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Human Germline Hedgehog Pathway Mutations Predispose to Fatty Liver

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Abstract

Background & Aims—Non-alcoholic fatty liver disease (NAFLD) is the most common form of liver disease. Activation of hedgehog (Hh) signaling has been implicated in the progression of NAFLD and proposed as a therapeutic target; however, the effects of Hh signaling inhibition have not been studied in humans with germline mutations affecting this pathway.

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Author contributions: BDS, PK, MJGS, KW and MM evaluated and recruited the patients at the NIH Clinical Center. RB, BDS and MM participated in study design, interpretation of the data and writing of the manuscript. RJL, MJGS and AFM participated in study design, mouse husbandry, data collection and interpretation, and writing of the manuscript. JLE participated in animal experiments. DK participated in the evaluation of human pathology samples. KKS and JMW participated in the evaluation of liver pathology samples.

Methods—Patients with holoprosencephaly (HPE), a disorder associated with germline mutations disrupting Sonic hedgehog (SHH) signaling, were clinically evaluated for NAFLD. A combined mouse model of Hh signaling attenuation (*Gli2* heterozygous null: *Gli2*^{+/-}) and diet-induced NAFLD was used to examine aspects of NAFLD and hepatic gene expression profiles, including molecular markers of hepatic fibrosis and inflammation.

Results—Patients with HPE had a higher prevalence of liver steatosis compared to the general population, independent of obesity. Exposure of *Gli2*^{+/-} mice to fatty liver-inducing diets resulted in increased liver steatosis compared to wild-type mice. Similar to humans, this effect was independent of obesity in the mutant mice and was associated with decreased expression of pro-fibrotic and pro-inflammatory genes, and increased expression of PPAR γ , a potent anti-fibrogenic and anti-inflammatory regulator. Interestingly, tumor suppressors p53 and p16INK4 were found to be downregulated in the *Gli2*^{+/-} mice exposed to high-fat diet.

Conclusions—Our results indicate that germline mutations disrupting Hh signaling promote liver steatosis, independent of obesity, with reduced fibrosis. While Hh signaling inhibition has been associated with a better NAFLD prognosis, further studies are required to evaluate the long-term effects of mutations affecting this pathway.

Keywords

non-alcoholic fatty liver disease; NAFLD; metabolic disease; hedgehog; SHH; GLI2; NASH; liver fibrosis; cirrhosis; hepatocellular carcinoma; holoprosencephaly; HPE

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common form of liver disease in the United States, affecting 20–30% of adults and 7.6% of children [1]. The severity of NAFLD ranges from simple steatosis (fat deposition without inflammation) to non-alcoholic steatohepatitis (NASH), which can progress to cirrhosis and hepatocellular carcinoma (HCC) [2, 3]. Although NAFLD is usually associated with obesity, type-2 diabetes, and hyperlipidemia [4]—primarily as a result of high intake of foods rich in sugar and saturated fats (e.g., Western-style diet) and inactive lifestyles—epidemiological studies, differences in prevalence among ethnicities, and evidence of familial aggregation suggest a genetic component [5–7].

Hedgehog (Hh) ligands are critical developmental morphogens necessary for embryonic patterning, growth and cell differentiation [8]. Beyond embryogenesis, several types of cells that reside in healthy adult livers are capable of producing and/or responding to Hh ligands and this pathway is activated in many types of acute and chronic liver injury, with tissue responses including expansion of liver progenitor populations, myofibroblast accumulation and fibrogenesis, repair-associated inflammation, and vascular remodeling [9]. In addition to its role in liver repair, several studies have also suggested Hh signaling involvement in the regulation of lipid metabolism in adipocytes [10, 11] and more recently, in hepatocytes [12]. This cumulative evidence suggests an important role for Hh signaling in liver function, but the effect of human germline Hh signaling mutations on the development and progression of NAFLD has not been investigated.

