Liver Cancer

Research Article

Liver Cancer 2023;12:57–71
DOI: 10.1159/000525546

Received: December 17, 2021
Accepted: June 4, 2022
Published online: September 16, 2022

Dietary Fat Composition Affects Hepatic Angiogenesis and Lymphangiogenesis in Hepatitis C Virus Core Gene Transgenic Mice

Pan Diao[a] Yaping Wang[a, b] Fangping Jia[a, c] Xiaojing Wang[a, d] Xiao Hu[a, e] Takefumi Kimura[f] Yoshiko Sato[g] Kyoji Moriya[h] Kazuhiko Koike[i] Jun Nakayama[g] Naoki Tanaka[j, k, l]

[a]Department of Metabolic Regulation, Shinshu University School of Medicine, Matsumoto, Japan; [b]Department of Basic Nursing, Hebei Medical University, Shijiazhuang, PR China; [c]State Key Laboratory for Diagnosis and Treatment of Infectious Disease, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Zhejiang Provincial Key Laboratory for Drug Clinical Research and Evaluation, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, PR China; [d]Department of Gastroenterology, Lishui Hospital, Zhejiang University School of Medicine, Lishui, PR China; [e]Department of Pathophysiology, Hebei Medical University, Shijiazhuang, PR China; [f]Department of Gastroenterology, Shinshu University School of Medicine, Matsumoto, Japan; [g]Department of Molecular Pathology, Shinshu University School of Medicine, Matsumoto, Japan; [h]Department of Infection Control and Prevention, The University of Tokyo, Tokyo, Japan; [i]Department of Gastroenterology, The University of Tokyo, Tokyo, Japan; [j]Department of Global Medical Research Promotion, Shinshu University Graduate School of Medicine, Matsumoto, Japan; [k]International Relations Office, Shinshu University School of Medicine, Matsumoto, Japan; [l]Research Center for Social Systems, Shinshu University, Matsumoto, Japan

Keywords
Growth factors · Angiogenesis · Lymphangiogenesis · Hypoxia-inducible factor · Hepatocellular carcinoma

Abstract
Introduction: Previous research has demonstrated that an isocaloric diet rich in trans-fatty acid (TFA), saturated fatty acid (SFA), and cholesterol (Chol) promoted steatosis-derived hepatic tumorigenesis in hepatitis C virus core gene transgenic (HCVcpTg) mice in different manners. Growth factor signaling and ensuing angiogenesis/lymphangiogenesis are key factors in hepatic tumorigenesis that have become recent therapeutic targets for hepatocellular carcinoma. However, the influence of dietary fat composition on these factors remains unclear. This study investigated whether the type of dietary fat would have a specific impact on hepatic angiogenesis/lymphangiogenesis in HCVcpTg mice. ***Methods:*** Male HCVcpTg mice were treated with a control diet, an isocaloric diet containing 1.5% cholesterol (Chol diet), or a diet replacing soybean oil with hydrogenated coconut oil (SFA diet) for a period of 15 months or with shortening (TFA diet) for 5 months. The degree of angiogenesis/lymphangiogenesis and the expression of growth factors, including fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF), were evaluated in non-tumorous liver tissues using quantitative mRNA measurement, immunoblot analysis, and immunohistochemistry. ***Results:*** Long-term feeding of SFA and TFA diets to HCVcpTg mice increased the expres-

Karger@karger.com
www.karger.com/lic

Correspondence to:
Naoki Tanaka, naopi@shinshu-u.ac.jp

sions of vascular endothelial cell indicators, such as CD31 and TEK receptor tyrosine kinase, in addition to lymphatic vessel endothelial hyaluronan receptor 1, indicating that angiogenesis/lymphangiogenesis were upregulated only by these fatty acid-enriched diets. This promoting effect correlated with elevated VEGF-C and FGF receptor 2 and 3 levels in the liver. c-Jun N-terminal kinase (JNK) and hypoxia-inducible factor (HIF) 1α, both key regulators of VEGF-C expression, were enhanced in the SFA- and TFA-rich diet groups as well. The Chol diet significantly increased the expressions of such growth factors as FGF2 and PDGF subunit B, without any detectable impact on angiogenesis/lymphangiogenesis. ***Conclusion:*** This study revealed that diets rich in SFA and TFA, but not Chol, might stimulate hepatic angiogenesis/lymphangiogenesis mainly through the JNK-HIF1α-VEGF-C axis. Our observations indicate the importance of dietary fat species for preventing hepatic tumorigenesis.

Introduction

Hepatocellular carcinoma (HCC) is a well-known hypervascular tumor and the fourth leading cause of cancer-related deaths worldwide [1, 2]. Hepatitis B virus, hepatitis C virus (HCV), and alcohol consumption have been recognized as the major risk factors for HCC [3], while nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) from excessive intake of dietary fat are becoming prominent causes of HCC as well [4–8]. Cholesterol (Chol) and saturated fatty acid (SFA) are typical lipids that are high in the diets of developed countries. As an essential structural component of animal cell membranes [9], Chol is abundant in animal foods, including eggs, cheese, shellfish, meats, sardines, and full-fat yoghurt [10]. SFA is a fatty acid lacking double carbon bonds that is contained mainly in animal and plant products, including dairy products, red meat, palm oil, and coconut oil [4]. Trans-fatty acid (TFA) is an unsaturated fatty acid with one or more unconjugated double bonds in the trans configuration. In the trans configuration, the two hydrogen atoms around the double bond point in opposite directions, whereas in the cis configuration, these atoms point in the same direction, leading to a curved conformation [5, 11]. TFA is naturally found in dairy products and animal meat in low amounts but can be produced in large quantities during the partial hydrogenation of vegetable oil, which is widely used in food manufacturing, commercial cooking, and frying [5, 11]. Previous studies have shown that excessive intake of Chol, SFA, and TFA in the daily diet may lead to metabolic syndrome, obesity, type 2 diabetes, cardiovascular disease, and NAFLD/NASH [12–14].

Our earlier research showed that the elevated consumption of Chol, SFA, and TFA all led to the occurrence of liver tumors in HCV core gene transgenic (HCVcpTg) mice [4–6]. Long-term feeding of Chol-, SFA-, and TFA-rich diets can enhance oxidative stress, endoplasmic reticulum stress, nuclear factor kappa B (NF-κB) signaling, the p62-nuclear factor erythroid 2-related factor 2 axis, and hepatocyte proliferation. It was noteworthy, however, that specific carcinogenic pathways were activated by different dietary fats. For instance, long-term excessive intake of Chol and TFA resulted in hepatic fibrosis, while that of SFA did not stimulate the fibrotic pathway. On the other hand, the hepatic de novo fatty acid (FA)-synthesizing pathway, a key component of the Warburg effect in cancer cells, was selectively enhanced in mice treated with long-term SFA and TFA diets. Additionally, β-catenin signaling, a key driver of liver tumorigenesis, was found to be highly expressed only in mice fed a TFA diet [4–6, 15]. The above results indicated that different types of dietary fats impacted various signaling/metabolic pathways in a type-specific manner.

The hypervascular nature of HCC underscores the importance of angiogenesis in liver tumorigenesis [2]. Lymphangiogenesis increases the metastasis and spread of HCC, which have a significant impact on tumor progression, recurrence, and outcome in HCC patients [16]. Several growth factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF), play a crucial role in hepatic angiogenesis, cell proliferation, and tumorigenesis, the signalings of which are now promising targets for treating HCC [2]. Indeed, sorafenib, a commonly used multi-kinase inhibitor for HCC, blocks VEGF receptor (VEGFR) and PDGF receptor (PDGFR)-β [17, 18]. Furthermore, lenvatinib, a second generation multi-kinase inhibitor, inhibits not only VEGFR and PDGFR-α but also FGF receptor (FGFR) [2, 19].

Previous studies have demonstrated that excessive intake of Chol, SFA, and TFA can promote the occurrence of liver tumors in HCVcpTg mice [4–6]. However, the influence of these fat-intensified diets on hepatic angiogenesis/lymphangiogenesis and the expression of growth factors remains unclear [4–6]. To address this issue, we evaluated the degree of angiogenesis/lymphangiogenesis and related growth factor changes in HCVcpTg mice fed isocaloric Chol-, SFA-, and TFA-rich diets.

Materials and Methods

Mice and Treatment

HCVcpTg mice were generated as reported previously [20, 21]. Male 8- to 12-week-old HCVcpTg mice weighing 25–30 g were treated for 15 months with a purified control (Con) diet (n = 17), Chol diet (n = 13), or SFA-rich diet (n = 10), as described previously [4, 6]. In addition, male HCVcpTg mice were treated for 5 months with a TFA-rich diet after 10-month Con diet feeding (n = 21) [5]. The detailed composition of the diets is shown in online Supplementary Table 3 (for all online suppl. material, see www.karger.com/doi/10.1159/000525546). All animal experiments were conducted in adherence to the animal research methods outlined in the "Guidelines for the care and use of laboratory animals" and this study protocol was reviewed and approved by the Shinshu University School of Medicine (approval number #190147). After treatment, the mice were sacrificed by CO_2 asphyxiation prior to blood and liver tissue collection. Blood samples were centrifuged at 3,000 rpm for 15 min twice to obtain serum, and all samples were stored at −80°C until use.

Quantification of mRNA Levels

Total RNA in non-tumorous liver tissue was extracted using an RNeasy Mini Kit (Qiagen, Tokyo, Japan), and 1 μg of total RNA was reverse-transcribed using oligo-dT and random primers with a PrimeScript RT Reagent Kit (Perfect Real Time, Takara Bio Inc., Shiga, Japan). mRNA levels were determined by real-time quantitative polymerase chain reaction (qPCR) using SYBR Premix Ex Taq II (Takara Bio Inc.) on an Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument (Thermo Fisher Scientific, Waltham, MA, USA) [4–6, 22]. The primer pairs used for qPCR are shown in online Supplementary Table 1. The data were normalized to those of 18S ribosomal RNA (18S rRNA) and the mRNA levels of the target molecules were expressed as fold-changes relative to those of the Con diet group.

