

Roles of E-cadherin in Hepatocarcinogenesis

Shin Maeda and Hayato Nakagawa

Abstract Loss of E-cadherin function has been reported to be associated with progression and poor prognosis of liver cancer. However, the precise role of E-cadherin in liver cancer development has not been elucidated. Thus, we generated liver-specific E-cadherin (*Cdh1*) knockout mice (*Cdh1*^{ΔLiv}) by crossing *Cdh1*^{lox/lox} mice with *albumin-Cre* transgenic mice. Interestingly, *Cdh1*^{ΔLiv} mice developed spontaneous inflammation in the portal areas, and then developed periductal onion skin-like fibrosis, which resembled primary sclerosing cholangitis. Microarray analysis showed that expression of stem cell markers such as CD44 and Sox9, and inflammatory cytokines such as IL-6 and TNF-α, are increased in *Cdh1*^{ΔLiv} liver compared with *Cdh1*^{lox/lox} liver. To investigate the role of E-cadherin in the liver tumorigenesis, we crossed *Cdh1*^{ΔLiv} mice with *lox-stop-lox Kras*^{G12D} mice (*Kras* + *Cdh1*^{ΔLiv}). *Kras* + *Cdh1*^{ΔLiv} mice developed liver tumors at age 28 weeks (8/8, 100 %), whereas *Kras* + *Cdh1*^{lox/+} mice did not develop any tumors. Histologically, these tumors were hepatocellular carcinomas with a small proportion of ductal lesions and strongly positive for progenitor cell markers such as CD44 and Sox9. Interestingly, epithelial to mesenchymal transition (EMT) was found in the tumors of *Kras* + *Cdh1*^{ΔLiv} mice. We also found that diethylnitrosamine-induced tumorigenesis was significantly accelerated in *Cdh1*^{ΔLiv} mice. In summary, loss of E-cadherin in the liver leads to sclerosing cholangitis and promotes tumorigenesis. Its tumor-promoting function seemed to be caused by gain of stem cell properties as well as induction of EMT.

Keywords Liver cancer • E-cadherin • Stem cell marker • Inflammation • Epithelial-mesenchymal transition (EMT) • Knockout mouse

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Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer deaths worldwide; therefore, improving the prognosis has become an important issue [1]. Major HCC risk factors include infection with hepatitis B (HBV) or C viruses (HCV), alcohol and non-alcoholic steatohepatitis (NASH). The prognosis of patients with HCC has improved recently because of progress in early diagnosis and treatment, but patients with advanced HCC are still incurable. Thus, understanding the carcinogenic mechanisms and exploring new therapeutic targets for HCC has become an important issue.

Hanahan et al. showed that all cancers share common hallmarks such as proliferation or anti-cell death that govern the transformation of normal cells to cancer cells [2]. Among them, invasion and metastasis play important roles in tumor promotion and progression, and are associated with prognosis of the patients. Dysregulation of E-cadherin has been reported to contribute to cancer progression [3]. It is reported that decreased expression of E-cadherin is associated with malignant progression in various kinds of cancer, such as gastric cancer or skin cancer [4]. In liver cancers, HCC or cholangiocarcinoma (CCC), E-cadherin expression is decreased by 20–60 % and is associated with invasiveness or poor prognosis [5, 6]. These findings suggest that E-cadherin may play an important role in tumor suppression in the liver. However, so far, the precise roles of E-cadherin in liver carcinogenesis remain unclear, especially *in vivo*; thus, we examined the role of E-cadherin in liver tumorigenesis by using liver-specific E-cadherin knockout mice.

