are important and influence the progression to cirrhosis.8 Some of these factors are age, gender, alcohol ingestion, obesity, diabetes, and co-infection with hepatitis B virus or human immunodeficiency virus.9 Hepatic steatosis is also considered a risk factor for HCC in humans with chronic HCV infection and may affect a patient’s response to treatment.10 Studies suggest that H. pylori may impact HCV disease based on detection of H. pylori or “H. pylori-like” DNA in liver tissues from HCV patients.11-13 Helicobacter spp. have been detected in bile and gallbladder tissues of chronic cholecystitis patients and the need for further research investigating a potential causal role of helicobacters in human liver disease has been proposed.14,15 H. pylori was isolated from the liver of a human with Wilson’s disease-associated liver cirrhosis.16 In vitro studies using a human hepatoma cell line (HuH7) have also documented that H. pylori induces cell arrest and apoptosis of infected hepatocytes and also induces hepatocyte malfunction associated with formation of podosomes.17,18 In addition, an enterohepatic helicobacter, H. bilis, can affect the modulation of various proteins including those involved with tumorigenesis in HuH7-derived cells transfected with HCV.19 H. hepaticus, an enterohepatic helicobacter of...
mice, can induce and promote HCC in A/JCr and HCV transgenic mice, respectively.\textsuperscript{21,22} The use of FL-N/35 (C57BL/6 background) mice has advanced the study of HCV pathogenesis. These mice are transgenic for FL-N which encodes the complete polyprotein of a genotype 1b strain of HCV. In humans, infection with the HCV genotype 1b strain appears to increase the risk of HCC development.\textsuperscript{23} FL-N/35 mice usually develop hepatic steatosis and HCC after 10 and 13 mo of age, respectively, and these hepatic lesions appear to be more common in male than female mice.\textsuperscript{24,25} These studies highlighted the role of structural and nonstructural viral proteins in HCV pathogenesis. Further studies using FL-N/35 mice have documented an attenuation of Fas-mediated apoptosis in transgenic hepatocytes, an increased risk of HCC by iron overload, and development of hepatocellular steatosis with decreased plasma triglycerides.\textsuperscript{26-28} In the current study, we used a F1 hybrid of transgenic FL-N/35 and C3H/HeNTac mice as a mouse model of HCV to investigate the impact of H. pylori infection on liver cancer. The use of C3H/HeNTac mice in the current study was based on findings by the National Toxicology Program that B6C3F1 mice were susceptible to H. hepaticus-associated hepatitis and liver cancer.\textsuperscript{29} Therefore, the F1 model was useful to investigate whether another species of Helicobacter (H. pylori) could influence the pathogenesis of liver cancer in mice.

**Results**

**HCV transgene increases liver weight in female mice**

The liver to body weight ratio (%) was significantly higher in WT male mice relative to WT female mice (p < 0.05) and in WT male Hp mice relative to WT female Hp mice (p < 0.01). In addition, the liver to body weight ratio (%) was significantly higher in HT female mice relative to WT female mice (p < 0.01) but not in HT male mice relative to WT male mice (Fig. 1A). Similar results were obtained when analyzing the absolute liver weight data. These findings suggest that male mice have a proportionately heavier liver than female mice and that the HCV transgene increases the liver weight in female mice. There were no significant differences in liver to body weight ratio (%) between HT male mice and HT male Hp mice, between HT female mice and HT female Hp mice, between WT male mice and WT male Hp mice, or between WT female mice and WT female Hp mice. Therefore, H. pylori infection did not increase liver weight in HT or WT mice.

**Figure 1.** (A) Liver to body weight ratio (%) in the different groups of mice. No liver or body weight was available for three mice in different groups including WT female Hp, HT female, and HT female Hp (\textsuperscript{†}, p < 0.05; \textsuperscript{‡}, p < 0.005). (B) Hepatic steatosis scores in the different groups of mice (\textsuperscript{†}, p < 0.05). (C) Normal liver in a hepatitis C virus transgenic male mouse infected with H. pylori (HT male Hp). (D) Mild fatty degeneration in the cytoplasm of centrilobular hepatocytes in a wild-type male mouse infected with H. pylori (WT male Hp). (C and D) Bar size 100 μm.
HCV transgene increases hepatic steatosis in female mice