Patients and Methods

Human subjects

Patients were recruited in accordance with the ethical guidelines and recommendations of the National Human Genome Research Institute (NHGRI) Institutional Review Board. An informed consent was obtained for all participants. Approximately 1,645 HPE cases and their biological relatives were enrolled under research protocol 98-HG-0249 ([clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00341978): NCT00341978) until June 2015. Each individual was tested for mutations in the four most common genes involved in nonchromosomal, nonsyndromic holoprosencephaly (HPE) (*SHH*, *ZIC2*, *SIX3*, and *TGIF1*) using previously described methods [13]. In addition to the standard evaluation described elsewhere [14], a subset of patients was examined at the NIH Clinical Center (NIH-CC) under NHGRI protocol 04-HG-00923 ([clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00088426): NCT00088426). Clinical testing included an abdominal ultrasound, fasting lipid profiles, and hepatic function tests. All individuals with a clinical diagnosis of HPE seen at the NIH-CC, including those with microform HPE, were included in the analysis. Additionally, 61 autopsy reports from individuals who succumbed to severe HPE were reviewed.

Mouse model

The animal protocol was approved by the NHGRI Animal Care and Use Committee (protocol G-13-2) in compliance with the ethical regulations stated in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The results are reported according to the ARRIVE guidelines [15]. *Gli2* heterozygous null (*Gli2*^{+/-}) mice were used as a model of attenuated Hh signaling. *GLI2* is a downstream target involved in Hh signaling [16], which allows us to investigate the convergence of multiple factors affecting this key pathway. Unlike humans, *Gli2*^{+/-} mice have not been previously reported to show any clinical phenotype [17], although a functional haploinsufficiency resulting in severe HPE has been demonstrated in gene-environment interaction studies [18, 19]. Additional information on this mouse model and specific experimental procedures can be found in the Supplementary Material.

Dietary interventions

At 4 weeks of age, initial weight was recorded and *Gli2*^{+/-} and wild-type (WT) mice were switched from standard breeder chow to: group 1) high-fat (HF) diet (60% kcal from fat) or a control diet (10% kcal from fat); or group 2) methionine- and choline-deficient (MCD) diet or a control diet. Mice in group 1 were exposed to diets for 12 weeks and mice in group 2 for 4 weeks. Animals were fed *ad libitum*. Weight was recorded again at the end of the intervention period, body fat composition determined and mice euthanized for blood and tissue collection (Supplementary Methods). The presence and severity of liver steatosis was assessed by histological evaluation and triglyceride quantification. A subset of livers was selected for RNA expression profiling, and a subset of pituitary glands was sent for histological evaluation. Because sex differences exist in the response of C57BL/6J mice to dietary models [20], we compared same-sex animals in experimental groups.

Details about histological, biochemical, gene expression, bioinformatics and statistical analyses can be found in the Supplementary Material.

Results

Fatty liver is more prevalent in patients with HPE, a disease caused by SHH signaling disruption

HPE is a disorder of forebrain development associated with genetic and teratogenic lesions disrupting Sonic hedgehog (SHH) signaling [18, 19, 21]. Clinical evaluation of 29 patients with HPE (18 children and 11 adults) at the NIH-CC showed sonographic evidence of fatty liver in 12 individuals (33.33% of children [$P=0.002$], and 54.50% of adults [$P<0.001$]) (Table 1). The mean age of individuals with NAFLD was 30.3 years ($P=0.03$) for adults and 5.5 years ($P=0.04$) for children. Three of the 6 adults (50%), but only 1 of the 6 children (16.67%) with fatty liver were obese. Hyperlipidemia, the most common morbidity associated with NAFLD, was significantly lower in patients with HPE than in the general population (16.67% versus 69.16%, $P=0.005$) (Table 1). Overall, there was no statistically significant correlation between the presence of fatty liver and the severity of brain malformation, age, diabetes, hypertension, obesity, dyslipidemia, or hepatic function tests (Tables S1 and S2). Example pedigrees of individuals with HPE and NAFLD are shown in Figure 1.