Immunoblot Analysis

After homogenizing 20 mg of non-tumorous liver tissue, the protein concentrations of whole lysates were measured as described previously [4]. Briefly, whole liver lysates (45 μg of protein) were loaded into each lane and separated by 7.5–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Afterward, the proteins were transferred to polyvinylidene fluoride membranes (G9903119, Amersham, Freiburg, Germany) or nitrocellulose filter membranes (G9935264, Amersham). The membranes were blocked by 6–10% nonfat dry milk or 10% bovine serum albumin in Tris-buffered saline for 1 h and incubated overnight with the appropriate antibodies shown in online Supplementary Table 2. Next, they were washed four times with Tris-buffered saline containing 1% Tween 20 and were incubated with horseradish peroxidase-conjugated secondary antibodies (#SA00001-2, Proteintech Group, Rosemont, IL, or #112-035-003, Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 1:10000 dilution for both antibodies) for detecting the chemiluminescent signal of target proteins using a myECL Imager System (Thermo Fisher Scientific). The positions of the target proteins were determined by co-electrophoresing a molecular weight marker (2.5 μL/well, PM2500, Smobio, Hsinchu, Taiwan). Based on the preliminary immunoblot analysis, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was adopted as a loading control due to minimal differences between the samples. NIH Image J software (National Institutes of Health, Bethesda, MD, USA) was used to quantify band intensity before normalizing to GAPDH and expressing as fold-changes relative to those of HCVcpTg mice fed the Con diet.

Immunohistochemistry

Liver tissues were fixed in 10% neutral formalin, embedded in paraffin, and cut into 4 μm sections. For immunostaining of CD31 and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1), antigen retrieval was carried out by microwaving tissue sections in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA for 30 min. The sections were, respectively, incubated for 1 h with CD31 antibody (DIA-310, Dianova, Hamburg, Germany, 1:100 dilution) and LYVE1 antibody (103-PA50AG, Relia Tech GmbH, Wolfenbüttel, Germany, 1:200 dilution). For secondary antibodies, Histofine Simple Stain Mouse MAX-PO (rat) and Histofine Simple Stain MAX-PO (R), both purchased from Nichirei (Tokyo, Japan) were, respectively, used. Peroxidase activity was visualized using a diaminobenzidine-hydrogen peroxide solution.

Biochemical Analysis

Serum insulin-like growth factor (IGF) 1 was evaluated using a mouse IGF1 ELISA kit (R&D Systems, Minneapolis, MN, USA) [22].

Statistical Analysis

All results were compared with the control group. Measured findings are expressed as the mean ± standard deviation. Two-tailed Student's t tests were conducted using SPSS statistics version 22 (IBM, Armonk, NY, USA). A p value of less than 0.05 was considered statistically significant.

Results

SFA and TFA Diets, but Not Chol Diet, Promoted Hepatic Angiogenesis in HCVcpTg Mice

Since the development of HCC is closely related to angiogenesis, we first evaluated angiogenesis markers by qPCR analysis. The expression of mRNA encoding *Cd34* was significantly increased in the Chol-, SFA-, and TFA-rich diet-treated HCVcpTg mice as compared with the Con diet, while the mRNA levels of *Cd31* and tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (*Tie1*) and TEK receptor tyrosine kinase *(Tie2*) were significantly increased by the SFA and TFA diets but not the Chol diet (Fig. 1a). The increases in CD31 and TIE2 proteins were confirmed by immunoblot analysis (Fig. 1b, c). Immunohistochemical analysis showed that CD31 was positive for endothelial cells and CD31-positive endothelial cells were increased following the SFA and TFA diets (Fig. 1d), which ruled out the possibility that CD31 was ectopically overexpressed, e.g., by hepatocytes or macrophages. The above results demonstrated that the SFA and TFA diets, but not the Chol diet, promoted angiogenesis in non-tumorous liver tissues.

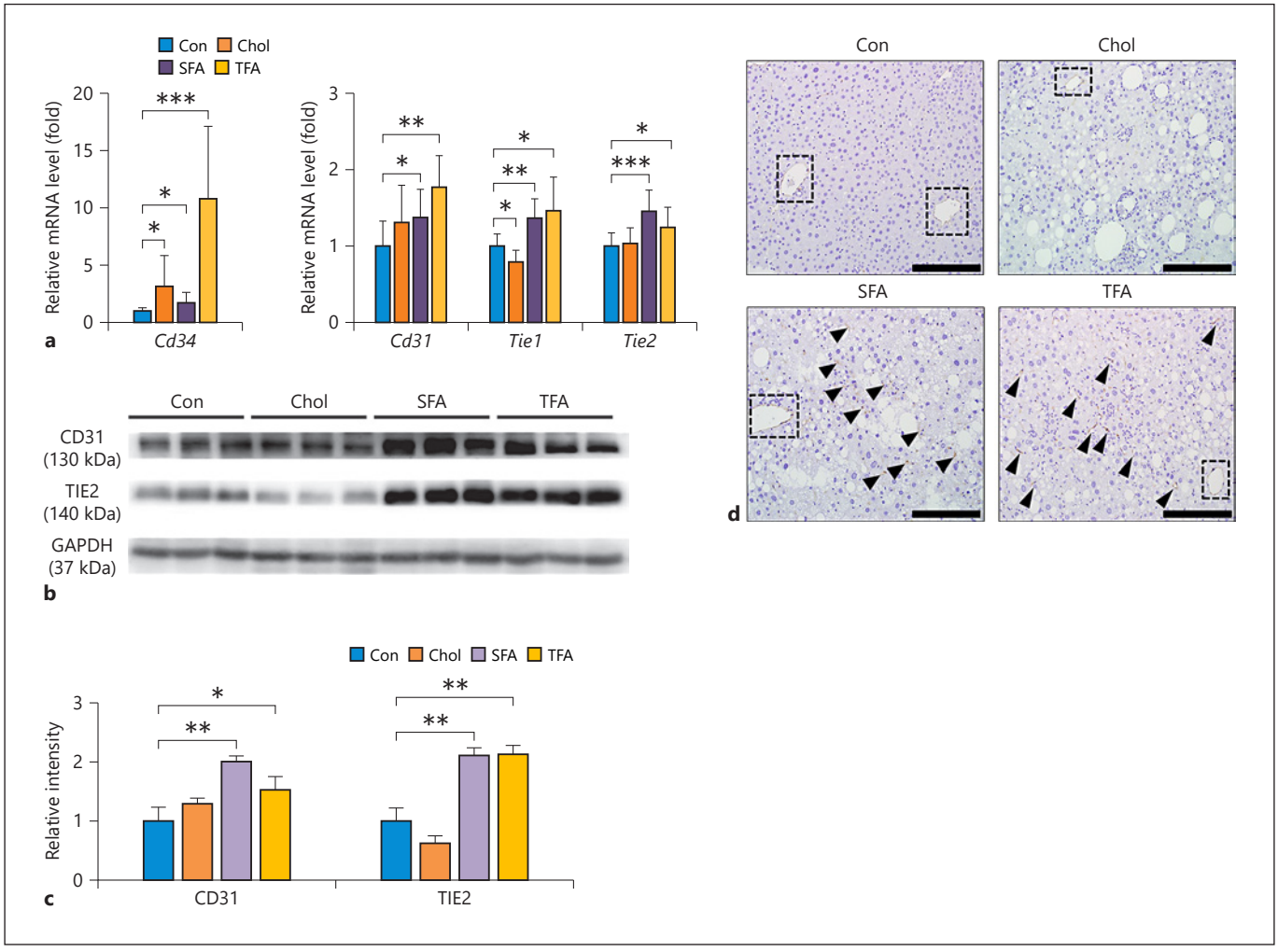


Fig. 1. SFA and TFA diets promote angiogenesis in non-tumorous liver tissues. **a** Hepatic mRNA levels of angiogenesis markers were quantified by qPCR, normalized to 18S rRNA, and expressed as values relative to HCVcpTg mice fed a control diet. The number of mice in the control diet-fed and Chol-, SFA-, and TFA-rich diet-fed groups was 8, 7, 8, and 8, respectively. **b**, **c** Immunoblot analysis of CD31 and TIE2. Whole-liver homogenates (45 μg of protein) were loaded into each well. GAPDH bands were used as the loading control. Band intensities were measured densitometrically, normalized to the loading control, and expressed as values relative to those of control diet mice. The samples from 3 mice in each group were adopted. Results were obtained from two independent immunoblot experiments. **d** Immunohistochemical analysis of CD31. Scale bar, 200 μm. CD31-positive endothelial cells were increased following SFA and TFA diets in comparisons of the areas indicated by arrowheads. Dashed squares indicate portal/central veins. Data are expressed as the mean ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ between control diet-fed and Chol-, SFA-, and TFA-rich diet-fed HCVcpTg mice. Con, control diet-fed HCVcpTg mice; Chol, cholesterol-rich diet-fed HCVcpTg mice; SFA, saturated fatty acid-rich diet-fed HCVcpTg mice; TFA, trans-fatty acid-rich diet-fed HCVcpTg mice; SD, standard deviation.

SFA and TFA Diets, but Not Chol Diet, Promoted Hepatic Lymphangiogenesis in HCVcpTg Mice

We next assessed the degree of lymphangiogenesis in animals fed the test diets. The expressions of mRNA encoding podoplanin (*Pdpn*) and ephrin-B2 (*Efnb2*) were similar among the groups (Fig. 2a), while the mRNA level of LYVE1 (*Lyve1*) was significantly increased by the SFA and TFA diets (Fig. 2a). Immunoblot analysis confirmed the increased expression of LYVE1 in the SFA and TFA groups (Fig. 2b, c). Immunohistochemical analysis revealed increased LYVE1-positive endothelial cells following the SFA and TFA diets (Fig. 2d). The SFA- and TFA-rich diets, but not the Chol-rich diet, were therefore thought to enhance hepatic angiogenesis/lym-

