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Brief Report

BRIEF REPORT

Liver-specific deficiency of TMEM30A develops spontaneous hepatocellular carcinoma

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Introduction

Hepatocellular carcinoma is the third leading cause of cancer deaths worldwide and mainly originates from chronic liver diseases [1]. It is essential to establish a stable and effective model for studying hepatocellular carcinomas. At present, chemical induction is the most common method to establish liver cancer models in mice, in which diethylnitrosamine (DEN) and carbon tetrachloride (CCl4) are the main inducers [2]. It simulates well the natural formation process of liver cancer, but the induction time is long. Human primary liver cancer organoids retain liver-tissue function and genetic stability [3]; however, the lack of other types of constituent cells cannot simulate the mechanism of cancer, which limits the range of its research. Transmembrane protein 30A (TMEM30A), a β-subunit of P4 ATPase family, catalyses the hydrolysis of ATP coupled to the transport of phosphatidylserine and phosphatidylethanolamine from the outer to the inner leaflet of various membranes and ensures the maintenance of asymmetric distribution of phospholipids [4]. *In vitro* studies have shown that TMEM30A promotes the absorption of anticancer drugs [5]. Deletion of TMEM30A can cause phosphatidylserine exposure, send out an "eat me" signal, and promote macrophage aggregation [6]. TMEM30A deficiency in hematopoietic stem cells impairs mouse fetal liver erythropoiesis [7]. In our previous work, we found that TMEM30A liver-specific knockout (LKO) mice suffered from severe cholestasis [8]. In this follow-up study, the results suggested that TMEM30A-deficient livers were accompanied by macrophage aggregation, increased inflammation, and disorder of fat metabolism. Hepatocellular carcinoma was initially observed in 14-month-old TMEM30A LKO mice. We further detailed the pathological and biochemical changes of TMEM30A-deficient mice so as to provide a mouse model for exploring the mechanism of liver metabolic disorder, inflammation, and liver cancer, as well as finding the preliminary basis for disease prevention and treatment.

To study the liver-specific role of TMEM30A, TMEM30A$^{F/F}$ mice were mated with Albumin (Alb)-Cre^{+} tool mice to obtain TMEM30A$^{F/F}$ Alb-Cre^{+} mice, i.e. TMEM30A LKO mice [8]. TMEM30A$^{F/F}$ mice were crossed with TMEM30A$^{F/F}$ Alb-Cre^{+} mice to further expand reproduction. TMEM30A$^{F/F}$ mice were used as littermate wild-type controls.

Results

For observing spontaneous tumor progressive development, we checked TMEM30A LKO and control mice livers every ~2 months according to the health status of the mice as well as

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animal ethics and welfare (approval No. HSD20150301). The liver histologic findings were examined because intrahepatic cholestasis often induces liver damage. We started to observe liver-cell proliferation and inflammatory cell infiltration in 2-month-old TMEM30A LKO mice (Figure 1A). Macrophage infiltration in the edge of livers (Supplementary Figure 1A) and liver fibrosis (Figure 1B) appeared in 2-month-old TMEM30A LKO mice. Ki67 immunohistochemistry showed signs of multinucleated cell infiltration and hepatocyte swelling in 9-month-old TMEM30A LKO mice (Figure 1A) and F4/80 immunohistochemistry analysis revealed obvious macrophage infiltration in 7-month-old TMEM30A LKO mice (Figure 1C). Wild-type controls showed almost no macrophage infiltration. Masson blue staining showed that liver fibrosis was distinctly aggravated in 9-month-old TMEM30A LKO mice (Figure 1B). Precancerous lesions were found in the liver of TMEM30A LKO mice at the age of 10 months (Figure 1D). Thus, hepatic TMEM30A deficiency induced inflammatory infiltration and liver fibrosis in mice.

In further research, multiple tumor nodules had appeared already on macroscopic examination of the livers in TMEM30A LKO mice after 14, 16, 20, and 23 months (Figure 1E). These spontaneous tumor nodules had obvious scattered inflammatory infiltration, accompanied by fat accumulation, smaller tumor nuclei, and apparent heterogeneity. At the border of the tumor nodules, hepatocytes shortened and the nuclear density increased. The adjacent hepatocytes became rounded, with hepatic plates disappeared and the structure disordered (Figure 1D). Proliferating liver cells (Supplementary Figure 1B) and the degree of liver fibrosis (Supplementary Figure 1C) increased obviously in tumor nodules of TMEM30A-deficient livers. These results suggested for the first time that liver-specific knockout of P4-type ATPase phospholipid invertase subunit TMEM30A causes primary hepatocellular carcinoma.