In addition, we evaluated 67 autopsy reports from individuals with severe HPE. Sixty-two reports were from patients who had fetal or immediate postnatal demise, but only 42 included a histological evaluation of the liver. Of those, 3 (7.32%) had fatty liver. Two of 6 (33%) individuals who survived the newborn period also had some degree of fatty liver (Table S3). These results suggest earlier onset and higher prevalence of fatty liver in our HPE cohort compared to the general population.

Hh signaling attenuation increases liver steatosis in mice

We used a *Gli2* heterozygous null (*Gli2*^{+/-}) mouse as a model of attenuated Hh signaling. In order to at least partly mimic Western-style dietary influences in humans, *Gli2*^{+/-} mice and their age-matched WT littermates were fed HF or control diet. Mice fed the HF diet showed a significant increase in body weight; body fat composition; serum levels of triglycerides, nonesterified fatty acids, total and LDL cholesterol; and increased hepatic steatosis (Figure S1, Table S4). These results are in agreement with previous animal studies showing similar metabolic effects of HF diets [20, 22], which validates our dietary model.

We next compared *Gli2*^{+/-} and WT on HF diet to determine the effect of Hh signaling attenuation on diet-induced fatty liver disease. While we were not able to detect any significant differences between strains in body weight, body fat composition, serum lipid profiles or liver triglyceride content (Figure S2 and S3A, Table S5), *Gli2*^{+/-} mice showed increased liver steatosis scores upon histological evaluation compared to their WT littermates (Figure 2A). This disagreement between liver triglyceride quantification and steatosis scores highlights the importance of histological evaluation in the diagnosis of NAFLD, as the pattern of fat deposition can vary from one individual to another. In general,

our mice exposed to HF diet for 12 weeks revealed a multifocal deposition phenotype, which limits our ability to accurately quantify triglycerides from liver hunks that may not be representative of the entire organ's lipid content.

The increased hepatic steatosis without body fat increase suggested that Hh signaling disruption might affect hepatic lipid metabolism independent of systemic adiposity. To test this hypothesis, we exposed *Gli2*^{+/-} and WT mice to an MCD diet, which is known to induce severe NASH in the presence of weight loss and does not induce features of metabolic syndrome, an important risk factor for NAFLD [2, 23]. Interestingly, *Gli2*^{+/-} mice still exhibited increased liver steatosis compared to WT animals (Figure 2B). Different from the HF diet, the livers of mutant mice exposed to MCD diet exhibited increased triglyceride content (Figure S3B) consistent with a more diffuse pattern of hepatic steatosis induced by this diet [24, 25]. These results indicate that Hh signaling defects can result in hepatic lipid accumulation in the absence of obesity.

Because *GLI2* mutations have been associated with pituitary anomalies in humans [16, 26], and this could independently influence energy metabolism, we examined basal pituitary, adrenal and thyroid secretory function in adult mice exposed to breeder chow. We did not find any differences between *Gli2*^{+/-} and WT mice in pituitary histology or serum levels of adrenocorticotrophic hormone (ACTH), growth hormone (GH), cortisone, triiodothyronine (T3) or thyroxine (T4) (Figures S4 and S5).

Hh signaling attenuation causes dysregulation of genes involved in hepatic energy metabolism

The increased liver steatosis seen in the *Gli2*^{+/-} mice suggests an intrinsic hepatic metabolic defect that predisposes to diet-induced NAFLD. To examine the molecular effects of Hh signaling attenuation alone, we compared the hepatic gene expression profiles of *Gli2*^{+/-} vs. WT mice exposed to control chow. Dysregulated genes are shown in Table S6. Gene network analysis identified key pathways in lipid and xenobiotic metabolism, lipid mobilization/transport and cellular migration (Figure S6A). To analyze and interpret the biological meaning of differential gene expression profiles we used Ingenuity Pathway Analysis (IPA) (see Supplementary Material). Interestingly, IPA Downstream Effects analysis identified fatty acid oxidation and immunological function as the most significantly decreased categories in the *Gli2*^{+/-} mice, suggesting that Hh attenuation predisposes to hepatic fat accumulation while decreasing inflammatory response (Figure 3). Upstream Regulator analysis predicted several regulators to be inhibited, such as peroxisome proliferator-activated receptor alpha (PPARα), nuclear receptor subfamily 1, group I, member 2 (NR1I2, also known as pregnane X receptor) and tumor suppressor p53 (TP53), while others were predicted to be activated, such as proto-oncogene c-Myc (Figure 4).