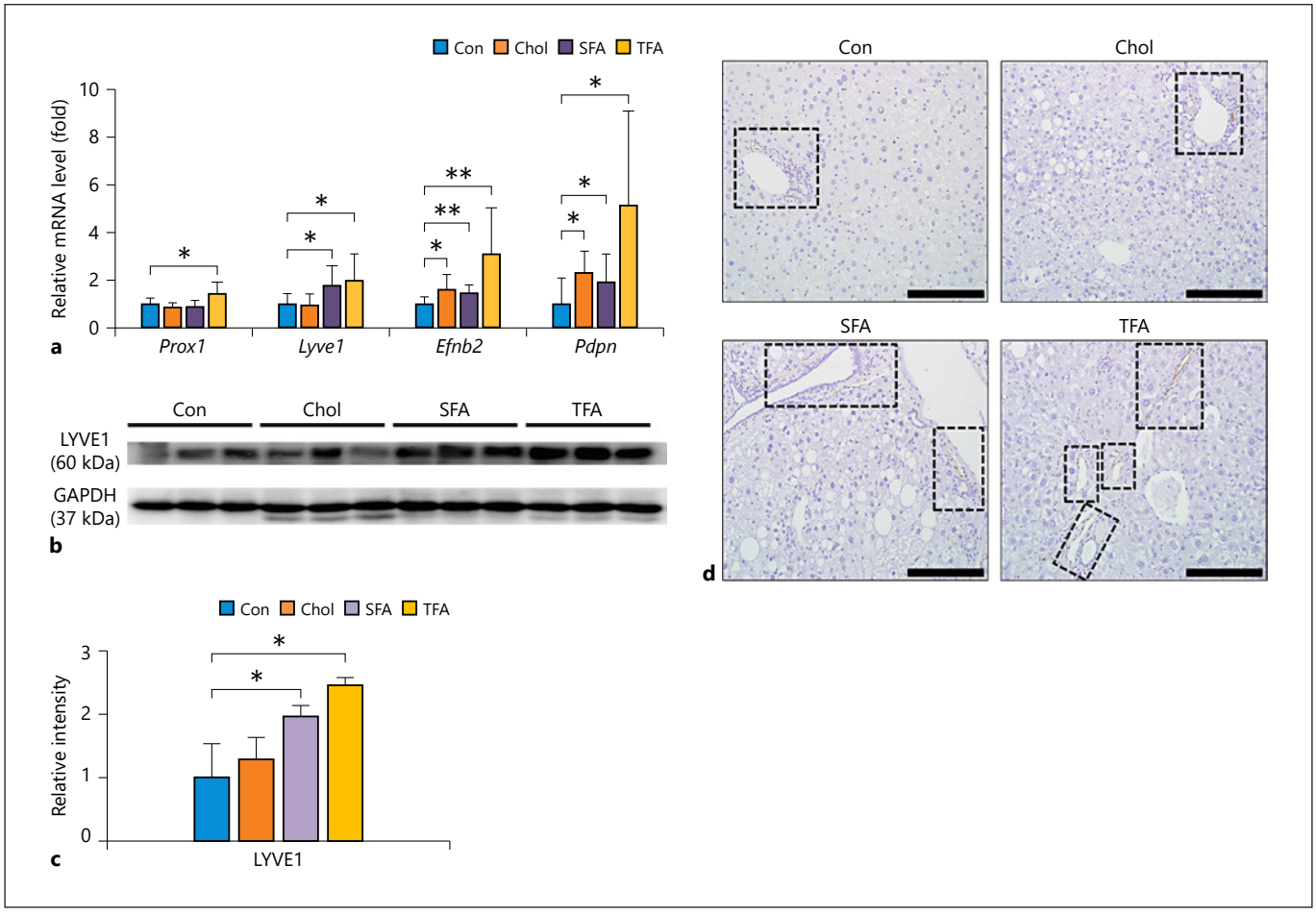


Fig. 2. SFA and TFA diets promote lymphangiogenesis in non-tumorous liver tissues. **a** Hepatic mRNA levels of lymphangiogenesis markers were quantified by qPCR, normalized to 18S rRNA, and expressed as values relative to HCVcpTg mice fed a control diet. The number of mice in the control diet-fed and Chol-, SFA-, and TFA-rich diet-fed groups was 8, 7, 8, and 8, respectively. **b**, **c** Immunoblot analysis of LYVE1. Whole-liver homogenates (45 μg of protein) were loaded into each well. GAPDH bands were used as the loading control. Band intensities were measured densitometrically, normalized to the loading control, and expressed as values relative to those of control diet mice. The samples from 3 mice in each group were adopted. Results were obtained from two independent immunoblot experiments. **d** Immunohistochemical analysis of LYVE1. Scale bar, 200 μm. LYVE1-positive endothelial cells were increased following SFA and TFA diets in comparisons of the areas indicated by dashed squares. Data are expressed as the mean ± SD. *$p < 0.05$ and **$p < 0.01$ between control diet-fed and Chol-, SFA-, and TFA-rich diet-fed HCVcpTg mice. Con, control diet-fed HCVcpTg mice; Chol, cholesterol-rich diet-fed HCVcpTg mice; SFA, saturated fatty acid-rich diet-fed HCVcpTg mice; TFA, trans-fatty acid-rich diet-fed HCVcpTg mice; SD, standard deviation.

phangiogenesis in non-tumorous liver tissues of HCVcpTg mice.

SFA and TFA Diets, but Not Chol Diet, Upregulated Hepatic VEGF-C Expression in HCVcpTg Mice

To investigate the mechanism of how the SFA and TFA diets exacerbated hepatic angiogenesis/lymphangiogenesis, the expressions of VEGF and their receptors in the liver were determined. Although *Vegfa* and *Vegfr1* mRNA levels were, respectively, increased by the TFA- and SFA-rich diets, the mRNA levels of *Vegfb*, *Vegfc*, *Vegfr2*, and *Vegfr3* were increased by all three diets (Fig. 3a). Immunoblot analysis confirmed the elevated hepatic expression of VEGF-C, a powerful driver of angiogenesis and lymphangiogenesis, by the SFA- and TFA-rich diets. the VEGFR2 protein level was only significantly increased in SFA-rich diet-fed HCVcpTg mice (Fig. 3b, c). Taken together, the augmentation of VEGF-C appeared to correlate with the enhancement of hepatic angiogenesis/lymphangiogenesis caused by

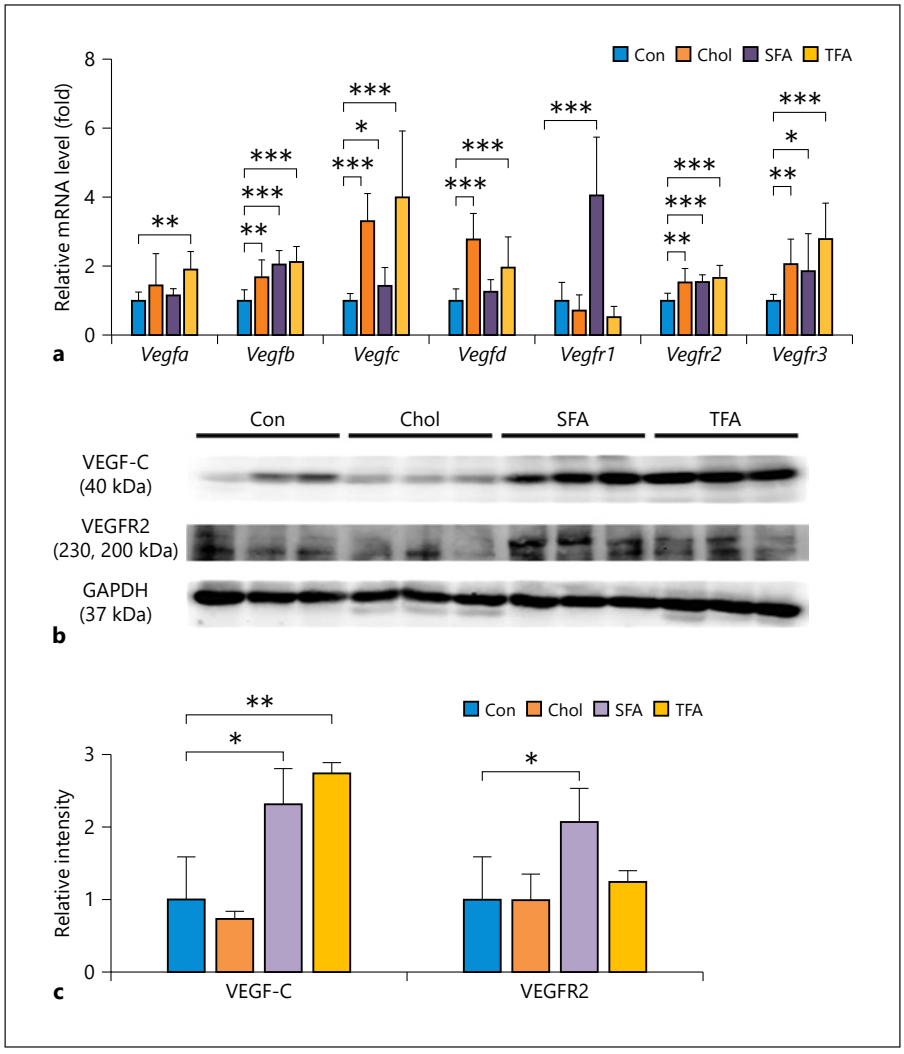


Fig. 3. Effect of dietary fats on hepatic VEGF signaling in HCVcpTg mice. **a** Hepatic mRNA levels of genes related to VEGF signaling were quantified by qPCR, normalized to 18S rRNA, and expressed as values relative to HCVcpTg mice fed a control diet. The same samples used in Figures 1 and 2 were adopted. The number of mice in the control diet-fed and Chol-, SFA-, and TFA-rich diet-fed groups was 8, 7, 8, and 8, respectively. **b**, **c** Immunoblot analysis of VEGF-C and VEGFR2. Whole-liver homogenates (45 μg of protein) were loaded into each well. GAPDH bands were used as the loading control. Band intensities were measured densitometrically, normalized to the loading control, and expressed as values relative to those of control diet mice. The samples from 3 mice in each group were adopted. Results were obtained from two independent immunoblot experiments. Data are expressed as the mean ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ between control diet-fed and Chol-, SFA-, and TFA-rich diet-fed HCVcpTg mice. Con, control diet-fed HCVcpTg mice; Chol, cholesterol-rich diet-fed HCVcpTg mice; SFA, saturated fatty acid-rich diet-fed HCVcpTg mice; TFA, trans-fatty acid-rich diet-fed HCVcpTg mice; SD, standard deviation.

persistent SFA- and TFA-rich diet consumption in HCVcpTg mice.

SFA and TFA Diets, but Not Chol Diet, Upregulated Hepatic FGFR2/FGFR3 Expression in HCVcpTg Mice

Since it was well recognized that FGF enhanced tumor cell survival and neoangiogenesis [23], the mRNA levels of FGF and their receptors were determined next. The levels of mRNA encoding *Fgf2* and *Fgfr2* were significantly increased in the Chol, SFA, and TFA groups. However, *Fgf15* mRNA expression was upregulated in the SFA-rich diet group alone. The mRNA level of *Fgfr3* was significantly increased in the SFA and TFA groups, while that of *Fgfr4* was unchanged (Fig. 4a). Immunoblot analysis detected increased FGF2 in the Chol and TFA groups and increased FGFR2/FGFR3 in the SFA and TFA groups (Fig. 4b, c). The increases in FGFR2/FGFR3 were considered to be associated with enhanced hepatic angiogenesis/lymphangiogenesis by persistent SFA- and TFA-rich diet feeding.