The hepatic steatosis score was significantly higher in HT female mice relative to WT female mice (p = 0.05) and in WT male Hp mice relative to HT male Hp mice (p = 0.05) (Fig. 1B, 1C, 1D). Histopathologic features of steatosis comprised hepatocellular cytoplasmic vacuolar change consistent with the formation of fat vacuoles. These findings suggest that the HCV transgene increases hepatic steatosis in HT female mice and decreases hepatic steatosis in HT male Hp mice. There were no significant differences in hepatic steatosis between HT male mice and HT male Hp mice, between HT female mice and HT female Hp mice, or between WT female mice and WT female Hp mice. The difference between WT male mice and WT male Hp mice could not be calculated due to the small sample size in the WT male group. Therefore, H. pylori did not modulate hepatic steatosis in HT mice or WT female mice.

**H. pylori infection does not promote hepatocellular tumors in HCV transgenic mice**

The percentage of mice in each group with microscopic pre-neoplastic (altered hepatocellular foci (AHF) and large foci of cellular alteration (LFCA)) and/or neoplastic (hepatocellular carcinoma (HCA)) liver lesions is detailed in Table 1. Multiplicity values for microscopic pre-neoplastic and neoplastic liver lesions were recorded in H. pylori-infected and uninfected HT and WT mice; however, there were no significant differences in multiplicity of microscopic pre-neoplastic and neoplastic liver lesions between HT male mice and HT male Hp mice (p = 0.50) and between HT female mice and HT female Hp mice (p = 0.80) (Fig. 2A). There were no significant differences in the number of liver lobes with dysplasia and neoplasia between HT male mice and HT male Hp mice (p = 0.50) and between HT female mice and HT female Hp mice (p = 0.50) (data not shown). These results indicate that H. pylori infection does not promote liver tumors in C57Bl/6-H2-TaqI-A(Taht)-HCV/N(355m) mice. HCCs were detected in livers of H. pylori-infected and uninfected HT and WT mice (Fig. 2B), and were characterized by poorly delineated proliferations of moderately-well differentiated, vacuolated, neoplastic hepatocytes, often organized in a trabecular pattern with minimal intervening stroma (Fig. 2C). The multiplicity of pre-neoplasia and/or neoplasia and the number of mice with microscopic neoplastic liver lesions did not differ significantly between groups. None of the groups of mice developed a median hepatic index score ≥ 4 and no significant differences in hepatitis index scores were observed in WT male Hp mice and HT female Hp mice relative to HT male mice and HT female mice, respectively (Supplementary Material).

**H. pylori colonizes the stomach but not the liver**

No H. pylori were detected in livers by quantitative PCR (qPCR). In the stomach, qPCR detected copies of H. pylori in all but three of the H. pylori-inoculated mice analyzed in this study (Table 2). In colonized mice, the values ranged from 28 to 165,862. H. pylori copes per μg of mouse DNA, qPCR did not detect H. pylori in gastric tissues from 2 WT female Hp and 1 HT female Hp mice. No significant differences in H. pylori colonization levels between WT female Hp mice and HT female Hp mice and between WT male Hp mice and HT male Hp mice. H. pylori were detected by fluorescent in situ hybridization (FISH) in the gastric corpus and antrum of selected gastric sections from infected mice. In gastric sections with high numbers of H. pylori, the antrum appeared to be colonized at a higher level than the corpus. H. pylori were detected on the apical surface of epithelial cells at the luminal edge and within crypts (Fig. 3B and 3C).