We next compared the hepatic gene expression profiles of *Gli2*^{+/-} vs. WT mice exposed to HF diet, as Western-style dietary influences impact liver metabolism and the development and progression of NAFLD in humans. Dysregulated genes are shown in Table S7. Gene network analysis identified pathways in cell signaling, nucleic acid metabolism, small molecule metabolism (in particular lipid and xenobiotic metabolism), amino acid metabolism and molecular transport (Figure S6B). Compared to control diet, the HF diet

showed statistically significant dysregulation of pathways related to liver proliferation, liver fibrosis and liver steatosis. Similar to control diet, IPA Downstream Effects analysis also predicted an inhibition of inflammatory response, but new functions included inhibited differentiation of connective tissue cells and inhibited differentiation of tumor cells (Figure 3). Upstream Regulator Analysis predicted inhibition of important regulators of inflammation (IL1 β , TNF α , INF γ and CXCL12) and cell cycle progression and apoptosis (p53) (Figure 4). These are interesting observations in view of the normal progression of NAFLD, which conversely involves increased inflammation and increased activation of hepatic stellate cells (HSC) leading to liver fibrosis and NASH cirrhosis [27]. Another interesting finding was the predicted inhibition of p53 and decreased tumor cell differentiation.

Hh signaling attenuation decreases liver inflammation and fibrogenesis

Gene expression analyses suggested that Hh signaling attenuation has a negative effect on liver reparative functions. Normally, liver damage as seen in steatosis and NASH induces the local production of Hh ligands that stimulate the proliferation and activation of cells involved in liver repair, including HSCs [9, 28]. Sustained hepatic damage induces the expression by HSCs of genes involved in extracellular matrix (ECM) remodeling, such as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) [29]. Pro-inflammatory cytokines also play a major role in liver regeneration and the pathogenesis of NAFLD, in particular the IL1 β inflammasome [30], as well as PPARs, which are master regulators of energy metabolism and overall liver function [31].

When exposed to control diet, we did not detect any differences between *Gli2*^{+/-} and WT mice in mRNA levels of fibrosis markers collagen type I, alpha-smooth muscle actin (α -SMA), vimentin, MMP-2 or TIMP-2 (Figure 5A). We did not detect any expression of TIMP-1. Likewise, mRNAs levels of pro-fibrogenic and pro-inflammatory cytokines TGF β 1, IL1 β and CCL2 remained unchanged. There was a slight increase in F4/80 mRNA expression, a macrophage surface marker. IL6 and TNF α mRNAs were not detectable in either strain. Contrary to IPA Upstream Regulator prediction (Figure 4), PPAR α remained unchanged. PPAR γ was also not detectable.

When exposed to HF diet, however, the *Gli2*^{+/-} mice showed reduced hepatic mRNA expression of collagen type I α -SMA, (Figure 5A) that was confirmed by immunohistochemistry and connective tissue staining (Figure 5B). No increase was observed in vimentin, MMP-2 or TIMP-2 mRNA levels, and TIMP-1 was still not detected, all of which are markers of HSC activation usually upregulated in NAFLD. IL1 β and IL6 mRNA expression was reduced but no difference was detected in TGF β 1, CCL or F4/80 levels (also usually upregulated in NAFLD). TNF α was still not detected. These results suggest an attenuated pro-fibrogenic and inflammatory response in the *Gli2*^{+/-} compared to WT mice. Interestingly, PPAR γ and PPAR α were upregulated in the *Gli2*^{+/-} mice, although PPAR γ expression was more pronouncedly increased. These results are in agreement with our IPA prediction of decreased inflammation and decreased differentiation of connective tissue (Figure 3), suggesting a new mechanism of NAFLD manifestation with increased steatosis but reduced or absent fibrosis and inflammation.