Effect of Dietary Fat Composition on Hepatic PDGF Signaling in HCVcpTg Mice

The expressions of mRNA encoding PDGF (*Pdgfa, Pdgfb, Pdgfc*, and *Pdgfd*) and their receptors (*Pdgfra* and *Pdgfrb*) were significantly increased in all three test diet-fed HCVcpTg mice as compared with the Con diet (Fig. 5a). The hepatic expression of PDGF-B was significantly upregulated in the Chol and TFA groups but not in the SFA group. The protein level of PDGFR-α was significantly elevated in HCVcpTg mice fed any of the test diets (Fig. 5b, c).

Liver Cancer 2023;12:57–71
DOI: 10.1159/000525546

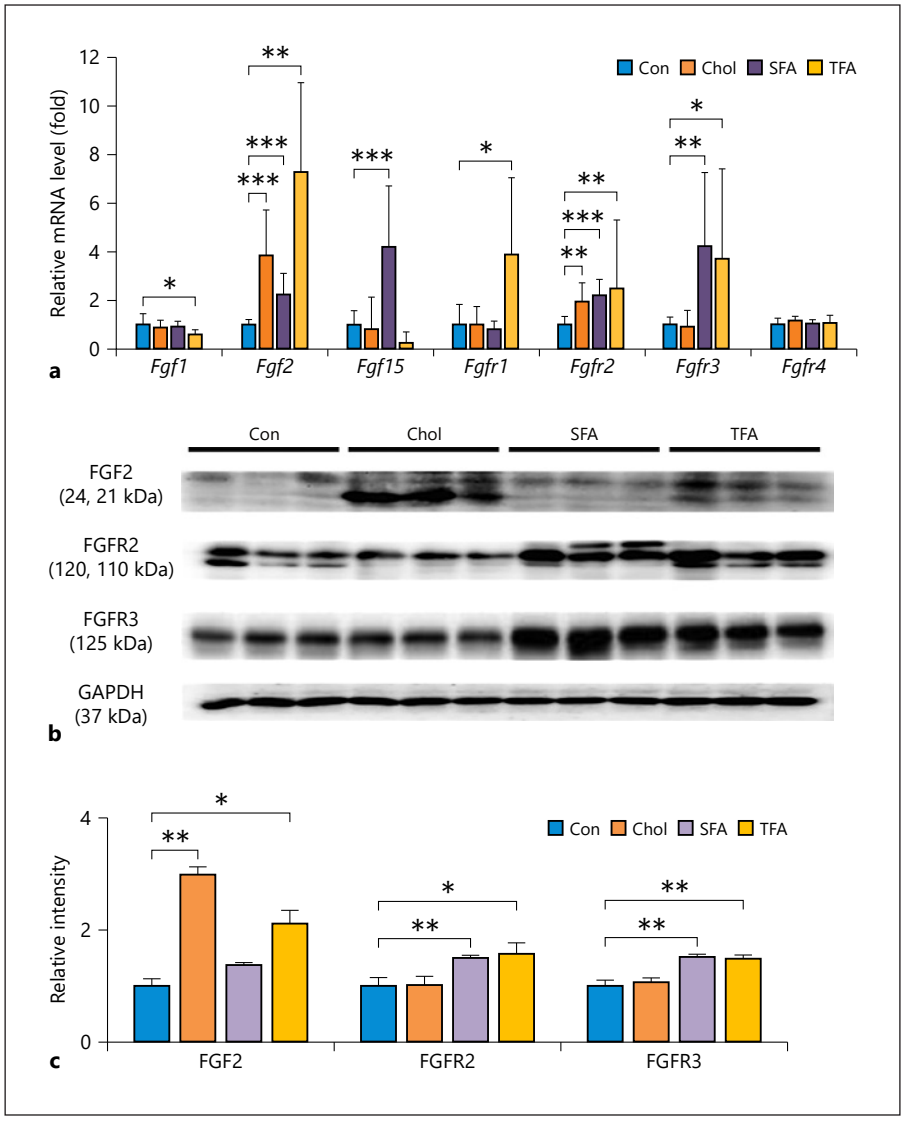


Fig. 4. Effect of dietary fats on hepatic FGF signaling in HCVcpTg mice. **a** Hepatic mRNA levels of genes related to FGF signaling were quantified by qPCR, normalized to 18S rRNA, and expressed as values relative to HCVcpTg mice fed a control diet. The same samples used in Figures 1 and 2 were adopted. The number of mice in the control diet-fed and Chol-, SFA-, and TFA-rich diet-fed groups was 8, 7, 8, and 8, respectively. **b**, **c** Immunoblot analysis of FGF2, FGFR2, and FGFR3. Whole-liver homogenates (45 μg of protein) were loaded into each well. GAPDH bands were used as the loading control. Band intensities were measured densitometrically, normalized to the loading control, and expressed as values relative to those of control diet mice. The samples from 3 mice in each group were adopted. Results were obtained from two independent immunoblot experiments. Data are expressed as the mean ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ between control diet-fed and Chol-, SFA-, and TFA-rich diet-fed HCVcpTg mice. Con, control diet-fed HCVcpTg mice; Chol, cholesterol-rich diet-fed HCVcpTg mice; SFA, saturated fatty acid-rich diet-fed HCVcpTg mice; TFA, trans-fatty acid-rich diet-fed HCVcpTg mice; SD, standard deviation.

Effect of Dietary Fat Composition on Other Growth Factor Signaling in HCVcpTg Mice

Changes in mRNA encoding *Igf1* and *Igf2* were detected only in the SFA group, while the levels of mRNA for IGF receptors were increased in all test groups (online suppl. Fig. S1A). However, there were no remarkable differences in circulating IGF1 levels (online suppl. Fig. S1B). The changes in the expressions of mRNA encoding hepatocyte growth factor (*Hgf*) as well as angiopoietin 1 (*Angpt1*) and 2 (*Angpt2*) were not consistent with the enhancement of hepatic angiogenesis/lymphangiogenesis observed in the SFA and TFA groups (shown in online suppl. Fig. S1C). The expression of mRNA encoding *Kit* was only increased in the SFA and TFA diet-treated HCVcpTg mice (online suppl. Fig. S1C).

SFA and TFA Diets Stimulated the JNK-HIF1α-VEGF-C Axis, Enhancing Hepatic Angiogenesis/Lymphangiogenesis in HCVcpTg Mice

VEGF-C was considered to be a key molecule in explaining the mechanism of how the SFA- and TFA-rich diets promoted hepatic angiogenesis/lymphangiogenesis. We next examined upstream of VEGF-C to elucidate the cause of VEGF-C augmentation, focusing on hypoxia-inducible factor (HIF) since the HIF pathway was closely associated with angiogenesis/lymphangiogenesis and tumorigenesis by modulating VEGF expression [24]. Indeed, the mRNA and protein levels of HIF1α were significantly increased in the SFA- and TFA-treated groups (Fig. 6a–c). The level of mRNA encoding aryl hydrocarbon receptor nuclear translocator (*Arnt*) was significant-

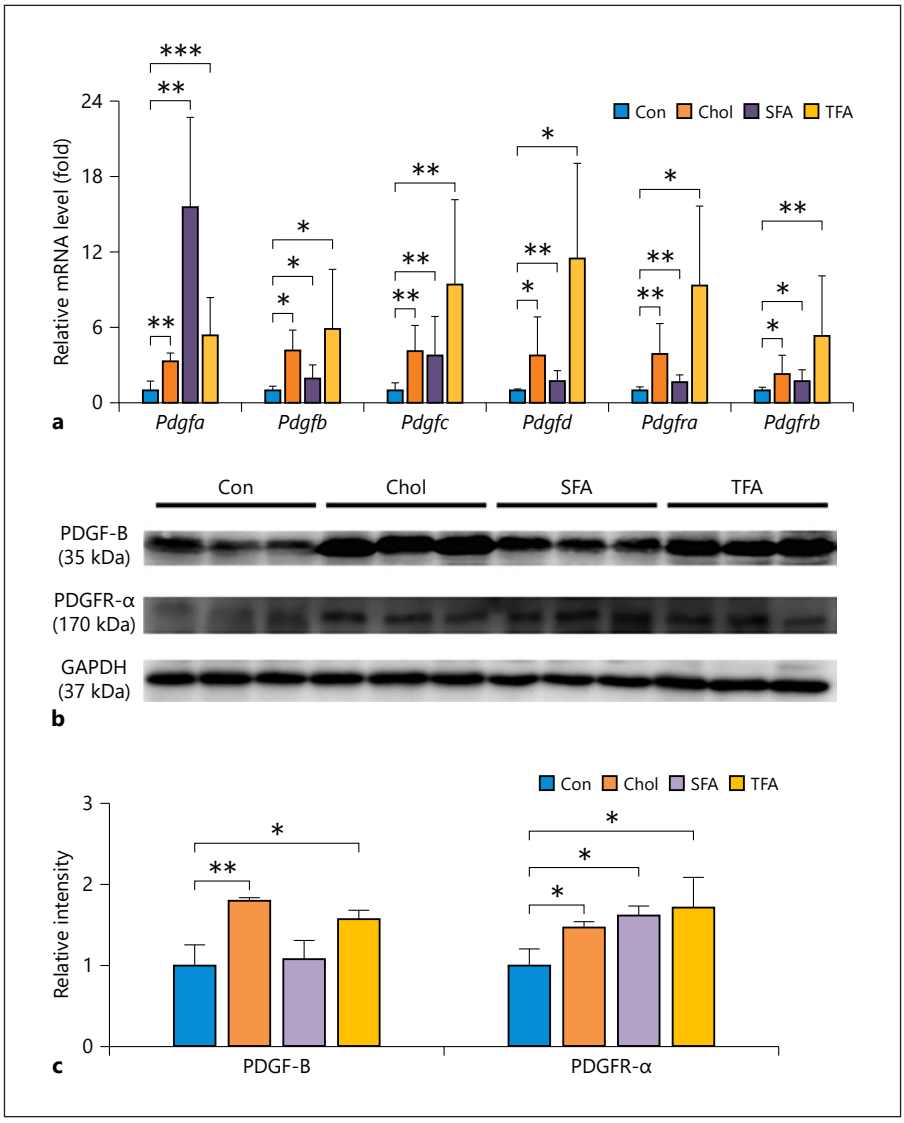


Fig. 5. Effect of dietary fats on hepatic PDGF signaling in HCVcpTg mice. **a** Hepatic mRNA levels of genes related to PDGF signaling were quantified by qPCR, normalized to 18S rRNA, and expressed as values relative to HCVcpTg mice fed a control diet. The same samples used in Figures 1 and 2 were adopted. The number of mice in the control diet-fed and Chol-, SFA-, and TFA-rich diet-fed groups was 8, 7, 8, and 8, respectively. **b**, **c** Immunoblot analysis of PDGF-B and PDGFR-α. Whole-liver homogenates (45 μg of protein) were loaded into each well. GAPDH bands were used as the loading control. Band intensities were measured densitometrically, normalized to the loading control, and expressed as values relative to those of control diet mice. The samples from 3 mice in each group were adopted. Results were obtained from two independent immunoblot experiments. Data are expressed as the mean ± SD. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ between control diet-fed and Chol-, SFA-, and TFA-rich diet-fed HCVcpTg mice. Con, control diet-fed HCVcpTg mice; Chol, cholesterol-rich diet-fed HCVcpTg mice; SFA, saturated fatty acid-rich diet-fed HCVcpTg mice; TFA, trans-fatty acid-rich diet-fed HCVcpTg mice; SD, standard deviation.