According to reports, aberrant NF-κB activation promotes bile-acid-related hepatocarcinogenesis [9]; p65 immunohistochemistry demonstrated that the expression level of p65 did not elevate in the livers of TMEM30A LKO mice (Supplementary Figure 1D). Real time-PCR results showed that the downstream proteins were regulated by farnesoid X-activated receptor alpha (FXRα), just as relative levels of tumor-suppressor genes *NDRG2* and *SHP* were significantly decreased (Supplementary Figure 1E), which may lead to spontaneous liver cancer in TMEM30A LKO mice.

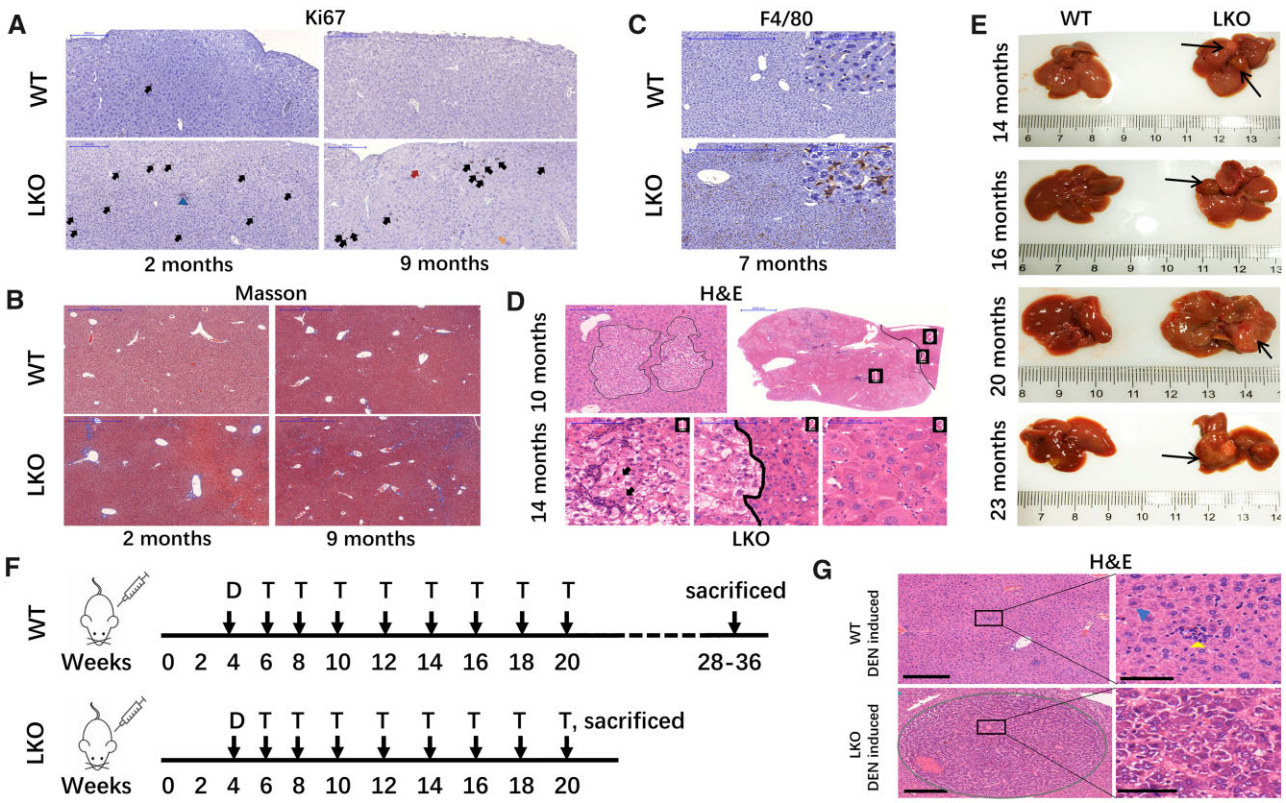

Figure 1. TMEM30A LKO mice develop hepatoma. (A) Immunohistochemistry staining for Ki67 in the livers of TMEM30A LKO mice and WT mice at the ages of 2 and 9 months. Ki67-positive cells (black arrow), inflammatory cell infiltration (blue triangle), multinucleated cell infiltration (orange arrow), liver-cell swelling (dark red arrow). (B) Masson blue staining showed the degree of liver fibrosis in TMEM30A LKO mice and WT mice at the ages of 2 and 9 months. (C) Immunohistochemistry staining for the F4/80 macrophage marker in livers of TMEM30A LKO mice and WT mice at the age of 7 months. (D) H&E staining showed the histological changes of liver tumors and paracancerous tissues. Premalignant lesions appear in TMEM30A-deficient livers at the age of 10 months (inside the black circle). Malignant lesions appear in TMEM30A-deficient livers at the age of 14 months. Tumor (1), the border of tumor nodules (2), adjacent tissue (3). (E) Macroscopic exogenous spontaneous liver tumors appear with age after 14, 16, 20, and 23 months in TMEM30A LKO mice. Hepatocellular carcinoma (black arrow). (F) DEN + TCPOBOP modeling diagram. The whole modeling process of DEN + TCPOBOP led to liver cancer in WT C57 mice and TMEM30A LKO mice. Intraperitoneal injection with 100 mg/kg DEN (D),with 3 mg/kg TCPOBOP (T). (G) H&E staining results of livers in DEN-induced WT mice and TMEM30A LKO mice of 20 weeks old. Inflammatory cell aggregation (yellow triangle), liver-cell swelling (blue arrow), and primary hepatocellular carcinoma (green circle). Scale bars: 100 μm (left) and 50 μm (right).
LKO, liver-specific knockout; WT, wild-type; H&E, hematoxylin and eosin; DEN, diethylnitrosamine; TCPOBOP, 1,4-Bis [2-(3,5-Dichloropyridyloxy)] benzene.

We next established a hepatocellular carcinoma model of TMEM30A LKO mice induced by DEN + TCPOBOP (1,4-Bis [2–(3,5-Dichloropyridyloxy)] benzene). Mice were intraperitoneally injected with DEN at 4 weeks old, followed by eight sequential injections with TCPOBOP biweekly from 6 to 20 weeks old to complete the modeling (Figure 1F). Mice were sacrificed at the age of 28–36 weeks. The liver form and histological changes were examined in a DEN + TCPOBOP induction