**H. pylori infection causes the most severe gastric lesions in the corpus of WT and HT female mice**

In the corpus, an increased gastric histologic activity index (GHA1) score was observed in WT female Hp mice relative to WT female mice (p < 0.05) and WT male Hp mice (p = 0.07) and in HT female Hp mice relative to HT female mice (p = 0.06) and HT male Hp mice (p < 0.01) (Fig. 4A-C). There were no

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**Table 1. Percentage of mice with microscopic preneoplastic and/or neoplastic liver lesions in each group**

| Group and gender (Hp) | Mice with no neoplasia | Mice with HCA only | Mice with HCC only | Total mice with HCA and HCC |
|-----------------------|------------------------|--------------------|--------------------|-----------------------------|
| WT female (n = 4)     | 1 (25.00%)             | 0 (0%)             | 0 (0%)             | 1 (25.00%)                  |
| HT female (n = 3)     | 0 (0%)                 | 2 (66.67%)         | 0 (0%)             | 2 (66.67%)                  |
| WT female Hp (n = 4)  | 0 (0%)                 | 0 (0%)             | 0 (0%)             | 0 (0%)                      |
| HT female Hp (n = 9)  | 1 (11.11%)             | 2 (22.22%)         | 2 (22.22%)         | 5 (55.56%)                  |
| WT male (n = 2)       | 0 (0%)                 | 1 (50.00%)         | 1 (50.00%)         | 2 (100.00%)                 |
| HT male (n = 4)       | 1 (25.00%)             | 2 (50.00%)         | 5 (125.00%)        | 8 (200.00%)                 |
| WT male Hp (n = 6)    | 2 (33.33%)             | 1 (16.66%)         | 3 (50.00%)         | 6 (100.00%)                 |
| HT male Hp (n = 10)   | 0 (0%)                 | 4 (40.00%)         | 4 (40.00%)         | 8 (80.00%)                  |

*No statistically significant differences were observed between the groups analyzed within each column. WT, wild-type; HT, hepatocytic C virus transgenic. Prenecrosis includes altered hepatocellular foci (AHF) and large foci of cellular alteration (LFCA); Neoplasia includes hepatocellular adenoma (HCA) and hepatocellular carcinoma (HCC).
significant differences in GHAI score between WT female Hp mice and HT female Hp mice and between WT male Hp mice and HT male Hp mice. The most common mucosal lesions were comprised of mild foveolar hyperplasia, oxyntic atrophy with mucous metaplasia, pseudopyloric intestinal metaplasia, epithelial dysplasia, and scattered inflammatory cell infiltrates; the latter of which also extended to involve the submucosa. The gastric GHAI scores were comparable between HT male and HT male Hp (p = 0.83). The difference between WT male mice and WT male Hp mice could not be calculated due to the small sample size in the WT male group.

Increased gastric lesions in the antrum, characterized by mucosal hyperplasia, were observed in WT male Hp mice relative to WT male mice (no P value due to small sample size in the WT male group) and in WT female Hp mice relative to WT female mice (p < 0.05). Gastric antral lesions were significantly greater in WT male Hp mice compared with HT male Hp mice (p < 0.05) (Fig. 5A-C). The antral histopathology index scores were comparable between HT male mice and HT male Hp mice (p = 0.40) and between HT female mice and HT female Hp mice (p = 0.19).

Table 2. Mouse strains, groups, and numbers in this study*

|                     | Sham | H. pylori (Hp)-infected |
|---------------------|------|------------------------|
|                     | Female | Male | Female | Male |
| C3B6F1*†           | 4 (4)  | 2 (4) | 4 (5)  | 6 (7) |
| C3B6F1-TgAb1-HCVCN35Sml‡ | 3 (4)  | 6 (6) | 9 (10) | 10 (12) |

*Number in parentheses indicates number of mice at the initiation of the study. One WT male mouse was euthanized at ~6 mo postinoculation and another mouse in this group was diagnosed with osteosarcoma at the end of the study. One WT female Hp mouse was diagnosed with histiocytic sarcoma at the end of the study. One WT male Hp mouse was found dead at ~12 mo postinoculation. Two HT male Hp mice were euthanized at ~3 to 4 mo and ~4 mo postinoculation, respectively. The three mice euthanized before the end of the study did not exhibit preneoplastic or neoplastic liver lesions. One HT female and one HT male Hp mouse were diagnosed with lymphoma at the end of the study. The mice with other tumors (osteosarcoma, histiocytic sarcoma, and lymphoma) and the mice that were euthanized or died before the end of the study were not included in the final analyses. † Wild-type (WT); ‡, HCVC transgenic (HVT).
In the antrum, exophytic, proliferative lesions were observed grossly in 2 of 6 (33%) WT male Hp mice. These exophytic antral lesions in WT male Hp mice were epithelial polyps, characterized by a proliferation of cells with antral epithelial differentiation (Fig. 5D). These lesions did not seem to be specific to WT male Hp mice as a microscopically similar antral polypoid lesion was diagnosed in a HT male Hp mouse that was euthanized at ~4 mo postinoculation.