ly increased in the Chol, SFA, and TFA groups (Fig. 6a). The level of mRNA for von Hippel-Lindau tumor suppressor (*Vhl*) was similar to that of *Hif1a* (Fig. 6a).

The activation of several mitogen-activated protein kinases (MAPKs) can induce HIF1α expression. The SFA and TFA diets increased the hepatic expression of total and phosphorylated c-Jun N-terminal kinase (JNK) and the degree of phosphorylation of the p54 component of JNK (Fig. 7a, b). The levels of the p46 component of JNK, the p42 component of total and phosphorylated extracellular regulated protein kinase (ERK), remained unchanged in all groups (Fig. 7a, b). The phosphorylation level of the p44 component of ERK was increased only in the TFA group. The increased JNK in total form was associated with an elevated *Jnk1* mRNA level (Fig. 7c). These results indicated that the JNK-HIF1α-VEGF-C axis contributed to the promotion of hepatic angiogenesis/lymphangiogenesis and ensuing tumor development by the SFA and TFA diets in HCVcpTg mice (Fig. 7d).

Discussion

The current study has demonstrated that angiogenesis/lymphangiogenesis were enhanced in SFA- and TFA-rich diet-fed HCVcpTg mice but not in Chol-rich diet-fed ones. These alterations were correlated with increased VEGF-C and FGFR2/FGFR3 in the liver, likely by stimu-

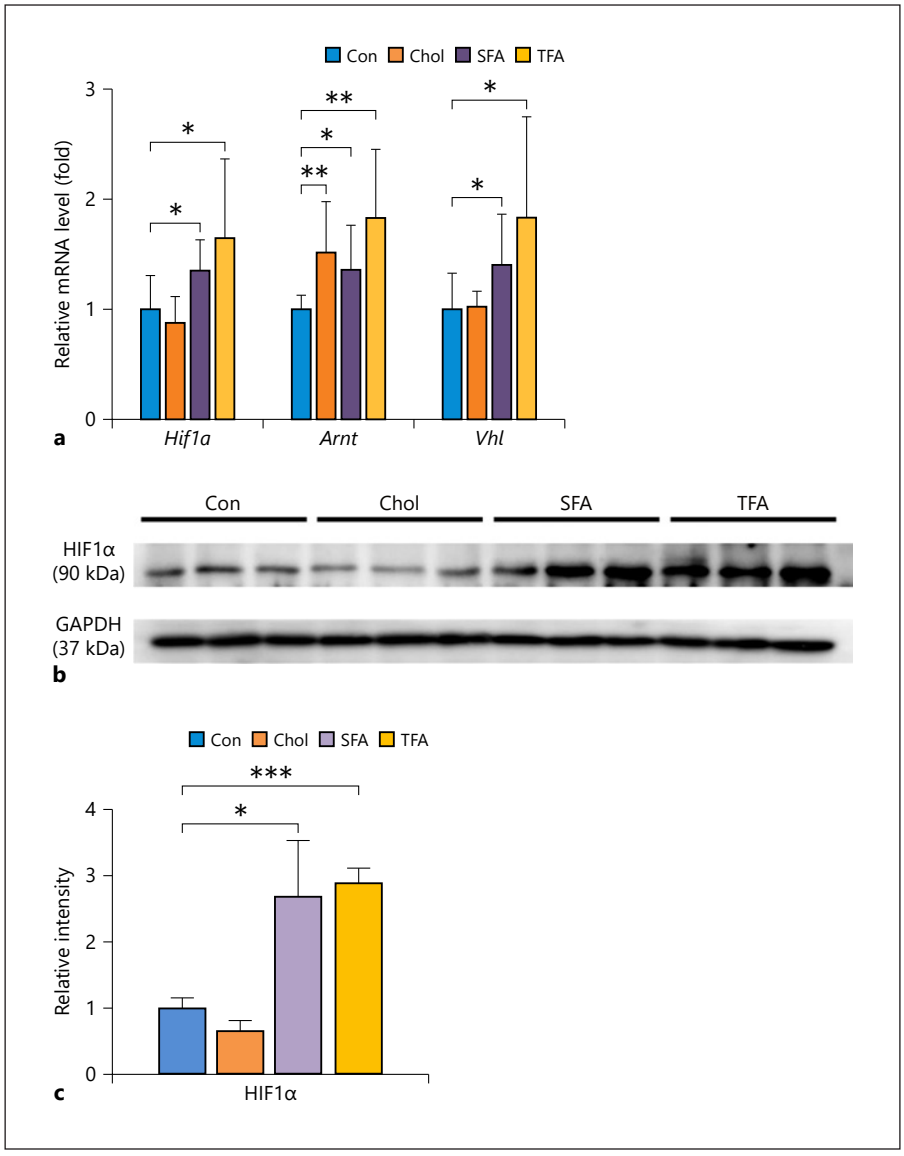


Fig. 6. Effect of dietary fat on HIF1α-related factors in HCVcpTg mice. **a** Hepatic mRNA levels of *Hif1a* and its related genes were quantified by qPCR, normalized to 18S rRNA, and expressed as values relative to HCVcpTg mice fed a control diet. The same samples used in Figures 1 and 2 were adopted. The number of mice in the control diet-fed and Chol-, SFA-, and TFA-rich diet-fed groups was 8, 7, 8, and 8, respectively. **b**, **c** Immunoblot analysis of HIF1α. Whole-liver homogenates (45 μg of protein) were loaded into each well. GAPDH bands were used as the loading control. Band intensities were measured densitometrically, normalized to the loading control, and expressed as values relative to those of control diet mice. The samples from 3 mice in each group were adopted. Results were obtained from two independent immunoblot experiments. Data are expressed as the mean ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ between control diet-fed and Chol-, SFA-, and TFA-rich diet-fed HCVcpTg mice. Con, control diet-fed HCVcpTg mice; Chol, cholesterol-rich diet-fed HCVcpTg mice; SFA, saturated fatty acid-rich diet-fed HCVcpTg mice; TFA, trans-fatty acid-rich diet-fed HCVcpTg mice; SD, standard deviation.

lation of the JNK-HIF1α cascade. Moreover, the expressions of VEGF, PDGF, and FGF varied by dietary fat composition in spite of similar calorie levels. To our knowledge, this is the first study to elucidate the differences in vasculogenesis and growth factor expression among dietary fats.

It has been documented that a high-fat diet in mice can induce inflammation and metabolic stress in the liver, leading to angiogenesis stimulation and lymphatic vessel dysfunction [25, 26]. Miura et al. [27] showed that a high-fat diet increased the number of CD31-positive endothelial cells in HCC tissues through higher circulating VEGF concentrations in hepatocyte-specific phosphatase and tensin homolog-deficient mice; however, it remains unclear whether CD31-positive endothelial cells are also increased in non-tumorous liver tissues. The current study revealed that diets rich in SFA and TFA, but not in Chol, stimulated the JNK-HIF1α-VEGF-C axis and augmented angiogenesis/lymphangiogenesis in the steatotic livers of HCVcpTg mice. Since obesity, fatty liver, and ensuing liver cancer are increasing worldwide [7, 8], our observations reveal the importance of not only dietary fat amount but also fat species, for suppressing aberrant angiogenesis/lymphangiogenesis and ensuing hepatic tumorigenesis.

