Increased liver tumor formation in neutral sphingomyelinase-2 deficient mice

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Abbreviations: Neutral sphingomyelinase-2, nSMase2; heptocellular carcinoma, HCC; sphingomyelin, SM; cancer stem-like cells, CSCs; signal transducer and activator of transcription 3, Stat3; sphingosine-1-phosphate, S1P; SM synthases, SMS; SM phosphodiesterase, Smpd; hematoxylin and eosin, HE; ceramide synthases, CerS.
Abstract

Sphingolipids are key signaling lipids in cancer. Genome-wide studies have identified neutral sphingomyelinase-2 (nSMase2), an enzyme generating ceramide from sphingomyelin (SM), as a potential repressor for heptocellular carcinoma. However, little is known about the sphingolipids regulated by nSMase2 and their roles in liver tumor development. We discovered growth of spontaneous liver tumors in 27.3% (9 of 33) of aged male nSMase2-deficient \( \textit{fro/fro} \) mice. Lipidomics analysis showed marked increase of SM in the tumor. Unexpectedly, tumor tissues presented with more than 7-fold increase of C16-ceramide, concurrent with upregulation of ceramide synthase 5 (CerS5). \( \textit{fro/fro} \) liver tumor, but not adjacent tissue, exhibited substantial accumulation of lipid droplet, suggesting nSMase2 deficiency is associated with tumor growth and increased neutral lipid generation in the tumor. Tumor tissue expressed significantly increased levels of CD133 and EpCAM mRNA, two markers of liver cancer stem-like cells (CSCs), and higher levels of phosphorylated signal transducer and activator of transcription 3 (pStat3), an essential regulator of stemness. CD133(+) cells showed strong labeling for SM and ceramide. In conclusion, these results suggest that SMase-2 deficiency plays a role in the survival or proliferation of CSCs leading to spontaneous tumors, which is associated with tumor-specific effects on lipid homeostasis.

(198 words)

Key words: Sphingolipids; lipid, ceramide, lipidomics, neutral sphingomyelinase-2; \( \textit{Smpd3} \); liver tumor; cancer stem-like cells; sphingomyelin; ceramide synthase 5, CerS5
Introduction

Sphingolipids, especially ceramide, sphingosine, and sphingosine-1-phosphate (S1P), play essential functions in cell signaling (1-5). They are modulators of various aspects of biology and pathology, including aging and cancer. In cancer, sphingolipids regulate tumor initiation, progression, metastasis, and drug resistance (1, 3, 5-9). Excess ceramide leads to cancer cell death, reduced growth, and senescence, and may serves as a real time predictive marker for radiation treatment response (6, 7, 9, 10). On the other hand, S1P promotes inflammation, metastasis, proliferation, vasculogenesis, and drug resistance (4, 5, 8, 11).

Cells mainly use three pathways to generate ceramide, namely the *de novo* pathway, the salvage pathway, and the sphingomyelin cycle (12-14). Several enzymes are involved in the sphingomyelin cycle including sphingomyelin (SM) synthases (SMS1 and SMS2) that catalyze the conversion of ceramide to SM, and several sphingomyelinases (SMases) that hydrolyze SM to generate ceramide (13, 15-18). Acid SMase, encoded by SM phosphodiesterase 1 (*Smpd1*), is a lysosomal enzyme the deficiency of which is associated with Niemann-Pick disease (19, 20). Four mammalian neutral SMases (nSMases) have been identified so far, among which nSMase2 (encoded by *Smpd3*) appears to be the predominant nSMase in various tissues and associated pathophysiologies (12, 15, 21). Mice deficient in nSMase2 have multiple developmental defects, including dwarfism and delayed puberty, attributed to hypothalamic pituitary deficiency (16-18). In addition, nSMase2 is implicated in diverse cellular functions, including inflammation, pathophysiology of pulmonary, circulatory, cardiac, and neurological systems (15). It is also implicated in cancer such as leukemia, breast cancer, and liver cancer (22, 23). A recent genome-wide study indicates that *Smpd3* is a potential repressor of hepatocellular carcinoma (HCC) (24).