Female gender in combination with H. pylori infection increases the gastric and hepatic expression of interferon gamma

In the stomach, significant upregulation of Ifng was observed in HT male Hp mice relative to HT male mice and in HT female Hp mice relative to HT female mice (Fig. 6A). In addition, significant upregulation of Ifng was observed in WT female Hp mice relative to WT male Hp mice and in HT female Hp mice relative to HT male Hp mice (Fig. 6A). In the liver, significant differences in Ifng expression were observed between WT female Hp mice and WT male Hp mice and between HT female Hp mice and HT male Hp mice (Fig. 6B). There were no significant differences in hepatic Ifng expression between HT male mice and HT male Hp mice and between HT female mice and HT female Hp mice. Gastric Tnf and Il10 and hepatic Il10 were significantly upregulated in H. pylori-infected female mice relative to H. pylori-infected male mice (Supplementary Material).

Hepatic Ifnb1 was significantly upregulated in HT female Hp mice relative to WT female Hp mice (Supplementary Material). The expression of gastric Tnf and Il10 and hepatic Il10 and Ifnb1 did not differ significantly between HT male mice and HT male Hp mice and between HT female mice and HT female Hp mice.

H. pylori increases serum Th1 and Th2 immune responses in female mice

Compared with uninfected controls, all mice infected with H. pylori developed similar, robust serum IgG titers to H. pylori (Fig. 7A-C). A significantly greater Th1-associated IgG2a antibody response was measured in WT female Hp mice relative to WT male Hp mice and in HT female Hp mice relative to HT male Hp mice. Th2-associated IgG1 responses were also significantly higher in HT female Hp mice compared with HT male Hp mice.
HCV transgene increases serum TNF-α during *H. pylori* infection in male mice

The serum concentration of TNF-α was significantly higher in HT male Hp mice relative to WT male Hp mice (185.5 ± 11.6 vs. 138.6 ± 17.1, p < 0.05) (Fig. 8). There were no significant differences in the serum concentration of TNF-α between HT male mice and HT male Hp mice and between HT female mice and HT female Hp mice. The serum concentration of IL1-β was significantly lower in HT female Hp mice relative to HT male Hp mice and the serum concentration of MCP-1 was significantly lower in WT male Hp mice relative to WT male mice. The serum IL1-β and MCP-1 findings were not consistent with the increased gastric corpus and antral pathology observed in HT female Hp and WT male Hp mice, respectively (Supplementary Material). No significant differences were observed in the serum concentration of IL-10 and IFN-γ when comparing the different groups of interest; however, the serum concentration of IL-17A was significantly higher in HT male mice relative to HT female mice (p < 0.01; data not shown) and the serum concentration of IL-6 was increased in WT female Hp mice relative to WT male Hp mice (p = 0.06; data not shown). There were no significant differences in the serum concentration of IL-1β, MCP-1, IL-10, IFN-γ, IL-17A, and IL-6 between HT male and HT male Hp mice and between HT female and HT female Hp mice. The serum concentration of IL1-β and MCP-1 findings were not consistent with the increased gastric corpus and antral pathology observed in HT female Hp and WT male Hp mice, respectively (Supplementary Material). No significant differences were observed in the serum concentration of IL-10 and IFN-γ when comparing the different groups of interest; however, the serum concentration of IL-17A was significantly higher in HT male mice relative to HT female mice (p < 0.01; data not shown) and the serum concentration of IL-6 was increased in WT female Hp mice relative to WT male Hp mice (p = 0.06; data not shown). There were no significant differences in the serum concentration of IL-1β, MCP-1, IL-10, IFN-γ, IL-17A, and IL-6 between HT male and HT male Hp mice and between HT female and HT female Hp mice.