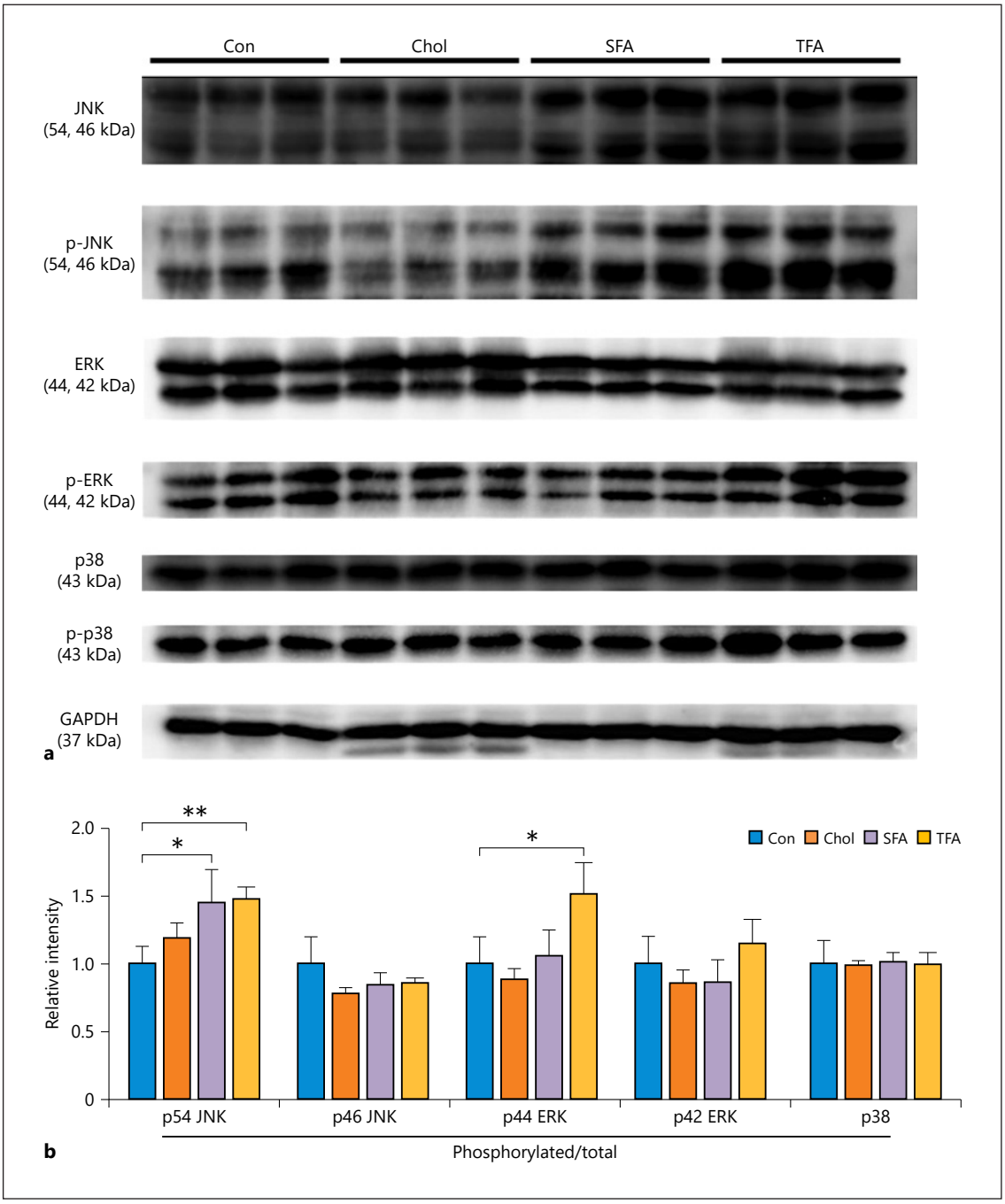
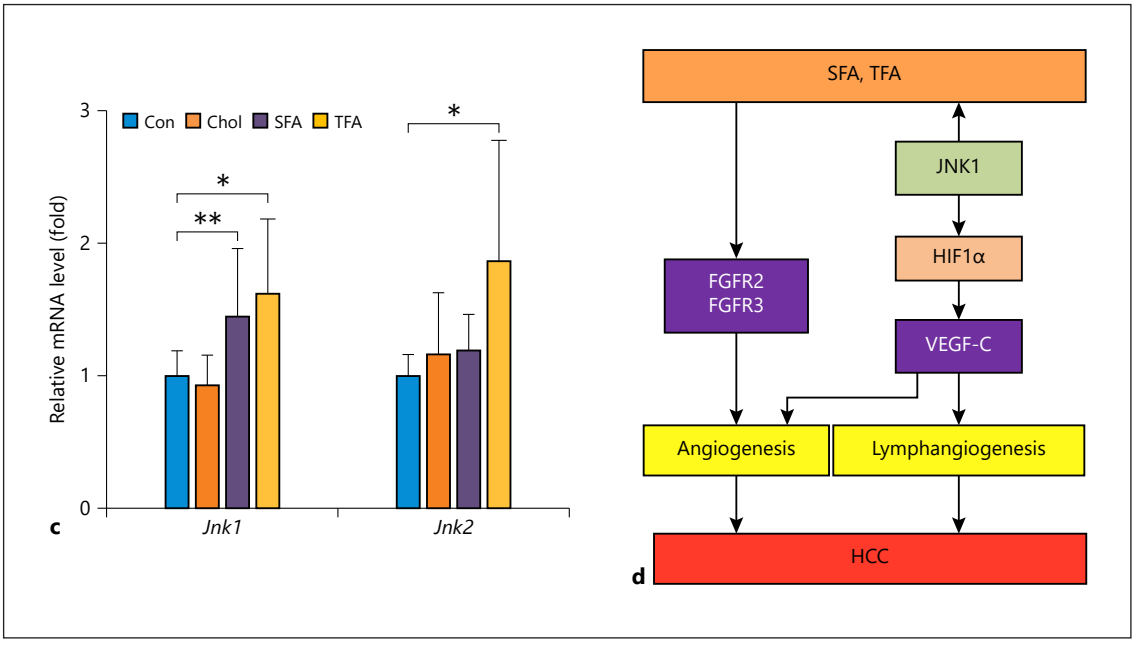


Fig. 7. Effect of dietary fat on MAPKs in HCVcpTg mice. **a**, **b** Immunoblot analysis of JNK, ERK, and p38. Whole-liver homogenates (45 μg of protein) were loaded into each well. GAPDH bands were used as the loading control. Band intensities were measured densitometrically, normalized to the loading control, calculated as phosphorylated/total ratio values, and expressed as values relative to those of control diet mice. The samples from 3 mice in each group were adopted. Results were obtained from two independent immunoblot experiments. **c** Hepatic mRNA levels of *Jnk1* and *Jnk2* were quantified by qPCR, normalized to 18S rRNA, and expressed as values relative to HCVcpTg mice fed a control diet. The same samples used in Figures 1 and 2 were adopted. The number of mice in the control diet-fed and Chol-, SFA-, and TFA-rich diet-fed groups was 8, 7, 8, and 8, respectively. **d** A proposed mechanism of how SFA-rich and TFA-rich diets enhance hepatic angiogenesis and lymphangiogenesis in HCV core gene transgenic mice. Data are expressed as the mean ± SD. $*p < 0.05$ and $**p < 0.01$ between control diet-fed and Chol-, SFA-, and TFA-rich diet-fed HCVcpTg mice. Con, control diet-fed HCVcpTg mice; Chol, cholesterol-rich diet-fed HCVcpTg mice; SFA, saturated fatty acid-rich diet-fed HCVcpTg mice; TFA, trans-fatty acid-rich diet-fed HCVcpTg mice; SD, standard deviation.

(Figure continued on next page.)



7

An earlier prospective study showed that excess SFA intake was positively associated with HCC in humans [28]. Another investigation revealed that the proportions of plasma saturated and monounsaturated FA in HCC patients were significantly increased compared with those of controls [29], with similar results found in breast cancer patients [30]. As the most abundant SFA in plasma, palmitate could induce VEGF secretion from human mesenchymal stem cells [31]. Elevated plasma nonesterified FA enhanced VEGF levels and increased renal CD31 expression as well [32]. Indeed, our previous study demonstrated fatty acid synthase (FASN) involved in de novo FA synthesis to be significantly increased in the liver after treatment with excessive SFA [4]. The inhibition of FASN reduced the proliferation of human dermal lymphatic endothelial cells, and a FASN inhibitor also decreased the production of VEGF-C in SK-Mel-25 human melanoma cells [33]. These findings collectively suggest significant crosstalk between FA metabolism and angiogenesis/lymphangiogenesis.

CD34, CD31, and TIE2 are all well-recognized markers of angiogenesis [34–36]. CD34 is a transmembrane phosphoglycoprotein first identified in hematopoietic stem and progenitor cells. However, it is expressed in a variety of cells, not only in vascular endothelial progenitors but also in hepatic stellate cells and epithelial progenitors [37, 38]. On the contrary, CD31 is highly expressed at endothelial cell-cell junctions where it functions as an adhesive stress-response protein to maintain endothelial cell junctional integrity [39]. TIE2 has also been identified as a receptor tyrosine kinase that is expressed principally on vascular endothelial cells but rarely on tumor-associated macrophages or tumor cells [40–42]. Therefore, CD31 and TIE2 are considered to be more specific markers of angiogenesis relative to CD34 [37, 43].

Lymphangiogenesis is also a crucial determinant of cancer cell spread and tumor aggressiveness [44]. LYVE1 is a lymphatic vessel endothelial hyaluronic acid receptor, a homolog of the CD44 hyaluronic acid receptor that belongs to the superfamily of link proteins [45], and is only expressed in lymphatic endothelial cells [46, 47]. While podoplanin is recognized as a marker of lymphangiogenesis as well [48, 49], its expression is upregulated in different cell types, including fibroblasts, macrophages, T helper cells, and epithelial cells, during inflammation and cancer [50]. LYVE1 therefore has greater specificity than podoplanin as an indicator of lymphangiogenesis [50, 51]. The increases in LYVE1 in addition to CD31 and TIE2 by the SFA- and TFA-rich diets corroborate a promoting effect on hepatic angiogenesis/lymphangiogenesis by excessive SFA and TFA intake.

Elevated circulating VEGF levels in HCC have been associated with tumor angiogenesis and progression [36]. VEGFR-2 is stimulated by binding to VEGF-A, VEGF-C, or VEGF-D to activate a phosphorylation cascade triggering a downstream cellular pathway that ultimately leads

to the vascular endothelial cell proliferation and tumor blood vessel formation/branching mandatory for tumor growth [2, 52]. The current study revealed overexpression of VEGF-C in the SFA- and TFA-rich diets, which has been correlated with hepatic angiogenesis/lymphangiogenesis. VEGF-C can not only promote the growth of lymphatic endothelial cells by activating ERK signaling [53] but is also able to enhance the mobility and invasiveness of cancer cells, thus promoting their metastasis. Indeed, plasma VEGF-C levels were negatively associated with the outcome of HCC patients after liver transplantation or resection [54], thereby demonstrating the importance of VEGF-C in HCC.

It was intriguing that VEGF-C overexpression by the SFA- and TFA-rich diets was mediated by HIF1α. The *Hif1a* and *Vegfc* genes possess an internal ribosome entry site that is crucial for translational activation by hypoxia [55, 56], and HIF1α and VEGF-C are positively correlated in various cancers [55, 57, 58]. Since the SFA and TFA diets, but not the Chol diet, augmented *Hif1a* mRNA, we considered that the FA-rich diets activated transcription factors regulating the transcriptional activity of *Hif1a*. Although NF-κB is a direct modulator of HIF1α expression, all test diets evoked NF-κB activation. Alternatively, MAPK signaling, including ERK, JNK, and p38, is located upstream of the *Hif1a* gene [24, 59–61]. The present results demonstrated that the HIF1α-VEGF-C axis was upregulated by increased *Jnk1* mRNA and JNK expression by the FA-rich diets in HCVcpTg mice. Overexpressions of JNK1 and HIF1α have been detected in human HCC samples and were correlated with unfavorable patient outcomes [62, 63]. FA reportedly activated JNK through SRC non-receptor tyrosine kinase and might be related to the Warburg effect [60, 64]; however, the precise mechanism of how FA enhances *Jnk1* mRNA expression requires clarification in future investigations.