Hh signaling attenuation alters the expression of cell cycle regulators

Gene network analyses predicted inhibition of p53 activity and tumor cell differentiation function in the *Gli2*^{+/-} mice. p53 is a master regulator of cell cycle progression and has been associated with the development of many cancer types including HCC [32, 33]. We confirmed that hepatic p53 mRNA was in fact reduced in the *Gli2*^{+/-} mice on HF diet compared to WT controls ($P = 5.3 \times 10^{-11}$) (Figure 6). HCC markers alpha-fetoprotein (*Afp*), heat-shock protein 70-1b (*Hspa1b*) and glypican 3 (*Gpc3*) were not detected in any group, indicating absence of carcinoma. No differences were detected in expression of mitochondrial pro-apoptotic genes *Bax* and *Bad*. Interestingly, mRNA expression of tumor suppressor p16INK4 was also reduced in the livers of *Gli2*^{+/-} mice fed the HF diet (Figure 6).

Evaluation of our HPE probands and their families did not reveal any evidence of HCC. However, given the young age of these individuals (oldest adults are now in their early 40's), follow-up and collection of additional family history related to liver disease outcomes are required.

Discussion

The genetic factors that contribute to the pathology of NAFLD remain largely unidentified. The research presented here shows for the first time that germline disruption of SHH signaling in humans promotes hepatic lipid accumulation. This observation was further supported by a combined mouse model of attenuated Hh signaling and diet-induced NAFLD. While normally indistinguishable from their wild type littermates, previous reports have shown that *Gli2*^{+/-} mice are sensitized to teratogen-induced HPE [18, 19, 21], demonstrating a functional GLI2 haploinsufficiency and providing an excellent model for the study of gene-environment interactions.

We found that germline Hh signaling attenuation predisposes mice to diet-induced liver steatosis independent of obesity. This is in agreement with our observation in HPE patients — most of whom are not overweight—and suggests a new mechanism of NAFLD manifestation. These findings are in concert with the recent work of Matz-Soja *et al.* [12] showing that conditional hepatocyte-specific deletion of Smoothened (a Patched-inhibited Hh co-receptor) in adult mice produces liver steatosis independent of insulin resistance. Previous studies have indicated that Hh signaling defects stimulate systemic adipogenesis [11, 34, 35], which can lead to metabolic complications such as dyslipidemia, type 2 diabetes and NAFLD. For instance, the study by Lee *et al.* [36] showed that mice lacking Hh co-receptor BOC display increased white adipose tissue. However, we did not see this effect in our *Gli2*^{+/-} mice. One possible explanation is that BOC-deficient mice are fundamentally different from our *Gli2*^{+/-} mice at least in terms of gene dosage (null vs. haploinsufficient, respectively), which requires additional experiments to examine the long-term effects of GLI2 haploinsufficiency and determine the gene expression threshold required for Hh-mediated dysregulation of adipogenesis.

Altered lipid homeostasis is considered the pathological hallmark of NAFLD [37]. Metabolic conditions leading to an imbalance between lipid acquisition and removal in

hepatocytes will result in liver steatosis. To investigate the molecular mechanism underlying the susceptibility to hepatic steatosis in the context of attenuated Hh signaling, we analyzed hepatic gene expression profiles of *Gli2*^{+/-} and WT mice exposed to low-fat (control) or HF caloric intakes. Interestingly, the livers of *Gli2*^{+/-} mice showed dysregulation of genes participating in key cellular processes implicated in the pathology of NAFLD. Gene network analyses revealed dysregulation of genes involved in fatty acid β - and ω -oxidation and sterol oxidation (*Cyp3a5*, *Cyp2c9*, *Sult2a1*, *Sult2a2*, *Acot2*) [38–41]; lipid mobilization (*Pltp* and *Fabp7*) [42, 43]; and glucose metabolism (*Pdk4*, *Pfkfb3*) [44, 45], indicating that Hh signaling disruption affects hepatic energy metabolism.