**Discussion**

Previous studies in humans infected with HCV suggested that *H. pylori* may play a role in the pathogenesis and progression of HCC. Direct and indirect mechanisms of helicobacter-induced liver damage involving toxin(s) and induction of pro-inflammatory cytokines, respectively, have been suggested. The present study, utilizing C3B6F1-Tg(Alb1-HCVN)35Sml mice, characterized by hepatic steatosis in transgenic female mice, demonstrated that *H. pylori* colonized the stomach, but not the liver, and provides indirect evidence that *H. pylori* does not promote HCC during HCV infection in humans. However, we cannot exclude the possibility that *H. pylori* or "*H. pylori-like" bacteria colonize the human liver during severe liver disease or cirrhosis as suggested by others. Hepatic *H. pylori* colonization and pathology
may also depend on the immune response of the human host and/or H. pylori virulence factors such as CagA or VacA. Since H. pylori SS1, used in the current study, appears to have a non-functional cag pathogenicity island, additional studies are needed in order to investigate the role of the H. pylori cag pathogenicity island in liver cancer. It is important to note that enterohelial Helicobacter spp. (EHS) may be involved in hepatobiliary diseases of humans. Although H. hepaticus was not detected in the stools of HCV or hepatitis B virus patients with HCC, a previous study from our laboratory indicated that H. hepaticus promoted liver cancer in B6C3F1-Tg(Alb1-HCVN)35Sml mice and also promoted aflatoxin-induced liver cancer in C3H/HeNCr mice with persistent intestinal colonization of H. hepaticus, despite the lack of the organism in the liver. The occurrence of HCCs in H. pylori-infected and uninfected HT and WT mice in our study is consistent with the natural susceptibility of aged B6C3F1 male mice to liver tumors.

Hepatic steatosis is frequently observed in humans with chronic HCV infection and may increase the risk of HCC through increased oxidative stress or by promoting fibrosis. The C3B6F1-Tg(Alb1-HCVN)35Sml mouse model used in the current study was characterized by increased hepatic steatosis in female mice relative to WT female mice and no significant development of liver tumors. In contrast, its parental transgenic strain, the C57BL/6-Tg(Alb1-HCVN)35Sml (FL-N/35) mouse model, was characterized by hepatic steatosis and liver cancer in male mice. This difference may be due in part to the mixed genetic background in our model since another HCV transgenic mouse model on B6C3F1 and B6C3F2 genetic backgrounds was characterized by more females than males developing hepatic steatosis. In addition, subsequent generations of FL-N/35 mice no longer developed spontaneous liver tumors. The tumor promoting effects of the HCV transgene depend on the host genetic background. In our study, however, the HCV transgene protected from hepatic steatosis in H. pylori-infected male mice and this decrease in steatosis correlated with a significant increase in serum levels of TNF-α. Our findings are in contrast with human data in which chronic HCV patients with hepatic steatosis (and unknown H. pylori infection status) have increased serum levels of TNF-α. However, TNF-α/β deficient mice (C57BL/6) background develop hepatomegaly and hepatic steatosis suggesting a role of TNF in control of lipid homeostasis. The role of H. pylori in hepatic steatosis associated with HCV in humans has been reported.
We observed that *H. pylori* infection induced increased gastric corpus pathology in C3B6F1 (WT and HT) female mice relative to male mice. This is consistent with previous studies utilizing C57BL/6j and C57BL/6 gpt delta mouse models which have documented that female mice develop more severe gastric lesions from *H. felis* and *H. pylori* infection, respectively.** H. pylori-infected female 129/Sv mice also showed increased gastric lesions compared with *H. pylori*-infected male 129/Sv mice.** Furthermore, HT male mice were protected from *H. pylori*-induced corpus lesions. In the gastric antrum, *H. pylori* induced gastric lesions in WT male and female mice. However, HT male and female mice were protected from *H. pylori*-induced gastric antral lesions and more antral lesions were observed in WT male *Hp* than in HT male *Hp* mice. These findings suggest that the HCV transgene protected the mice against gastric corpus and antral lesions during *H. pylori* infection. However, studies in humans with HCV-associated chronic hepatitis did not find a correlation between liver inflammation and gastric mucosal lesions.**