Sphingolipids play essential functions in stem cell and cancer stem-like cell (CSC) biology (2, 25-29). CSCs, also termed cancer initiating cells or tumor initiating cells, are a subpopulation of cells in the tumor that possess stem cell characteristics (30, 31). They are derived from either reactivation of dormant progenitor cells or dedifferentiation of somatic cells (30-32). Mounting evidence has demonstrated that CSCs contribute significantly to tumor initiation, metastasis, treatment resistance, and relapse in many cancer types including liver cancer (30,
31). Thus, identifying and targeting CSCs is a new promising strategy for cancer therapy (33). Signal transducer and activator of transcription 3 (Stat3) is a key transcription factor in maintaining mouse embryonic stem cell self-renewal and CSC stemness (34-37). The activated Stat3 translocates into the nucleus and binds to promoters of stemness genes, including the liver CSC marker protein CD133, leading to their expression (30, 35).

We report here the potential function of sphingolipids in liver tumor development using the nSMase2-deficient (fragilitas ossium, fro/fro) mouse line (38, 39). This mouse line was discovered in a random-bred stock of mice after treatment with the chemical mutagen tris(1-aziridinyl) phosphine-sulphine (40), which caused a deletion in the Smpd3 gene leading to loss of enzyme activity (38). These mice show similar phenotypes as nSMase2 knockout mice generated through gene-targeting approaches (16-18). In our study, liver tumors were observed in 27.3% of aged male fro/fro mice (9 out of 33), accompanied by increased proliferation and apoptosis. Lipidomics studies demonstrate marked increase of C_{16}-SM, C_{18}-SM, C_{16}-ceramide, and dihydro(dh)C_{16}-ceramide in the tumor tissue. The number of CSCs is increased in fro/fro liver tumors as determined by quantitative PCR and immunocytochemistry of marker genes CD133 and/or Epcam. Interestingly, CD133 (+) cells were labeled for high levels of SM and ceramide. Furthermore, fro/fro liver tumors exhibited significant increase of ceramide synthase 5 (CerS5) expression and lipid droplet content, suggesting that the lack of nSMase2 was associated with a compensatory upregulation of sphingolipid and neutral lipid synthesis, which may play a role in CSC proliferation/survival and tumor formation in the fro/fro mouse.
Materials and Methods

Materials Oil Red O, propylene glycol, and fluoroshield supplemented with DAPI were from Sigma-Aldrich (St. Louis, MO, USA). Anti-Ki67 was from Novocastra (Buffalo Grove, IL, USA). Lysenin and Anti-lysenin antibody were from Peptide Institute, Inc (Osaka, Japan). Anti-CD133 was from EMD Millipore (Billerica, MA, USA). Anti-pStat3 and anti-tStat3 were from Cell Signaling Technology (Danvers, MA, USA). Anti-ceramide rabbit IgG was generated in our laboratory as described previously (41). Fluorophore-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The in situ terminal dUTP nick-end labeling (TUNEL) fluorescence staining kit was from Roche (Indianapolis, IN, USA).

Animals The nSMase2 deficient (fro/fro) mouse line (C3H and C57Bl6 mixed background), carrying a deletion in the sphingomyelin phosphodiesterase-3 (Smpd3) gene, was a gift from Dr. Christophe Poirier, Indiana University, Indianapolis (38, 39). These mice were maintained in the Laboratory Animal Service facility of Medical College of Georgia at Augusta University according to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Augusta University Institutional Animal Care and Use Committee.