Injured hepatocytes produce Hh ligands to initiate progenitor-cell mediated liver regeneration. In this process, Hh signaling activates quiescent HSC resulting in ECM deposition [28, 46]. However, in order to maintain hepatic tissue homeostasis, a healthy balance between matrix breakdown mediated by MMPs and matrix protein synthesis (mainly collagen deposition) is required. In NAFLD, this balance is disrupted resulting in excess ECM. Contrary to classical NAFLD molecular pathogenesis, the *Gli2*^{+/-} mice showed reduced expression of pro-fibrogenic markers when exposed to HF diet. Based on our results, this could be mechanistically explained by a combination of reduced collagen deposition mediated by PPAR γ , and increased ECM degradation by MMPs due to decreased expression of TIMPs [29]. Counter-intuitively, MMP-2 is considered a profibrogenic MMP, as it regulates the degradation of basal lamina allowing replacement by fibrillar collagens during fibrogenesis [47].

In addition to its widely recognized requirement for normal adipocyte function and lipid metabolism [48], PPAR γ plays a central role in liver repair and the development and progression of NAFLD [31]. Some studies have proposed that increased PPAR γ expression promotes liver steatosis and have attributed a causal role to PPAR γ through mechanisms involving activation of lipogenic genes and *de novo* lipogenesis [12, 31]. Furthermore, it has been demonstrated that PPAR γ inhibits monocyte and macrophage inflammatory responses and prevents myofibroblastic transdifferentiation of HSC [31], which explains the reduced expression or lack of induction of fibrosis markers and pro-inflammatory cytokines. Our results are supported by recent reports showing decreased inflammatory response upon inhibition of Hh signaling [49].

Another interesting finding was the dysregulation of molecules involved in cell cycle control and apoptosis. p53 and p16INK4 were downregulated in our *Gli2*^{+/-} mice exposed to HF diet. About 50% of human tumors carry mutant p53, and many p53 mutants facilitate oncogenic functions such as increased proliferation, survival, and metastasis [33]. Mutations in p53 are found in 12–48% of HCC cases [50, 51] and are particularly frequent in hepatitis B-related HCC [50, 52], where tumorigenesis may arise in non-cirrhotic livers and display overexpression of hepatic progenitor cell genes [32]. Similarly, p16INK4 inactivation has been associated with the development of many human cancers, including HCC [53, 54], frequently associated with reduced or absent expression of the gene (34–48% of cases) [55]. However, these findings are counterintuitive in view of the established paradigm of otherwise activated Hh signaling during NAFLD progression and hepatocarcinogenesis [56].

Based on the results of this study, we suggest a novel mechanism of NAFLD manifestation in the context of Hh signaling attenuation and exposure to Western-style diets (Figure 7). Additional studies are required to evaluate the long-term effects of Hh signaling inhibition on liver phenotype and determine the physiological consequences of increased liver steatosis. More immediately, our results have important clinical implications for the management and care of patients with HPE and their mutation-positive relatives, as this disorder has traditionally been considered as an “above the neck” diagnosis. NAFLD is associated with other health risks that might have historically been neglected in families affected by HPE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
HCC	hepatocellular carcinoma
Hh	hedgehog
HPE	holoprosencephaly
SHH	sonic hedgehog
GLI2	GLI family zinc finger 2
ZIC2	Zic family member 2
SIX3	SIX homeobox 3
TGIF1	TGFB induced factor homeobox 1
NIH-CC	National Institutes of Health Clinical Center
HF	high fat
MCD	methionine and choline deficient