Results

Physiological Function of E-cadherin in the Liver

To determine the role of E-cadherin in the liver, we generated liver-specific E-cadherin knockout mice by crossing *Cdh1^{F/F}* and albumin-Cre transgenic mice (both from Jackson Laboratories). In *Cdh1^{F/F}* control mice, E-cadherin was expressed on the membrane of hepatocytes and interlobular biliary epithelial cells. In contrast, in *Cdh1^{ΔL}* mice, the expression was completely deleted in both hepatocytes and biliary epithelial cells (Fig. 1a). Histologically, *Cdh1^{ΔL}* liver was shown to be almost normal at 1 month; however, at 2 month of age, *Cdh1^{ΔL}* mice spontaneously developed inflammation around the portal area, and at 8 month of age, periductal onion skin-like fibrosis, which resembled human primary sclerosing cholangitis, was observed in *Cdh1^{ΔL}* mice (Fig. 1b).

According to the periductal inflammation, we hypothesized that the bile transport system might be impaired by the E-cadherin deletion from the biliary epithelial cells. To prove the hypothesis, fluorescent-labeled bile acid was injected into *Cdh1^{F/F}* and *Cdh1^{ΔL}* mice. After 15 min, in control *Cdh1^{F/F}* mice, we could see a

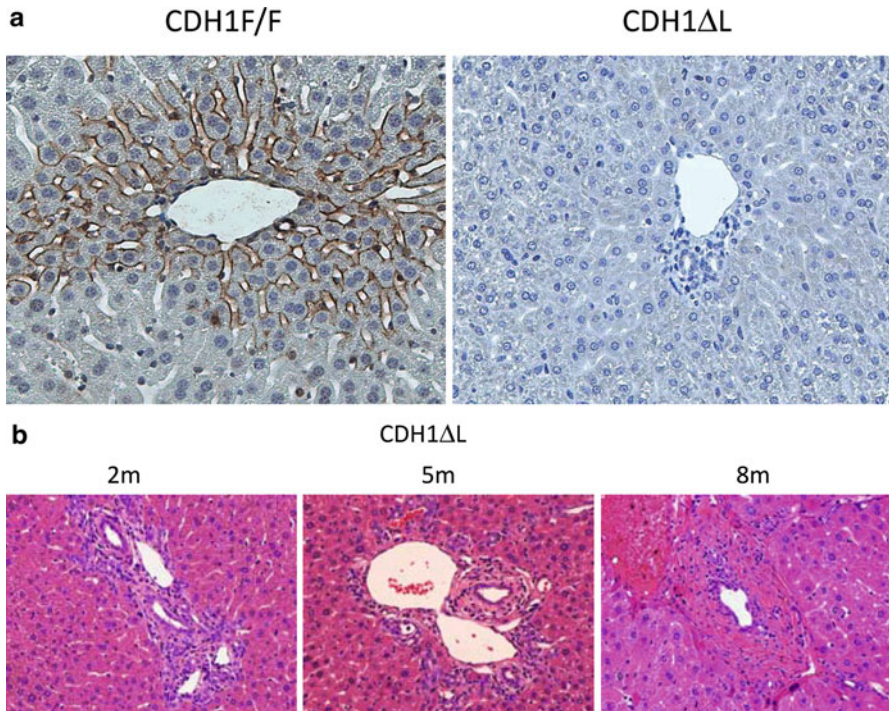


Fig. 1 (a) Analysis of E-cadherin expression by immunohistochemistry of liver sections ($\times 200$) obtained from 1-month-old $CDH1^{F/F}$ and $CDH1^{\Delta L}$ mice. (b) H&E staining of 2-, 5-, and 8-month-old $CDH1^{\Delta L}$ mice ($\times 200$)

clear canalicular pattern and bile acid was smoothly transported into the bile duct. In contrast, in $Cdh1^{\Delta L}$ mice, the canalicular staining pattern was very fuzzy and bile acid did not reach the bile duct lumen. These observations suggest that the bile canalicular network may be functionally impaired in $Cdh1^{\Delta L}$ mice, and this leads to liver injury and subsequent inflammation.