Methods

Tissue collection Tumor and adjacent normal tissue were collected immediately after surgery, by either snap freeze in -80°C or fixation with 4% PFA. Frozen tissues were never allowed to thaw after initial freezing until used for further experiments. Fixed tissues were either embedded in Optimal Cutting Temperature media or paraffin for sectioning. Hematoxylin & Eosin (H&E) staining and RNA analysis were used for quality control of the integrity of the samples.

Histology, immunohistochemistry, and confocal laser microscopy For histology studies, paraffin-embedded tissues were sectioned into 10-µm slices and stained by HE in the Electron Microscopy & Histology Core Laboratory at Augusta University (directed by Dr. Sylvia B. Smith). For immunohistochemistry, frozen or
paraffin sections were immunolabeled with antibodies listed and images taken using a Zeiss LSM 510 upright or a Zeiss LSM 780 inverted confocal laser scanning microscope equipped with a two photon argon laser at 488 nm (Cy2), 543 nm (Cy3), and 633 nm (Cy5, Alexa Fluor 647), respectively. LSM 510 Meta 3.2 software was used for image acquisition and processing. Samples labeled with antibodies against lipids were only PFA-fixed and frozen for cryosectioning. Images obtained with secondary antibodies only were used as negative controls to correct for background intensity of a particular laser channel. Antigen-specific immunolabeling was quantified by counting cells that showed signals two-fold or more above background fluorescence. The percentage of Ki67 and TUNEL positive cells was recorded at ×400 magnification in at least 5 random fields where tumor exists.

**Oil Red O staining** Fixed frozen sections were washed with distilled water. Excess water was drained and depleted by rinsing twice using propylene glycol. Oil Red O was applied and incubated for 7 minutes at 60°C. Specimens were destained with 85% propylene glycol, rinsed in distilled water, and counterstained using hematoxylin. After sufficient washing with water, specimens were mounted with aqueous mounting media and microscopic images taken using an epifluorescence microscope.

**RNA extraction, RT-PCR, and qPCR** RNA was extracted using the TriZol reagent (Invitrogen). First strand cDNA was generated by the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). Real time qPCR was performed using SYBR green/Rox qPCR master mix on the CFX96 Touch Real Time System (BioRad). The primers for qPCR were: mCerS5-forward, TAGAGAGCAGCTGAGAGGAAGAAGA; mCerS5-reverse, GAACCCAGAGTCTCAAGAGCCATGGC; mCD133-forward, CAGCAATCACTGA AATTTGTG; mCD133-reverse, ACATCCTCTGAATCCATCCTG; mEpCAM-forward, TATT TTGAAAAAGATGTGAAG; mEpCAM-reverse, ATTAAGCTCTCTGTGGATCTC.

**Sphingolipid measurement** Liver tissues were analyzed at the Lipidomics Core of the Medical University of South Carolina according to published protocols on a ThermoFisher TSQ Quantum triple quadrupole mass spectrometer, operating in a Multiple Reaction Monitoring (MRM) positive ionization mode(42). Briefly, similar
amounts of tissue were homogenized, protein concentration determined, aliquots of the homogenates equivalent to 1.5 mg protein fortified with internal standards (ISs), and lipids from the fortified homogenates were extracted twice with 2 ml ethyl acetate, isopropanol, and water (60:30:10 v/v/v) solvent. Lipid extracts were evaporated and reconstituted in 150 µl of 1 mM ammonium formate in 0.2% formic acid in methanol before subjected to LC/MS/MS on the HP1100/TSQ Quantum LC/MS/MS system. The samples were gradient-eluted from the BDS Hypersil C8, 150 x 3.2 mm, 3 µm particle size column, with 1.0 mM methanolic ammonium formate/2 mM aqueous ammonium formate mobile phase system. Peaks corresponding to the target analytes and internal standards (ISs) were collected and processed using the Xcalibur software system. Quantitative analysis was based on the calibration curves generated by spiking an artificial matrix with the known amounts of the target analyte synthetic standards and an equal amount of the ISs. The target analyte/IS peak areas ratios were plotted against analyte concentration. The target analyte/IS peak area ratios from the samples were similarly normalized to their respective IS and compared to the calibration curves, using a linear regression model. Results were normalized to lipid phosphate.