model. TMEM30A-deficient liver tissues were swollen and white cancerous lesions could be obviously seen by nude eyes (Supplementary Figure 1F). After the modeling, hematoxylin and eosin (H&E) staining revealed there were liver histomorphology disorder, inflammatory cell aggregation, and hepatocytes swelling, but no significant tumor-like cell lesion showed in the wild-type controls of the model induced by DEN + TCPOBOP. Primary hepatocellular carcinomas appeared in TMEM30A LKO mice of this model and were characterized by hepatocytes morphological disorder, pathological karyokinesis, and vacuolar cells (Figure 1G). These results showed that the DEN + TCPOBOP-induced hepatocellular carcinoma model could establish in 20-week-old TMEM30A LKO mice.

Serum biochemistry variables were also studied after DEN + TCPOBOP treatment. Liver swelling was one of the clinical manifestations of hepatocellular carcinoma. Liver-to-body weight ratios in TMEM30A LKO mice were higher than that in the controls (Supplementary Figure 1G). In addition, TMEM30A LKO mice showed anemia with decreased leukocytes and erythrocytes (Supplementary Figure 1G). Compared with the control group, cholyglycine levels were significantly increased in the TMEM30A LKO group (Supplementary Figure 1G), suggesting liver injury such as inflammation and necrosis. Serum total bile acid levels in the TMEM30A LKO group were also elevated compared with the control group (Supplementary Figure 1G), indicating that the liver cholestasis of TMEM30A LKO mice still exists in DEN + TCPOBOP modeling. From these findings, hepatic deletion of TMEM30A also resulted in liver damage and impaired liver function after DEN + TCPOBOP modeling.

Discussion

In summary, the results demonstrated that primary hepatocellular carcinoma was observed at the beginning of 14 months old in TMEM30A LKO mice. After DEN + TCPOBOP treatment, hepatocellular carcinoma was successfully induced within 4.5 months. Additionally, the characteristics of cholestasis, inflammatory cell aggregation, and metabolic disorder in TMEM30A LKO mice are closer to the pathogenesis of clinical patients with primary liver cancer. It enriches the model of metabolic-related primary hepatocellular carcinoma and expands the application scope of TMEM30A LKO mice as a model of liver cancer.

Supplementary Data

Supplementary data is available at *Gastroenterology Report* online.

Authors' Contributions

L.Z., L.S., and Y.Z. designed the experiments; L.L. was responsible for maintenance of the experimental mice; L.L., Z.X., and J.S. performed the histologic and immunohistochemistry, the RT-qPCR experiments, and serum biochemical analysis; Z.X. and L.Z. wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest

None declared.

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