FGF is involved in several tumorigenic processes, including stemness, proliferation, anti-apoptosis, drug resistance, and angiogenesis [65]. FGF signaling is also responsible for adaptive responses and tissue repair to maintain tissue homeostasis [66]. In particular, the combination of FGF and FGFR in promoting HCC angiogenesis has received recent widespread attention [67]. FGFR2 and FGFR3 can act as regulators of cell differentiation and proliferation, respectively. FGFR2 also promotes the growth of human liver tumors and angiogenesis through the PI3K-AKT pathway [68]. FGFR2/FGFR3 was found to be significantly overexpressed in human HCC samples and was closely related to the overall survival of HCC patients [69, 70]. There are no reports to date showing that JNK and HIF1α can directly upregulate FGFR2/FGFR3 expression. Further studies are necessary to clarify the molecular mechanism of FGFR2/FGFR3 upregulation by SFA- and TFA-rich diets.

Lastly, PDGF can activate hepatic stellate cells and promote fibrogenesis as well as induce the proliferation and migration of tumor cells [71]. In this study, PDGF-B was increased in the TFA and Chol groups, which was consistent with previous studies showing significant liver fibrosis by those diets [72, 73]. The absence of correlations between PDGF and angiogenesis/lymphangiogenesis markers prompted us to consider a minor contribution of PDGF signaling to angiogenesis/lymphangiogenesis observed in the SFA- and TFA-rich diet-fed HCVcpTg mice.

There are several limitations to this study. First, the effect of dietary fats on hepatic angiogenesis/lymphangiogenesis should be verified using other hepatocarcinogenesis models. Second, we could not assess if the dietary fats in this study directly influenced hepatic angiogenesis/lymphangiogenesis. Evaluation using a coculture system including hepatocytes, endothelial cells, and non-parenchymal cells or with a hepatic organoid might solve this question. Third, we speculate that the duration of dietary intervention may have affected the results of the current study; similar experiments of different durations will validate our hypothesis. Lastly, identifying the significance of VEGF-C and FGFR2/FGFR3 in the pathogenesis of steatosis-derived HCC will be useful toward the early diagnosis and prediction of HCC occurrence in NAFLD/NASH patients.

In conclusion, dietary fat composition affected the expression of VEGF, FGF, and PDGF in a fat-specific manner. Particularly, SFA- and TFA-rich diets promoted hepatic angiogenesis/lymphangiogenesis mainly through stimulation of the JNK-HIF1α-VEGF-C axis. Growth factor signaling related to angiogenesis is a crucial target for treating HCC and dietary fat composition may modulate this aspect in the context of steatosis-derived hepatocarcinogenesis caused by HCV core protein. Clinically, this study prompts us to emphasize that patients with hepatic steatosis should not only reduce dietary fat consumption but also pay attention to dietary fat composition. This discovery may contribute to new preventive strategies for steatosis-derived HCC.

Acknowledgments

We thank Dr. Takero Nakajima and Dr. Ruan Guo (Department of Metabolic Regulation, Shinshu University School of Medicine) and Ms. Hitomi Imamura (Department of Molecular Pa-

thology, Shinshu University School of Medicine) for their invaluable help, advice, instruction, and encouragement, and Mr. Trevor Ralph for his editorial assistance. We also thank the Research Center for Supports to Advanced Science (Shinshu University) for technical support and animal care.

Statement of Ethics

All animal experiments were conducted in adherence to the animal research methods outlined in the "Guidelines for the care and use of laboratory animals" and this study protocol was reviewed and approved by the Shinshu University School of Medicine (approval number #190147).

Conflict of Interest Statement

The authors have declared no conflict of interest.

Funding Sources

The authors have declared no financial support.

Author Contributions

Study design: Naoki Tanaka; writing the paper: Pan Diao and Naoki Tanaka; obtaining data: Pan Diao, Yaping Wang, Fangping Jia, Xiaojing Wang, Xiao Hu, Takefumi Kimura, Yoshiko Sato, and Naoki Tanaka; analyzing data: Pan Diao and Naoki Tanaka; supervision: Takefumi Kimura, Kyoji Moriya, Kazuhiko Koike, and Jun Nakayama. All the authors have read and approved the final manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material. Further inquiries can be directed to the corresponding author.

References

1 Chen Z, Xie H, Hu M, Huang T, Hu Y, Sang N, et al. Recent progress in treatment of hepatocellular carcinoma. Am J Cancer Res. 2020;10(9):2993–3036.
2 Morse MA, Sun W, Kim R, He AR, Abada PB, Mynderse M, et al. The role of angiogenesis in hepatocellular carcinoma. Clin Cancer Res. 2019;25(3):912–20.
3 Jin YJ, Byun S, Han S, Chamberlin J, Kim D, Kim MJ, et al. Differential alternative splicing regulation among hepatocellular carcinoma with different risk factors. BMC Med Genomics. 2019;12(Suppl 8):175.
4 Diao P, Wang X, Jia F, Kimura T, Hu X, Shirotori S, et al. A saturated fatty acid-rich diet enhances hepatic lipogenesis and tumorigenesis in HCV core gene transgenic mice. J Nutr Biochem. 2020;85:108460.
5 Hu X, Wang X, Jia F, Tanaka N, Kimura T, Nakajima T, et al. A trans-fatty acid-rich diet promotes liver tumorigenesis in HCV core gene transgenic mice. Carcinogenesis. 2020;41(2):159–70.
6 Wang X, Tanaka N, Hu X, Kimura T, Lu Y, Jia F, et al. A high-cholesterol diet promotes steatohepatitis and liver tumorigenesis in HCV core gene transgenic mice. Arch Toxicol. 2019;93(6):1713–25.
7 Tanaka N, Aoyama T, Kimura S, Gonzalez FJ. Targeting nuclear receptors for the treatment of fatty liver disease. Pharmacol Ther. 2017;179:142–57.
8 Tanaka N, Kimura T, Fujimori N, Nagaya T, Komatsu M, Tanaka E. Current status, problems, and perspectives of non-alcoholic fatty liver disease research. World J Gastroenterol. 2019;25(2):163–77.
9 Cerqueira NMFSA, Oliveira EF, Gesto DS, Santos-Martins D, Moreira C, Moorthy HN, et al. Cholesterol biosynthesis: a mechanistic overview. Biochemistry. 2016;55(39):5483–506.
10 Zhong VW, Van Horn L, Cornelis MC, Wilkins JT, Ning H, Carnethon MR, et al. Associations of dietary cholesterol or egg consumption with incident cardiovascular disease and mortality. JAMA. 2019;321(11):1081–95.
11 Oteng AB, Kersten S. Mechanisms of action of trans fatty acids. Adv Nutr. 2020;11(3):697–708.
12 Vancells Lujan P, Vinas Esmel E, Sacanella Meseguer E. Overview of Non-Alcoholic Fatty Liver Disease (NAFLD) and the role of sugary food consumption and other dietary components in its development. Nutrients. 2021;13(5):1442.
13 Green CJ, Hodson L. The influence of dietary fat on liver fat accumulation. Nutrients. 2014;6(11):5018–33.
14 Riccardi G, Giacco R, Rivellese AA. Dietary fat, insulin sensitivity and the metabolic syndrome. Clin Nutr. 2004;23(4):447–56.
15 Tanaka N, Moriya K, Kiyosawa K, Koike K, Gonzalez FJ, Aoyama T. PPARalpha activation is essential for HCV core protein-induced hepatic steatosis and hepatocellular carcinoma in mice. J Clin Invest. 2008;118(2):683–94.
16 Thiele W, Sleeman JP. Tumor-induced lymphangiogenesis: a target for cancer therapy? J Biotechnol. 2006;124(1):224–41.
17 Furuse J. Sorafenib for the treatment of unresectable hepatocellular carcinoma. Biologics. 2008;2(4):779–88.
18 Wilhelm SM, Adnane L, Newell P, Villanueva A, Llovet JM, Lynch M. Preclinical overview of sorafenib, a multikinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signaling. Mol Cancer Ther. 2008;7(10):3129–40.
19 Matsuki M, Hoshi T, Yamamoto Y, Ikemori-Kawada M, Minoshima Y, Funahashi Y, et al. Lenvatinib inhibits angiogenesis and tumor fibroblast growth factor signaling pathways in human hepatocellular carcinoma models. Cancer Med. 2018;7(6):2641–53.
20 Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med. 1998;4(9):1065–7.
21 Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, et al. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J Gen Virol. 1997;78(Pt 7):1527–31.
22 Jia F, Diao P, Wang X, Hu X, Kimura T, Nakamuta M, et al. Dietary restriction suppresses steatosis-associated hepatic tumorigenesis in hepatitis C virus core gene transgenic mice. Liver Cancer. 2020;9(5):529–48.
23 Gauglhofer C, Sagmeister S, Schrottmaier W, Fischer C, Rodgarkia-Dara C, Mohr T, et al. Up-regulation of the fibroblast growth factor 8 subfamily in human hepatocellular carcinoma for cell survival and neoangiogenesis. Hepatology. 2011;53(3):854–64.