Dysplasia in the gastric antrum has been observed in trefoil factor family A knockout male and female mice (on a mixed B6129Sv background) infected with *H. pylori* and this finding correlated with upregulation of gastric *Ifng.* During *H. pylori* infection the expression of *Ifng* was significantly higher in the stomach and liver of female mice relative to male mice. Similarly, in Mongolian gerbils infected with *H. pylori* SS1, the gastric *Ifng* expression was higher in females than in males at 36 weeks post-infection and females developed more gastric antral and corpus pathology than males at 36 weeks post-infection.** In our study, the upregulation of *Ifng* in the stomach was associated with a significant increase in Th1 immune response against *H. pylori*, an increase in gastric corpus pathology, and a decrease in *H. pylori* colonization levels.** These findings are consistent with the absence of *H. pylori* in the stomach of three female mice originally inoculated with *H. pylori*. Interferon gamma mediates inflammation during *H. pylori* infection with a subsequent reduction in *H. pylori* colonization.** Increased gastric pathology in female C57BL/6 gpt delta mice at 6 mo postinfection has been associated with decreased *H. pylori* colonization levels.** *H. pylori* also modulates the hepatic expression of innate immunity-, inflammation-, and fibrogenesis-associated genes in female C57BL/6 mice.** Increased hepatic expression of *Ifng* in female mice in our study may contribute to the activation of NF-κB with a resulting pro-inflammatory response.**

A potential beneficial effect of *H. pylori* infection has been documented in humans with HCV. Increased levels of iron in the body may contribute to HCC development.** *H. pylori*-positive HCV patients have lower levels of hepatic iron deposits than *H. pylori*-negative HCV patients.** This finding may be consistent with the association of *H. pylori* infection and iron deficiency anemia.** Interestingly, an excess-iron diet increased the risk of HCC in FL-N/35 mice whereas an iron-depleted diet increased the risk of gastric cancer in a gerbil model of *H. pylori* infection.** In addition, chronic *H. felis* infection induced iron deficiency in hypergastrinemic INS-GAS mice.** Whether *H. pylori* infection can decrease the risk of HCC in FL-N/35 mice fed a high iron diet requires additional studies.

*H. pylori* infection may prolong viral infection by decreasing the cytotoxic T cell response.** Recent studies also suggest that the DNA of *H. pylori* includes a high ratio of immunoregulatory to immunostimulatory sequences that are associated with decreased type I interferon (IFN-α) and amelioration of colitis.** However, in the context of HCV infection, decreased IFN-α signaling could promote chronic infection by decreasing the host immune response against the virus.** *Ifnb1*, the type I interferon gene encoding IFN-β, induces degradation of HCV proteins through autophagy.** In addition, through nucleotide-binding oligomerization domain 1, *Ifnb1* also partially protects the infected host by decreasing *H. pylori* gastric colonization.** We observed significant upregulation of hepatic *Ifnb1* in HT female *Hp* mice relative to WT female *Hp* mice suggesting that the HCV transgene contributed to a type I interferon response during *H. pylori* infection. Since the transgenic HCV mouse model used in the current study does not involve viral infection, it is not possible to determine the effect of *H. pylori* infection on HCV replication. The high prevalence of

**Figure 6. Gastric and hepatic gene expression in the different groups of mice: (A) Gastric interferon gamma (Ifng). (B) Hepatic Ifng. *, p < 0.05; **, p < 0.01.
Figure 7. Serological immune response against H. pylori in the different groups of mice: (A) Total IgG. (B) Th1 (IgG2c). (C) Th2 (IgG1). OD, optical density.

*, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.
H. pylori infection in humans justifies the need for future studies designed to investigate if H. pylori and/or its DNA affect HCV replication in animal models. Mouse models with “humanized” livers and/or their DNA affect HCV infection may provide novel opportunities for delineating the effect of H. pylori on liver disease and cancer in humans.12-14

