INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is becoming increasingly common, due in part to the wide spectrum of disease pathologies, ranging from simple liver fat deposition to more severe presentations such as nonalcoholic steatohepatitis (NASH), liver cirrhosis (LC), and hepatocellular carcinoma (HCC). It is estimated that 25% of the world’s population has NAFLD.1 The etiology of the disease is related to diet, body mass index, gut microbiota, and genetic factors.2

Liver cancer is one of the major types of cancer-related death worldwide.3 Common forms include HCC and cholangiocellular...
progression in the liver. Furthermore, liver-specific deletion of E-cadherin has been used as a model for examining tumor progression of various cancers, including primary sclerosing cholangitis (PSC), viral infection (hepatitis B virus [HBV] and hepatitis C virus [HCV]), LC, and biliary stone disease. Metabolic conditions including diabetes and obesity have also been identified as risk factors for CCC.

Recent studies have reported several mechanisms, such as the epithelial-mesenchymal transition (EMT), which may promote carcinogenesis. Mutation or decreased expression of E-cadherin is associated with malignant progression of various cancers, including HCC and CCC. As deletion of E-cadherin is known to promote invasiveness and EMT, liver-specific knockout of E-cadherin has been used as a model for examining tumor progression in the liver. Furthermore, liver-specific deletion of E-cadherin was shown to induce spontaneous periportal inflammation resembling PSC, indicating that these mice can also be used as a model of cholangitis.

Nonalcoholic fatty liver disease is associated with several important comorbidities, including hepatic inflammation, fibrosis, and the development of HCC, however the role of NAFLD in cholangitis, as well as periductal fibrosis, which resembles PSC. To determine the role of NAFLD in cholangitis and the development of CCC remains poorly understood. This study investigated whether HFD promotes cholangitis and the development of CCC in mice.

2 | MATERIALS AND METHODS

2.1 | Animals

CDH1F/F, Alb-Cre, Alb-CreERT, CK19-CreERT, KrasLSLG12D, and Rosa26tdTomato mice were purchased from the Jackson Laboratory (Bar Harbor, MN, USA). All knockout strains were developed on the C57BL/6 genetic background. Mice were maintained in filter-topped cages on autoclaved food and water at Yokohama City University. All animal experiments were approved by the Ethics Committee for Animal Experimentation of Yokohama City University and conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals.

2.2 | Reagents

The following antibodies were used in the experiments: anti-CD44 (AbD Serotec); anti-Sox9 and anti-K19 (Santa Cruz Biotechnology), anti-F4/80 (Caltag), anti-Ki67 (Gene Tex), anti-HNF4α (Abcam), and anti-RFP (Rockland).

2.3 | High-fat diet

Mice were fed with a normal or high-fat diet (HFD) (40% kcal fat [22% trans-fat and 26% saturated fatty acids by weight], 22% fructose, 10% sucrose, 2% cholesterol; D09100301, Research Diets).

2.4 | Immunohistochemical analyses

Liver tissue was fixed in 10% formaldehyde, dehydrated, embedded in paraffin, and sectioned (5 μm thickness). Sections were deparaffinized, rehydrated, treated with 3% H2O2 in PBS, and incubated overnight at 4°C with the appropriate antibodies. Binding of the primary antibody was detected using biotin-labeled anti-rabbit IgG or anti-rat IgG antibodies (1:500 dilution; Vector Laboratories), followed by the streptavidin-horseradish peroxidase (HRP) reaction and visualization with 3,3-diaminobenzidine (DAB; Sigma) and counterstaining with hematoxylin. For the double staining, sections were incubated with 1st primary antibody (1:500) overnight at 4°C, and the immunoreactivity was visualized with DAB (brown staining) with a peroxidase-based Histofine Simple Stain Kit (MAX PO R, Nichirei). For the immunoreactivity to another target, 2nd primary anti (1:500) was incubated overnight at 4°C and visualized with Fast Red II Substrate (Nichirei) using an alkaline phosphatase-based Histofine Simple Stain Kit (AP R, Nichirei).

2.5 | Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Significant differences were determined using Student t test. P-values ≤ .05 were considered significant.