Progenitor Cell Proliferation in $CDH1^{\Delta L}$ Mice

To characterize the phenotype of $CDH1^{\Delta L}$ mice, microarray analysis was performed using whole-liver samples obtained from $CDH1^{F/F}$ and $CDH1^{\Delta L}$ mice. The expression of several hepatic progenitor cell markers, such as Sox9, CD44, or Epcam, was upregulated in $CDH1^{\Delta L}$ mice compared with $CDH1^{F/F}$ mice. The results were confirmed by immunohistochemical analysis in $CDH1^{F/F}$ and $CDH1^{\Delta L}$ mice. We also found a lot of ductal cells expressing these progenitor cell markers in the periportal area. These results suggest that ductal cells with progenitor potential are proliferating in the portal area of $CDH1^{\Delta L}$ mice.

Loss of E-cadherin Accelerates Oncogene-Addicted Liver Carcinogenesis

Because Ras signaling is frequently active in human HCC, we crossed *CDH1 Δ L* mice with active *Kras* conditional knockin (*LSL-Kras^{G12D}*) mice (*Kras/CDH1 Δ L*). All male *Kras/CDH1 Δ L* mice developed multiple liver tumors at 8 months of age ($n=10$), whereas only 4 of 10 male albumin-Cre/*LSL-Kras^{G12D}/CDH1* wild-type mice (*Kras/CDH1^{+/+}*) developed a few visible tumors (Fig. 2a). Most of the tumors arising in *Kras/CDH1 Δ L* mice were AFP-positive HCC and ranged from a well to a poorly differentiated type. On the other hand, tumors in the *Kras/CDH1^{+/+}* mice were mostly AFP-negative dysplastic nodules or well-differentiated HCC. These results suggest that loss of E-cadherin accelerates Ras-addicted liver cancer development.

We assessed activation of extracellular signal-regulated kinase (ERK), which is a major downstream transducer of Ras, in non-tumor tissue. Strong ERK phosphorylation was observed in *Kras/CDH1 Δ L* livers compared with that in *Kras/CDH1^{+/+}* livers. We thought that the increased ERK activation was one of the mechanism for the tumor acceleration in *CDH1 Δ L* mice.

Epithelial to mesenchymal transition (EMT) is considered a key process for tumor invasiveness, and loss of E-cadherin expression is a hallmark of EMT [7]. Interestingly, in some tumors in *Kras/CDH1 Δ L* mice, HCC cells gradually transformed into fibroblast-like cells, and these cells were positive for the mesenchymal marker vimentin, indicating that spontaneous EMT occurred in the tumors of these mice. EMT was shown to be associated with a gain of stem cell properties [7]. Indeed, evident expression of two stem cell markers, CD44 and Sox9, was positive in tumor cells undergoing EMT.

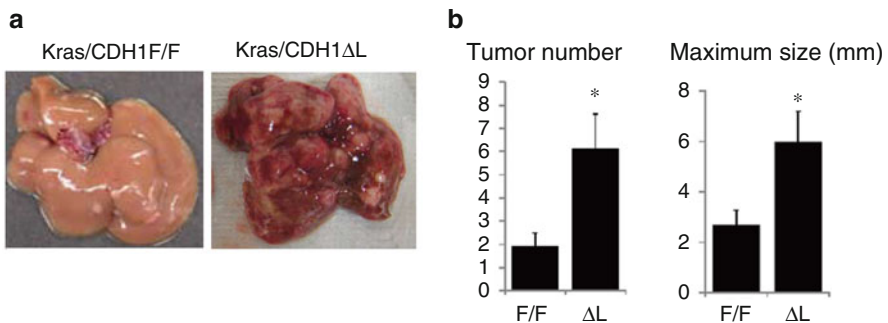


Fig. 2 (a) Representative images of the liver from 8-month-old *Kras/CDH1^{+/+}* and *Kras/CDH1 Δ L* mice. (b) Bar graphs of tumor number and tumor size in each mouse after 8 months of diethylnitrosamine (DEN) injections are shown. Data are expressed as means \pm SEMs ($n=10$ per group, $*p<0.05$)

In female *Kras/CDH1^{ΔL}* mice, only two of eight mice developed tumors by 12 months of age. This indicates gender disparity in this model of liver cancer susceptibility, as was shown in other mouse HCC models [8].