24 Masoud GN, Li W. HIF-1α pathway: role, regulation and intervention for cancer therapy. Acta Pharm Sin B. 2015;5(5):378–89.
25 Plaza A, Naranjo V, Blonda AM, Cano V, Gonzalez-Martin C, Gil-Ortega M, et al. Inflammatory stress and altered angiogenesis evoked by very high-fat diets in mouse liver. Endocrinol Diabetes Nutr. 2019;66(7):434–42.
26 Byun KA, Oh S, Son M, Park CH, Son KH, Byun K. Dieckol decreases caloric intake and attenuates nonalcoholic fatty liver disease and hepatic lymphatic vessel dysfunction in high-fat-diet-fed mice. Mar Drugs. 2021;19(9):495.
27 Miura K, Ohnishi H, Morimoto N, Minami S, Ishioka M, Watanabe S, et al. Ezetimibe suppresses development of liver tumors by inhibiting angiogenesis in mice fed a high-fat diet. Cancer Sci. 2019;110(2):771–83.
28 Freedman ND, Cross AJ, McGlynn KA, Abnet CC, Park Y, Hollenbeck AR, et al. Association of meat and fat intake with liver disease and hepatocellular carcinoma in the NIH-AARP cohort. J Natl Cancer Inst. 2010;102(17):1354–65.
29 Jiang J, Nilsson-Ehle P, Xu N. Influence of liver cancer on lipid and lipoprotein metabolism. Lipids Health Dis. 2006;5:4.
30 Kumar K, Sachdanandam P, Arivazhagan R. Studies on the changes in plasma lipids and lipoproteins in patients with benign and malignant breast cancer. Biochem Int. 1991;23(3):581–9.
31 Yaghooti H, Mohammadtaghvaei N, Mahboobnia K. Effects of palmitate and astaxanthin on cell viability and proinflammatory characteristics of mesenchymal stem cells. Int Immunopharmacol. 2019;68:164–70.
32 Sun X, Yu Y, Han L. High FFA levels related to microalbuminuria and uncoupling of VEGF-NO axis in obese rats. Int Urol Nephrol. 2013;45(4):1197–207.
33 Bastos DC, Paupert J, Maillard C, Seguin F, Carvalho MA, Agostini M, et al. Effects of fatty acid synthase inhibitors on lymphatic vessels: an in vitro and in vivo study in a melanoma model. Lab Invest. 2017;97(2):194–206.
34 Germano D, Daniele B. TIE2-expressing monocytes as a diagnostic marker for hepatocellular carcinoma correlates with angiogenesis. Hepatobiliary Surg Nutr. 2014;3(4):166–7.
35 Alghzzawy ZM, Elmaghraby TK, El-Hamid Hagag SA, Awwad MH. Combretastatin A-4 disodium phosphate and low dose gamma irradiation suppress hepatocellular carcinoma by downregulating ROCK1 and VEGF gene expression. Mol Biol Rep. 2020;47(3):1883–93.
36 Pang R, Poon RTP. Angiogenesis and antiangiogenic therapy in hepatocellular carcinoma. Cancer Lett. 2006;242(2):151–67.
37 Sidney LE, Branch MJ, Dunphy SE, Dua HS, Hopkinson A. Concise review: evidence for CD34 as a common marker for diverse progenitors. Stem Cells. 2014;32(6):1380–9.
38 Suskind DL, Muench MO. Searching for common stem cells of the hepatic and hematopoietic systems in the human fetal liver: CD34+ cytokeratin 7/8+ cells express markers for stellate cells. J Hepatol. 2004;40(2):261–8.
39 Lertkiatmongkol P, Liao D, Mei H, Hu Y, Newman PJ. Endothelial functions of platelet/endothelial cell adhesion molecule-1 (CD31). Curr Opin Hematol. 2016;23(3):253–9.
40 Peters KG, Kontos CD, Lin PC, Wong AL, Rao P, Huang L, et al. Functional significance of Tie2 signaling in the adult vasculature. Recent Prog Horm Res. 2004;59:51–71.
41 Duran CL, Borriello L, Karagiannis GS, Entenberg D, Oktay MH, Condeelis JS. Targeting Tie2 in the tumor microenvironment: from angiogenesis to dissemination. Cancers. 2021;13(22):5730.
42 Hughes DP, Marron MB, Brindle NPJ. The antiinflammatory endothelial tyrosine kinase Tie2 interacts with a novel nuclear factor-kappaB inhibitor ABIN-2. Circ Res. 2003;92(6):630–6.
43 Liao Y, Wang C, Yang Z, Liu W, Yuan Y, Li K, et al. Dysregulated Sp1/miR-130b-3p/HOXA5 axis contributes to tumor angiogenesis and progression of hepatocellular carcinoma. Theranostics. 2020;10(12):5209–24.
44 Pereira ER, Jones D, Jung K, Padera TP. The lymph node microenvironment and its role in the progression of metastatic cancer. Semin Cell Deve Biol. 2015;38:98–105.
45 Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, et al. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. J Cell Biol. 1999;144(4):789–801.
46 Prevo R, Banerji S, Ferguson DJ, Clasper S, Jackson DG. Mouse LYVE-1 is an endocytic receptor for hyaluronan in lymphatic endothelium. J Biol Chem. 2001;276(22):19420–30.
47 Al-Rawi MAA, Mansel RE, Jiang WG. Molecular and cellular mechanisms of lymphangiogenesis. Eur J Surg Oncol. 2005;31(2):117–21.
48 Kong LL, Yang NZ, Shi LH, Zhao GH, Zhou W, Ding Q, et al. The optimum marker for the detection of lymphatic vessels. Mol Clin Oncol. 2017;7(4):515–20.
49 Raica M, Cimpean AM, Ribatti D. The role of podoplanin in tumor progression and metastasis. Anticancer Res. 2008;28(5B):2997–3006.
50 Quintanilla M, Montero-Montero L, Renart J, Martin-Villar E. Podoplanin in inflammation and cancer. Int J Mol Sci. 2019;20(3):E707.
51 Jackson DG. Biology of the lymphatic marker LYVE-1 and applications in research into lymphatic trafficking and lymphangiogenesis. APMIS. 2004;112(7–8):526–38.
52 Su JL, Yang PC, Shih JY, Yang CY, Wei LH, Hsieh CY, et al. The VEGF-C/Flt-4 axis promotes invasion and metastasis of cancer cells. Cancer Cell. 2006;9(3):209–23.
53 Yu S, Lv H, Zhang H, Jiang Y, Hong Y, Xia R, et al. Heparanase-1-induced shedding of heparan sulfate from syndecan-1 in hepatocarcinoma cell facilitates lymphatic endothelial cell proliferation via VEGF-C/ERK pathway. Biochem Biophys Res Commun. 2017;485(2):432–9.
54 Duda DG, Dima SO, Cucu D, Sorop A, Klein S, Ancukiewicz M, et al. Potential circulating biomarkers of recurrence after hepatic resection or liver transplantation in hepatocellular carcinoma patients. Cancers. 2020;12(5):E1275.
55 Schoppmann SF, Fenzl A, Schindl M, Bachleitner-Hofmann T, Nagy K, Gnant M, et al. Hypoxia inducible factor-1alpha correlates with VEGF-C expression and lymphangiogenesis in breast cancer. Breast Cancer Res Treat. 2006;99(2):135–41.
56 Morfoisse F, Renaud E, Hantelys F, Prats AC, Garmy-Susini B. Role of hypoxia and vascular endothelial growth factors in lymphangiogenesis. Mol Cell Oncol. 2014;1(1):e29907.
57 Liang X, Yang D, Hu J, Hao X, Gao J, Mao Z. Hypoxia inducible factor-alpha expression correlates with vascular endothelial growth factor-C expression and lymphangiogenesis/angiogenesis in oral squamous cell carcinoma. Anticancer Res. 2008;28(3A):1659–66.
58 Tao J, Li T, Li K, Xiong J, Yang Z, Wu H, et al. Effect of HIF-1alpha on VEGF-C induced lymphangiogenesis and lymph nodes metastases of pancreatic cancer. J Huazhong Univ Sci Technol. 2006;26(5):562–4.
59 Bao Y, Suvesh M, Li X, Bai X, Li H, Li X, et al. Ebp1 p48 promotes oncogenic properties in hepatocellular carcinoma through p38 MAPK/HIF1α activation and p53 downregulation. Mol Carcinog. 2021;60(4):252–64.
60 Chen Z, Zuo X, Zhang Y, Han G, Zhang L, Wu J, et al. MiR-3662 suppresses hepatocellular carcinoma growth through inhibition of HIF-1α-mediated Warburg effect. Cell Death Dis. 2018;9(5):549.
61 Tacchini L, Dansi P, Matteucci E, Desiderio MA. Hepatocyte growth factor signalling stimulates hypoxia inducible factor-1 (HIF-1) activity in HepG2 hepatoma cells. Carcinogenesis. 2001;22(9):1363–71.
62 Chen F, Beezhold K, Castranova V. JNK1, a potential therapeutic target for hepatocellular carcinoma. Biochim Biophys Acta. 2009;1796(2):242–51.
63 Ding ZN, Dong ZR, Chen ZQ, Yang YF, Yan LJ, Li HC, et al. Effects of hypoxia-inducible factor-1α and hypoxia-inducible factor-2α overexpression on hepatocellular carcinoma survival: a systematic review with meta-analysis. J Gastroenterol Hepatol. 2021;36(6):1487–96.
64 Holzer RG, Park EJ, Li N, Tran H, Chen M, Choi C, et al. Saturated fatty acids induce c-Src clustering within membrane subdomains, leading to JNK activation. Cell. 2011;147(1):173–84.

65 Katoh M, Nakagama H. FGF receptors: cancer biology and therapeutics. Med Res Rev. 2014;34(2):280–300.
66 Grose R, Dickson C. Fibroblast growth factor signaling in tumorigenesis. Cytokine Growth Factor Rev. 2005;16(2):179–86.
67 Sandhu DS, Baichoo E, Roberts LR. Fibroblast growth factor signaling in liver carcinogenesis. Hepatology. 2014;59(3):1166–73.
68 Fang X, Yang D, Luo H, Wu S, Dong W, Xiao J, et al. SNORD126 promotes HCC and CRC cell growth by activating the PI3K-AKT pathway through FGFR2. J Mol Cell Biol. 2017;9(3):243–55.
69 Lee HJ, Kang HJ, Kim KM, Yu ES, Kim KH, Kim SM, et al. Fibroblast growth factor receptor isotype expression and its association with overall survival in patients with hepatocellular carcinoma. Clin Mol Hepatol. 2015;21(1):60–70.
70 Qiu WH, Zhou BS, Chu PG, Chen WG, Chung C, Shih J, et al. Over-expression of fibroblast growth factor receptor 3 in human hepatocellular carcinoma. World J Gastroenterol. 2005;11(34):5266–72.
71 Lv X, Fang C, Yin R, Qiao B, Shang R, Wang J, et al. Agrin para-secreted by PDGF-activated human hepatic stellate cells promotes hepatocarcinogenesis in vitro and in vivo. Oncotarget. 2017;8(62):105340–55.
72 Diao P, Jia F, Wang X, Hu X, Kimura T, Nakajima T, et al. Mechanisms of steatosis-derived hepatocarcinogenesis: lessons from HCV core gene transgenic mice. Engineering. 2021;7(12):1797–805.
73 Jia F, Hu X, Kimura T, Tanaka N. Impact of dietary fat on the progression of liver fibrosis: lessons from animal and cell studies. Int J Mol Sci. 2021;22(19):10303.