3 | RESULTS

3.1 | NAFLD exacerbates cholangitis caused by E-cadherin deletion

Previously, we have shown that liver-specific E-cadherin knockout mice (CDH1ΔL) developed spontaneous periportal inflammation as well as periductal fibrosis, which resembles PSC. To determine the role of NAFLD in cholangitis, CDH1ΔL mice were fed an HFD for 20 wk to induce NAFLD CDH1ΔL mice fed the HFD exhibited significant increases in body weight and fat deposition, similar to HFD-administered CDH1ΔL control mice (Figure 1A,B, Figure S1). HFD-administered CDH1ΔL mice exhibited steatosis, ballooning, and inflammation similar to that of CDH1ΔF mice, and the cholangitis in the periportal area was more severe compared with that seen in mice fed a ND (Figure 1B and Figure S1). To better assess the inflammatory
response, we performed immunostaining of F4/80 myeloid cells in CDH1ΔL mice, and observed a significant increase in the number of F4/80-positive cells in the periportal area of mice fed an HFD relative to ND controls (Figure 1C). CD45- and CD3-positive lymphoid cells were also increased in HFD mice, although the overall number of CD45-positive cells was relatively small compared with F4/80 myeloid cells (data not shown). We observed increased ALT expression in both CDH1ΔL and CDH1F/F control mice fed the HFD, with no significant difference between strains. Serum levels of total alkaline phosphatase (ALP) and bile acid were increased in CDH1ΔL mice compared with CDH1F/F control mice, however the ALP and total bile acid levels were not different between ND and HFD mice (Figure 1D).

CDH1ΔL showed numerous primitive duct cells expressing progenitor markers, such as Sox9 and CD44, in the periportal area.31 CDH1ΔL HFD mice showed increased numbers of Sox9- and CD44-positive cells compared with ND mice (Figure 2A,B). CK19-positive ductular cells were also increased by HFD (Figure 2A,B). Interestingly, Sox9-positive cells were observed not only in zone 1, but also in in zone 2 of CDH1ΔL HFD mice compared with only zone 1 in the CDH1ΔL ND mice (Figure 2A,B). CDH1F/F HFD mice showed slightly but not significantly increased numbers of Sox9, CD44 and CK19-positive cells compared with ND mice (Figure S2). We performed double staining of Sox9/CK19 and Sox9/CD44 (Figure 2B), and found that Sox9 and CK19 were stained in almost the same cells. Most of the Sox9-positive cells were positive for CD44, however CD44 single positive cells were frequently found, possibly because of the positivity of CD44 in mesenchymal cells in addition to immature cholangiocytes. In contrast, CDH1F/F HFD mice showed slightly, but not significantly, increased numbers of Sox9-, CD44-, and CK19-positive cells (Figure 2C). These results suggested a significant increase in the extent of cholangitis and the number of bile ductules in CDH1ΔL HFD mice relative to CDH1ΔL ND mice. Fibrosis, as determined using α-smooth muscle actin (α-SMA) immunostaining, was slightly increased in CDH1ΔL HFD mice relative to ND controls (data not shown).

3.2 | The NAFLD-induced exacerbated ductular reaction might be irreversible

To determine whether the ductular reaction seen in these mice is reversible, mice fed the HFD diet for 5 mo were switched to the ND for 3 wk. We found that severe cholangitis and fat deposition in CDH1ΔL were significantly reversed after 3 wk; however, the increased ductular reaction, as well as the increased numbers of Sox9, CD44 and CK19-positive cells were not reversed (Figure 3A,B). In contrast, the number of F4/80 myeloid cells was reduced in these mice (Figure 3C), suggesting that the NAFLD-induced bile duct reaction may be irreversible over short time periods.

**Figure 1** NAFLD exacerbates cholangitis caused by E-cadherin deletion. A, Body weight curve of CDH1ΔL mice (n = 5) and CDH1F/F (n = 5) after HFD. B, H&E staining of the liver in ND and HFD CDH1ΔL mice and CDH1F/F HFD mice. ×100 (left panel) and ×200 (right panel) magnification. C, Immunostaining of F4/80 in CDH1ΔL ND and HFD mice. ×200 magnification. D, Serum ALT, ALP, and total bile acid levels of ND and HFD mice of each genotype (both n = 5). Values represent the mean ± standard error of the mean (SEM). *P < .05 as determined by Student t test.
3.3 Increased ductular reaction induced by HFD may be due to bile duct epithelial cells