WT	wild type
ACTH	adrenocorticotrophic hormone
GH	growth hormone
T3	triiodothyronine
T4	thyroxine
IPA	Ingenuity Pathway Analysis
PPARα	peroxisome proliferator-activated receptor alpha
TP53	tumor protein p53
IL1β	interleukin 1 beta
TNFα	tumor necrosis factor alpha
INFγ	interferon gamma
CXCL12	C-X-C motif chemokine ligand 12
CCL2	C-C motif chemokine ligand 2
HSC	hepatic stellate cells
PPARγ	peroxisome proliferator-activated receptor gamma
mRNA	messenger RNA
α-SMA	alpha-smooth muscle actin
TGFβ1	transforming growth factor beta 1
IL6	interleukin 6
PCR	polymerase chain reaction
p16INK4	cyclin dependent kinase inhibitor 2A (CDKN2A)
MMP-2	matrix metalloproteinase 2
TIMP-1	inhibitor of matrix metalloproteinase-1
TIMP-2	inhibitor of matrix metalloproteinase-2
ECM	extracellular matrix

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Lay summary

Non-alcoholic fatty liver disease (NAFLD) is characterized by excess fat deposition in the liver predominantly due to high-calorie intake and a sedentary life style. NAFLD progression is usually accompanied by activation of the Sonic hedgehog (SHH) pathway leading to fibrous buildup (scar tissue) and inflammation of the liver tissue. In this research, the authors show for the first time that patients with holoprosencephaly, a disease caused by SHH signaling mutations, have increased liver steatosis independent of obesity. This observation was recapitulated in a mouse model of attenuated SHH signaling that also showed increased liver steatosis but with decreased fibrosis and inflammation. While SHH inhibition is associated with a good NAFLD prognosis, this increase in liver fat accumulation in the context of SHH signaling inhibition must be studied prospectively to evaluate its long-term effects, especially in individuals with Western-type dietary habits.

Highlights

- Humans with holoprosencephaly, a disorder of Sonic hedgehog signaling disruption, have a higher incidence of fatty liver
- Mutant mice with attenuated Hedgehog signaling are predisposed to develop fatty liver when exposed to fatty liver-inducing diets.
- Contrary to the normal progression of non-alcoholic fatty liver disease, mutant mice with attenuated Hedgehog signaling have reduced expression of pro-fibrogenic and pro-inflammatory markers.
- Mutant mice with attenuated Hedgehog signaling have decreased expression of cell cycle regulators p53 and p16.

Figure 1.

Example of families segregating mutations that perturb SHH signaling. Families 1 and 4 correspond to kindreds segregating heterozygous *SHH* mutations. The majority of affected individuals in these two families have microform HPE (mutation positive individuals with subtle midline facial differences), though both families have deceased individuals who succumbed to severe HPE. The child with HPE and the mutation-positive mother in family 1 had fatty liver at the time of examination. The grandmother in family 4 also carried the mutation and had a history of fatty liver, but was not available for examination. In family 2, both the proband and her mother carry a *SIX3* nonsense mutation; the proband has frank HPE, while the mother (as well as other family members who are not shown), demonstrate microform features only.

Figure 2.

Dietary interventions in C57BL/6J-*Gli2*^{+/-} and C57BL/6J-WT littermates. Animals were exposed to (A) a high-fat (HF) diet for 12 weeks (females) or (B) a methionine- and choline-deficient (MCD) diet for 4 weeks (males). While there were no differences in body mass and body fat between strains fed the HF diet (see also Table S5), *Gli2*^{+/-} mutants showed an increase in hepatic steatosis compared to WT. *Gli2*^{+/-} mice on the MCD diet also showed an increase in macrovesicular liver steatosis but in the presence of weight loss. Results are expressed as the mean $\pm$ SEM. ** $P < 0.01$. Student *t* test with Holm-Sidak correction for multiple comparisons was used for statistical analysis. Magnification: top panel, 400 $\times$; bottom panel, 100 $\times$. H&E, hematoxylin and eosin staining. ORO, Oil Red O staining.

Figure 3.

IPA Downstream Effects analysis of genes differentially expressed in the livers of C57BL/6J-*Gli2*^{+/-} mice (n=4) compared to C57BL/6J-WT (n=4) fed control or HF diet (females). Molecules in green and red correspond to down- and upregulated genes from our dataset, respectively.