Loss of E-cadherin Promotes Chemical-Induced HCC

To further examine the role of E-cadherin in hepatocarcinogenesis, we used diethylnitrosamine (DEN) to induce a hepatocyte-derived HCC [9]. *CDH1^{F/F}* mice and *CDH1^{ΔL}* mice were injected with 25 mg/kg DEN on postnatal day 14 [10]. After 8 months, *CDH1^{ΔL}* mice showed a significantly increased number and size of liver tumors compared with *CDH1^{F/F}* mice. In addition, *CDH1^{ΔL}* mice developed histologically more advanced tumors (Fig. 2b). As in *Kras/CDH1^{ΔL}* mice, strong ERK phosphorylation was observed in tumors of DEN-treated *CDH1^{ΔL}* mice. Some tumors in *CDH1^{ΔL}* mice strongly expressed CD44 and vimentin, whereas very few tumors in *CDH1^{F/F}* mice expressed these markers. These results confirmed that loss of E-cadherin enhances activation of ERK and expression of stem cell and EMT markers in a chemically induced HCC model.

Relationship Between E-cadherin Loss and Mesenchymal and Stem Cell Markers in Human HCC

To investigate whether E-cadherin loss correlates with mesenchymal and stem cell markers in human HCC, we examined the expression of E-cadherin, CD44, and vimentin in human HCC cell lines. Significant inverse correlations were observed, particularly between E-cadherin and CD44. Among these cell lines, we chose three that expressed E-cadherin, Hep3B, HuH7, and PLC/PRF/5, and we examined the effect of E-cadherin knockdown with siRNA. All three cell lines exhibited elevated expression of mesenchymal markers such as N-cadherin and vimentin, and showed an elongated mesenchymal-like appearance. In addition, invasion capacity was significantly increased by E-cadherin knockdown, suggesting that loss of E-cadherin can be a causal factor of EMT and invasive phenotype of HCC.

Discussion

Our current data strongly suggest that E-cadherin is a tumor suppressor in the liver. Although various kinds of epithelial tumors showed decreased E-cadherin expression, there have been few reports of direct connections between E-cadherin loss and tumor progression, especially in vivo [11, 12]. In this study, when combined with

Ras activation or chemical carcinogen administration, *CDH1^{ΔL}* display markedly accelerated carcinogenesis and an invasive phenotype. Although it has been unclear whether loss of E-cadherin is a consequence or a cause of EMT, we have demonstrated its causal role *in vivo* and *in vitro*. Recent reports have established a direct link between EMT and a gain of stem/progenitor cell properties [7], which is supported by our mouse models since tumor cells undergoing EMT clearly expressed stem cell markers. The expression of stem cell markers such as CD44 and Sox9 has been reported to be associated with a poor prognosis in patients with HCC [13].

The cellular source of liver cancers still remains unclear. Recent studies suggested that mature hepatocytes could translate into not only HCC but also CCC [14]. We speculated that tumors in *Kras/CDH1^{ΔL}* mice originated from two different cell types on the basis of the pathological findings and distribution of the tumors—proliferating duct cells including progenitor cells induced by loss of E-cadherin, and mature hepatocytes transformed by Ras activation. However, to reach a firm conclusion, further analyses such as cell lineage tracing are needed, and we consider this an important future issue.

In summary, loss of E-cadherin in the liver causes impairment of the intrahepatic biliary network and subsequent inflammatory reactions. In mature hepatocytes, loss of E-cadherin leads to EMT induction, upregulation of stem cell markers, and ERK activation, which eventually results in enhanced carcinogenesis and an invasive phenotype [15]. Thus, E-cadherin plays critical roles in maintaining homeostasis and suppressing carcinogenesis in the liver.

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