We have previously reported that loss of E-cadherin in bile duct epithelial cells (BECs) rather than hepatocytes induces cholangitis and ductular reaction in CDH1ΔL. We also showed that E-cadherin was deleted both in hepatocytes and BECs of CDH1ΔL mice. Therefore, we wanted to determine whether the increased ductular reaction associated with HFD was also associated with BECs. To delete E-cadherin only in BECs, we crossed CDH1ΔL mice with K19CreERT mice expressing a tamoxifen (TAM)-inducible Cre ERT in the endogenous K19 locus (CDH1ΔL/K19CreERT; CDH1Δich) (Figure 4A). At 12 wk after TAM injection, increased cholangitis (F4/80-positive cells), ductular reaction (CK19-positive), and Sox9-positive staining were observed in ND mice, as described previously (Figure 4B). In CDH1Δich mice, HFD increased cholangitis (F4/80-positive cells), ductular reactivity...
exacerbation of the ductular reaction may be irreversible. A, H&E staining of CDH1\textsuperscript{ΔL} (n = 4) and CDH1\textsuperscript{Δ}\textsuperscript{F} (n = 4) mice fed an HFD for 5 mo before being switched to ND for 3 wk. B, Sox9, CD44, and CK19 immunostaining and quantification. ×200 magnification. C, Quantification of F4/80-positive cells.

(CK19-positive cells), and Sox9-positive staining compared with ND mice (Figure 4B,C). These observations suggested that the increased cholangitis and ductular reactivity induced by HFD were dependent on BECs (Figure 4A–C). However, compared with CDH1\textsuperscript{ΔL} HFD mice, CK19- and Sox9- positive cell expression in CDH1\textsuperscript{Δ}\textsuperscript{ich} HFD mice was relatively limited around zone 1. To confirm that the increased ductular reaction was derived from E-cadherin-deleted BECs, we crossed Rosa26\textsuperscript{tdTomato} mice (\textit{tdTomato} mice) with CDH1\textsuperscript{Δich} mice and found that most of the ductular cells were \textit{tdTomato}-positive, suggesting that the increase in ductular cells was derived from CK19-positive BECs in HFD-administered CDH1\textsuperscript{Δich} mice (Figure 4D).

**3.4 Increased primitive cell expression induced by HFD was also due to hepatocytes**

Biphenotypic hepatocytes, also known as ductular hepatocytes, express ductal markers and give rise to ductal cells, and have been reported in cholestatic liver injury models. 12-14 Furthermore, pre-existing populations of Sox9-expressing periportal hepatocytes and other bile-duct-enriched genes have shown extensive proliferation after chronic injuries. 15 In CDH1\textsuperscript{ΔL} mice, an HFD increased Sox9-positive cells, not only in zone 1, but also in zone 2 (Figure 2A). Therefore, we wanted to determine whether hepatocytes other than BECs were the source of Sox9-positive cells. To generate hepatocyte-specific E-cadherin knockout mice, we crossed CDH1\textsuperscript{F/F} mice with Alb-\textit{CreERT} mice expressing a TAM-inducible Cre ERT in the endogenous albumin locus (CDH1\textsuperscript{F/F}/Alb-\textit{CreERT}; CDH1\textsuperscript{Δihep}). CDH1\textsuperscript{Δihep} and control mice were injected with TAM at 6 wk of age and fed with the HFD or ND for 12 wk (Figure 5A). No obvious phenotype was found in CDH1\textsuperscript{Δihep} ND mice, consistent with our previous report of no apparent periportal inflammation associated with the injection of adenovirus-expressing Cre recombinase in CDH1\textsuperscript{ΔF} mice. 11 Interestingly, CDH1\textsuperscript{Δihep} HFD mice showed increased numbers of small Sox9-positive hepatocyte-like cells, with no such phenotype seen in control HFD mice (Figure 5B–D). As shown in
Figure 5C, Sox-9 cells were located in both zone 1 and zone 2. CK19-positive cells were slightly increased in CDH1∆ihep HFD mice relative to both CDH1∆ihep ND and CDH1F/F HFD mice.

To determine the source of the Sox9-positive cells in CDH1∆ihep HFD mice, these mice were crossed with tdTomato mice, as described above. We found that the Sox-9-positive cells in zone 2 were derived from Alb-positive hepatocytes. These observations indicated that E-cadherin deletion in hepatocytes led to the production of hepatoblast-like cells by steatosis. These results suggested that increased Sox9-positive cell expression in CDH1∆ihep was due to both hepatocytes and BECs.

3.5 High-fat diet promotes tumorigenesis in CDH1∆L

Previously, we showed that a small percentage of male CDH1∆L mice (2/12, 16.7%) spontaneously developed liver tumors by 11 mo of age.11 In this study, we analyzed tumorigenesis in 8-mo-old CDH1∆L HFD mice over 6 mo. The overall tumor incidence was 2/11 (22.2%), representing a small, but noticeable, difference compared with CDH1∆L ND mice (0/8; 0%). The majority of the tumors in the CDH1∆L HFD mice contained both hepatocellular and ductular (cholangiocellular) components (Figure 6A, left panel); the others contained only ductular or hepatocellular component (Figure 6A, right panel). As only 2 CDH1∆L HFD mice developed spontaneous tumors, it was difficult to determine whether mixed type, hepatocellular, or cholangiocellular tumors were more common in this group.