Figure 4. IPA network analysis of upstream regulators identified in the C57BL/6J-*Gli2*^{+/-} (n=4) compared to C57BL/6J-WT (n=4) mice fed control or HF diet (females). Molecules in green and red correspond to down- and upregulated genes from our dataset, respectively.

Figure 5.

Expression of hepatic fibrosis and inflammation genes in the C57BL/6J-*Gli2*^{+/-} (n=8) and C57BL/6J-WT (n=11) mice fed HF or control diet for 12 weeks (females). **(A)** Relative mRNA expression for α -SMA (*Actb*), collagen type I (*Col1a2*), vimentin (*Vim*), MMP-2 (*Mmp2*), TIMP-2 (*Timp2*), PPAR γ (*Pparg*), PPAR α (*Ppara*), TGF β 1 (*Tgfb1*), IL1 β (*Il1b*), IL6 (*Il6*), CCL2 (*Ccl2*), and F4/80 (*Adgre1*). **(B)** Masson's trichrome and Sirius red staining of connective tissue, and immunohistochemistry of α -SMA, collagen type I and PPAR γ proteins in mice fed the HF diet. Results are expressed as the mean $\pm$ SEM. * P <0.05, ** P <0.01 and *** P <0.001. Student *t* test with Holm-Sidak correction for multiple comparisons was used for statistical analysis. Scale bar: 100 μ m. ns: not significant. nd: not detected.

Figure 6.

mRNA expression of cell cycle regulation, apoptosis, and carcinogenesis markers in the C57BL/6J-*Gli2*^{+/-} (n=8) and C57BL/6J-WT (n=11) mice (females). No differences were observed between strains when fed control chow. Expression of p53 and p16INK4 mRNAs was significantly downregulated in *Gli2*^{+/-} mice fed HF diet. HCC markers alpha-fetoprotein (*Afp*), heat-shock protein 70 (*Hspa1b*) and glypican 3 (*Gpc3*) were not detectable. Results are expressed as the mean $\pm$ SEM. *** P <0.001. Student *t* test with Holm-Sidak correction for multiple comparisons was used for statistical analysis. ns: not significant.

Figure 7.

Proposed mechanism of NAFLD progression in the context of SHH signaling and exposure to Western-style diets. In a normal liver (top panel), prolonged exposure to high-fat (HF) diet results in fatty liver with a concurrent activation of SHH and PPAR γ pathways. The liver becomes chronically inflamed and the SHH ligand produced by ballooned hepatocytes activates quiescent hepatic stellate cells to differentiate into myofibroblasts with increased production of pro-fibrogenic markers (e.g., collagen type I and α -SMA) and pro-inflammatory cytokines (e.g., IL1 β , IL6). About 20% of patients with NASH eventually develop cirrhosis, which prevents the liver from functioning properly. They may also develop hepatocellular carcinoma. In the presence of HF diet and attenuated SHH signaling (bottom panel), there is increased susceptibility to fat accumulation but decreased deposition of fibrous tissue and inflammation due to overactivation of PPAR γ , which reduces the risk of cirrhosis. The course of NAFLD in this scenario remains to be elucidated.

Table 1

Comparison of individuals with NAFLD in the HPE cohort compared to the general population

	Population estimates	HPE cohort	P value
Adults with NAFLD (n=6)			
Percentage	24.13 [4]	54.50	<0.001
Age (y)	40.10 [57]	30.30	0.026
Obesity	51.34 [4]	50.00	1
BMI* (Kg/m ²)	28.20 [57]	27.98	0.960
Diabetes	22.51 [4]	16.67	1
Dyslipidemia	69.16 [4]	16.67	0.005
Hypertension	39.34 [4]	25.00	1
Children with NAFLD (n=6)			
Percentage	7.60 [1]	33.33	0.002
Age	11 [58]	5.50	0.037

* Body mass index was calculated as weight (kg)/height² (m²). *P* compared to population estimates using two-sided *t* tests.