In summary, utilizing a transgenic mouse model of HCV pathogenesis and hepatic steatosis, we ascertainment that H. pylori was not able to colonize the liver nor did the organism promote liver cancer. In contrast, previous studies using B6AF1 and B6D2F1, B6C3F1-Tg(Alb1-HCVN)35Sm1, and C3;B6 mice infected with the prototype EHS, H. hepticus, demonstrated that this bacterium induced persistent hepatitis and/or liver tumors.15-17 Our results therefore would argue that EHS rather than H. pylori, are more likely to be associated with human hepatobiliary diseases including cancer. Our experimental evidence is supported by studies in which EHS have been identified in humans with inflammatory and neoplastic conditions including cholecystitis, bile duct and gallbladder cancer as well as by studies suggesting the presence of non-H. pylori helicobacters in patients with hepatobiliary diseases including primary biliary cirrhosis and primary sclerosing cholangitis.18-20 Future studies in humans with HCV and other hepatobiliary diseases should attempt to culture helicobacters not only in liver, but also the gastrointestinal tract, and compare isolates by detailed molecular techniques to ascertain their taxonomic classifications.11,17

Materials and Methods
Mice and experimental design
FL/N/55 mice [C57BL/6-Tg(Alb1-HCVN)35Sm1; Mouse Genome Informatics ID: MGI:3513779] were obtained from Stanley M. Lemon (University of North Carolina School of Medicine). Male C57BL/6-Tg(Alb1-HCVN)35Sm1 mice were bred to C3H/HeNTac female mice and the C3B6F1-Tg(Alb1-HCVN)35Sm1 [HCV transgenic (HT)] and C3B6F1 wild-type (WT) progeny were used for the current study. C3B6F1 mice were genotyped for the transgene using primers HCV-F (5'-CAACCTTACCTAACGGCTG-3') and HCV-R (5'-GGTAGTCAACCTGATGCAC-3') and the following polymerase chain reaction (PCR) conditions: 1 cycle of 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 52 °C for 45 s, and 72 °C for 1 min, and a final cycle of 72 °C for 10 min. Prior to experimental inoculation, C3B6F1 mice were determined to be free of Helicobacter spp. by PCR of their fecal DNA using primers that detect Helicobacter spp.11,13 Specific pathogen-free WT and HT mice were assigned to groups based on genotype and gender and were orally-inoculated every day for three days with 0.2 mL of media only (sham) or media with H. pylori SS1 (~2 × 10^8 organisms per inoculum) at ~2 mo of age. The sham inoculated groups are designated: WT female, HT female, WT male, and HT male. The H. pylori-inoculated groups are designated: WT female Hp, HT female Hp, WT male Hp, and HT male Hp (Table 2). Mice were housed in Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facilities and fed Purina® RMH 3000 (LabDiet®, SP00) and provided reverse osmosis water ad libitum. Mice were euthanized at ~12 mo postinoculation. This study was approved by the Massachusetts Institute of Technology Committee on Animal Care.

Histopathological evaluation
Immediately following euthanasia by CO2 inhalation, mouse body weight was recorded and blood was collected by cardiac puncture. A complete necropsy was performed and liver weight was measured. The stomach was collected and incised along the line of the greater gastric curvature, luminal contents were removed, and the mucosa was rinsed with sterile PBS. For histopathologic evaluation, linear strips extending from the gastric squamocolumnar junction to the proximal duodenum were taken along the lesser curvature. The liver was separated into left, right, median, and caudate lobes, and a section from each was collected. Stomach and liver sections were fixed in 10% neutral-buffered formalin and were routinely processed for histology. Sections of stomach and liver were also frozen in liquid nitrogen and stored at ~−80°C for DNA extraction and at ~−20°C for RNA extraction. Sera were also stored at ~−20°C.

Five-micrometer-thick sections were stained with hematoxylin and eosin (H&E) for histopathological evaluation by a board-certified veterinary pathologist (N.M.A.P.) blinded to treatment groups. H&E-stained stomach sections were scored for corpus and antral pathology. Lesions in the corpus were scored according to a previously described scoring system, on an ascending scale of 0 to 4 for inflammation, epithelial defects, hyperplasia, dysplasia, atrophy and intestinal metaplasia; a GHAI was calculated as the sum of the scores for all parameters listed.17 Lesions in the antrum were scored based on a modification of the system used for lower intestinal lesions. The degree and frequency of epithelial defects, inflammation, and hyperplasia were scored on a scale of 0 to 4 with ascending severity (0, none; 1, minimal; ©2013 Landes Bioscience. Do not distribute
2. mild; 3. moderate; and 4. severe). Epithelial dysplasia was also graded using a scale of 0 to 4 (0, normal; 1, mild dysplasia; 2, mild; 3, moderate; and 4, severe). Epithelial dysplasia or carcinoma in situ; and 4, invasive carcinoma. A total of 5 or more lesions was defined as severe dysplasia or carcinoma in situ. A total of 5 or more lesions was defined as severe dysplasia or carcinoma in situ.