As the incidence of tumors in CDH1∆L HFD mice was too low to analyze further, we expanded our analysis to tumor incidence in liver-specific Kras-expressing mice (Alb-cre/KrasLSLG12D.Kras). At 5 mo of age, there were no visible tumors in any of the Kras ND mice (0/8; 0%). Kras mice fed the HFD (starting at 2 mo of age) sacrificed at 5 mo old (3-mo HFD) showed visible white lesions containing histologically confirmed hepatocellular tumors (8/8/10%; Figure 6B, right panel). The number of tumor foci was increased in Kras HFD mice compared with ND controls (Figure 6D). These
results suggested that Kras HFD mice can be used as a model for hepatocarcinogenesis.

To analyze the role of cholangitis in HFD-induced hepatocarcinogenesis, Kras/CDH1ΔL mice were transitioned to an HFD at 2 mo and analyzed after 3 mo (5 mo of age). Kras/CDH1ΔL ND mice developed a small number of visible liver tumors at 5 mo of age, however the Kras/CDH1ΔL HFD group exhibited a significant increase in tumor burden compared with Kras/CDH1+/+ mice (Figure 6C,D). Histological analyses revealed a variety of tumor types, including cholangiocellular (CC) (34%), cholangiocellular/hepatocellular (Mixed) (42%) and hepatocellular (24%) (HC) tumors (Figure 6F) determined using immunostaining of CK19 for CC and HNF4α for HC tumors (Figure 6G). These results suggested that loss of E-cadherin exacerbated HFD-related increases in Ras signaling, resulting in enhanced development of both hepatocellular and cholangiocellular tumors.

**DISCUSSION**

In this study, we observed a significant increase in the extent of cholangitis and fibrosis, and in the number of bile ductules in CDH1Δliv mice fed an HFD for 7 mo compared with ND-administered mice (Figure 7). The numbers of CD44- and Sox9-positive stem cell-like cells were also increased in HFD mice.

Overweight and obese PSC patients have been found to exhibit more advanced fibrosis, as well as more rapid progression of fibrosis, compared with normal weight patients.16 Several other liver diseases are known to elicit ductular reactions such as chronic viral hepatitis.17 Therefore, an increased ductular reaction in response to HFD may be more common than previously thought and may be associated with cancer development.

The mechanism by which obesity or NAFLD leads to more aggressive inflammation and an increased ductular reaction is unknown.
We have shown that the ductular reaction in response to E-cadherin deletion is dependent on impairment of the intrahepatic biliary network. In this study, we did not observe any increase in the number of tubules in HFD-fed CDH F/F mice, suggesting that HFD itself is not sufficient to induce a ductular reaction. In contrast, CDH1\(^{\Delta L}\) showed an increased ductular reaction in response to HFD. Recent reports have suggested that pathogen-associated molecular pattern (PAMP)-sensing receptors, such as Toll-like receptors, and the subsequent expression of cytokines triggered by their activation, are important for increased ductular reaction.\(^{18,19}\) PAMPs are known to activate inflammatory cells, such as Kupffer cells and macrophages, in the liver and also induce the production of inflammatory cytokines and growth factors, such as tumor necrosis factor (TNF)-\(\alpha\) and interleukin (IL)-6. Although inflammation is an important factor seen in association with ductule formation, the factors driving their proliferation remain poorly understood. We aimed to induce the ductular reaction in TNF-\(\alpha\) knockout mice using antibiotics; however, no significant effect was seen (data not shown).

Based on these observations, we aimed to determine whether this enhanced ductular response promoted CCC. We showed that liver-specific LSL-Kras\(^{G12D}/CDH1^{\Delta L}\) with ND revealed 2-10 macroscopically visible tumors containing both hepatocellular and cholangiocellular components, and HFD administration induced the development of more numerous and aggressive CCCs. To investigate the source of cholangiocellular tumors, we crossed the CDH1\(^{\Delta L}\) and LSL-Kras\(^{G12D}\) mice, and maintained them on an HFD for 3 mo. Small precancerous lesions were found in all mice, suggesting that the cholangiocellular tumors in Kras\(^{+/}\)/CDH1\(^{\Delta L}\) mice were at least partially due to the increased expression of CK-19-positive ductular cells. The model CK19-CreERT showed relatively low efficiency of knockdown and CDH1 was expressed in around 70% of the cholangiocytes. We assume that this potentially explains the low number of tumors in the mouse line when crossed with Kras. In contrast, a previous mouse model of cholangiocarcinoma, in which hepatocytes and cholangiocytes were labeled with heritable, cell type-specific reporters, found that cholangiocarcinomas were

FIGURE 6 High-fat diet promotes tumorigenesis in CDH1\(^{L}\). A, Tumors in 8-mo-old CDH1\(^{L}\) mice fed an HFD for 6 mo (\(\times 100\) magnification). B, Tumors of Alb-cre/LSL-KrasG12D (Kras\(^{L}\)) HFD mice. Kras\(^{L}\) mice were started on an HFD at 2 mo of age and sacrificed at 5 mo (3-mo HFD). C, Macroscopic features of Kras\(^{L}\)/CDH1\(^{L}\) and Kras\(^{L}\)/CDH1\(^{L}\) mice fed the HFD. D, The number of tumor foci in Kras\(^{L}\)/CDH1\(^{L}\) (\(n = 5\)) and Kras\(^{L}\)/CDH1\(^{L}\) (\(n = 7\)) mice fed the HFD for 3 mo, starting at 2 mo of age. E, H&E staining of CC and Mixed tumors of Kras\(^{L}\)/CDH1\(^{L}\) HFD mice (\(\times 100\) magnification). F, Tumor histology in the tumors of Kras\(^{L}\)/CDH1\(^{L}\) and Kras\(^{L}\)/CDH1\(^{L}\) HFD mice. G, HNF4\(\alpha\) and CK19 immunostaining in the HC (left panel) and CC (right panel) tumors of Kras\(^{L}\)/CDH1\(^{L}\) HFD mice (\(\times 100\) magnification).
In this study, Alb-CreERT/Kras (Kras\textsuperscript{hap}) mice showed no visible tumors, but histologically small foci were observed in all mice. However, there were more than 10 visible tumors in the Kras\textsuperscript{hap}/CDH1\textsuperscript{hap} HFD mice (Figure 6D). Furthermore, the number of Sox9-positive cells was increased in the background and tumors of Kras\textsuperscript{hap}/CDH1\textsuperscript{hap} mice. To trace these cells, tdTomato mice were crossed with Kras\textsuperscript{hap}/CDH1\textsuperscript{hap}. Histological analyses showed that the majority of these cells were Tomato-positive, suggesting that Sox9 positive cells of Kras\textsuperscript{hap}/CDH1\textsuperscript{hap} mice originated from hepatocytes. Most of the tumors were AFP-positive HCC, with only a small proportion of CK19-positive CCC cells. These observations suggested that E-cadherin deleted hepatocytes, which exhibit a Sox9-positive premature NAFLD phenotype, may represent an important source of tumors.

The bile canalicular reaction is caused by the proliferation of liver stem or progenitor cells, such as oval cells. A typical bile duct reaction is characterized by an increase in relatively well-shaped bile ducts with a clear lumen, usually associated with rapid biliary obstruction. Conversely, an atypical bile duct reaction is an irregular form of bile duct hyperplasia often observed in various pathological conditions, such as cirrhosis, alcoholic liver injury, and fulminant hepatitis with inflammation and fibrosis. The atypical bile duct reaction is caused by the proliferation of existing BECs, although the underlying cause of this reaction remains unclear. However, many researchers consider the cause to be the proliferation of stem cells activated by injury, as well as the proliferation of oval cells.

Loss of E-cadherin expression is associated with mutations of the E-cadherin gene CDH1, loss of heterozygosity in 16q22.1, or a transcriptional down-regulation of CDH1 by promoter methylation.\textsuperscript{21,22} Clinically, a decrease or deletion of E-cadherin has been reported in some liver diseases. For example, both HCV and HBV proteins were shown to induce promoter hypermethylation of E-cadherin, resulting in decreased expression and induction of EMT.\textsuperscript{23,24} The presence of hypermethylated E-cadherin in serum has been linked to HCC in patients with HCV-related cirrhosis.\textsuperscript{25}

In summary, NAFLD exacerbes cholangitis and becomes a strong promoter not only of HCC, but also of CCC in mice (Figure 7). As the number of cancer patients with fatty liver continues to rise, a better understanding of the precise molecular mechanisms underlying tumor growth in both normal and NAFLD livers is needed to improve the diagnosis and treatment of these patients.

ACKNOWLEDGMENTS

SM was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (#19K08373 and #19K34567).

DISCLOSURE

All authors have no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.