H. pylori colonization was assessed by qPCR. DNA was extracted from a longitudinal section of stomach using TRIzol® Reagent (Invitrogen-Life Technologies). Five micrograms of RNA were converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gastric and hepatic gene expression of Tumor necrosis factor-α (Tnfrf) (Assay ID: Mm00999908_m1), Interferon gamma (Ifng) (Assay ID: Mm01603394_m1), Interferon β 1, Bcl-2 (Bcl2), and Bcl-2 associated X protein (Bax) (Assay IDs: Mm00439546_s1, Mm00436696_m1, Mm01288368_m1) were measured using commercial TaqMan® probes-probe sets (Applied Biosystems-Life Technologies). Duplicate reactions were performed for each sample. Gene expression was determined using the comparative C method relative to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems, 4352661), per PE Applied Biosystems’ User Bulletin # 2 ABI PRISM 7700 Sequence Detection System and using uninfected WT male and WT female mice as controls for male and female mice, respectively.

Enzyme-linked immunosorbent assay (ELISA) for serum antibody to H. pylori antigens. Serum IgG, IgM-associated IgG2c, and Ig2-associated IgG1 responses to outer membrane antigens of H. pylori were measured by ELISA as previously described.79,80 Antigen was coated on Immunolon II plates (Thermo Fisher Scientific, 3459) at a concentration of 1 μg/ml (IgG) or 10 μg/ml (IgM) with sera diluted 1:100. Biotinylated secondary antibodies included goat anti-mouse IgG (Southern Biotech, 1030–03) and monoclonal anti-mouse antibodies produced by clones A85–1 and 5.7 (BD Biosciences, 553441 and 555004, respectively) for detecting IgG1 and IgG2c, respectively. Incubation with ExtrAvidin®-Peroxidase (Sigma, E2886) was followed by 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS®) substrate (Kirkegaard and Perry Laboratories, 50–65–02 and 50–64–02) and 1:1000 H2O2. Absorbance (optical density) development at 405/590 nm was recorded by an ELISA plate reader (Dyntech MR7000, Dyntech Laboratories, Inc.).

Serum cytokine assay (xMAP® Technology-Luminex). Serum samples were analyzed using the Bio-Plex Pro™ Mouse Cytokine Td7 panel A 6-Plex Group 1 (BIO-RAD, M60–0000(70)) which included IL-1β, IL-10, IL-6, IL-17A, IFN-γ, and TNF-α. In addition, MCP-1 (MCAF; BIO-RAD, 171-50S059M) was added to the assay following the manufacturer’s recommendations. Most samples were analyzed in duplicate. Four serum samples were not analyzed (samples were hemolyzed).

Statistical analyses. Statistical analyses were performed using GraphPad Prism version 5.03 for Windows (GraphPad Software). The combined multiplicity of hepatic preneoplasia and neoplasia was defined as the number of preneoplastic (AHF and LFCA) and neoplastic (HCA and HCC) microscopic liver lesions in each group divided by the total number of mice in the group.76,80 The Mann Whitney test (2 tailed) was used for analysis of outcomes.
Results are shown as median with interquartile range (for hepatic steato- 
sis, corpus' GHAI, antrum's total histopathology index, and hepato-
choroiditis index), mean ± standard error of the mean [for liver to body 
weight ratio (%), number of liver lobes with dysplasia and neoplasia, 
multiplicity of microscopic preneoplastic and neoplastic 
liver lesions, and the numbers of mice with microscopic preneoplastic 
lesions and the presence of Helicobacter pylori]. Results were 
considered significant if p ≤ 0.05.

Disclosure of Potential Conflicts of Interest
No potential conflict of interest was disclosed.

Supplemental Materials
All supplemental materials may be found here: 
http://www.landesbioscience.com/journals/gutmicrobes/ 
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