**Statistics** The statistical significance was calculated using student $t$-test with Excel or one-way ANOVA and Tukey’s post hoc test with GraphPad Prism. $P<0.05$ was considered significant.
Results

Aged fro/fro male mice show liver tumors

To determine a potential involvement of nSMase2 and sphingolipids in hepatic cancer, we analyzed liver tissue in aged fro/fro mice (38, 39). Spontaneous hepatic tumors were observed in 9 out of 33 (27.3%) aged (18-24 months old) male fro/fro mouse livers (38) (Table 1 and Fig. 1A). The phenomena appeared to be age- and sex-dependent since no tumors were detected in young (less than 10 months old) or in age-matched female (0 of 31) fro/fro mice (Table 1). The incidence in aged-matched male controls (fro/+) was also 0% (0 of 21) (Table 1). The liver tumors contained heterogeneous proliferation nodules sized 0.1-5 mm in diameter (Fig. 1A). The color of many nodules was pale, suggestive of focal fat accumulation (Fig. 1A).

H&E staining of the tumor sections revealed many well-defined proliferation nodules of varying sizes (T1 and T2, Fig. 1B). Higher magnification of the inset α in nodule T1 indicated a clear boundary between the tumor tissue and liver parenchyma (Fig. 1C). The hepatocytes within the tumor showed enlarged and vacuolated cytoplasm, suggestive of fat buildup (Fig. 1C). Pronounced cell hyperplasia (arrowheads) (Fig. 1D) and penetration of lymphocytes along the portal vein (arrow, Fig. 1E) were observed within the tumor.

Enhanced proliferation and apoptosis in fro/fro liver tumors

Dysregulated cell proliferation and death is a hallmark of cancer (43). We detected the rate of cell proliferation and death in the fro/fro liver tumors. The number of Ki67 positive cells was markedly increased in the tumor when compared to normal adjacent tissues (Fig. 2A and 2C), indicating cell proliferation is enhanced. On the other hand, the number of TUNEL positive cells was also markedly increased, indicating augmented apoptosis (Fig. 2B and 2C). These results are consistent with clinical findings that, although evasion of cell death pathways is a major attribute of cancer, as tumor cells grow and exhaust nutritional supply from blood circulation, dead cells remain a common feature of many malignancies (44, 45).

Increased number of cancer stem-like cells in fro/fro liver tumors

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Cancer stem-like cells (CSCs) contribute to liver carcinogenesis (46-48). *fro/fro* liver tumors contained pockets of proliferating cells (Fig. 1D and 2A), which could be due to the existence of CSCs. To dissect the cell source of tumor formation, we tested for the presence of CSCs in *fro/fro* liver tumors. RT-PCR and qPCR results showed that the message levels of CD133 and EpCAM, two markers of liver CSCs (30, 35), were increased by 15- and 54-fold, respectively, in *fro/fro* liver tumors when compared to adjacent normal tissues (Fig. 3A and 3B).

The IL6/JAK/Stat3 pathway is essential for stem cell pluripotency and CSC stemness (34-37). Sphingolipids have been shown to regulate multiple pluripotency pathways, including the Akt/GSK-3β and IL6/JAK/Stat3 pathways (3, 49-51). We tested whether nSMase2 deficiency in *fro/fro* mice has an effect on these pathways. Western blot analysis showed that the phosphorylated Stat3 (pStat3) level was significantly increased in *fro/fro* liver tumors when normalized to total Stat3 (tStat3) as compared to adjacent normal tissue, indicating that the IL6/JAK/Stat3 pathway was activated in *fro/fro* liver tumors (Fig. 3C and 3D). However, the Akt/GSK-3β pathway was not activated (not shown). To further elucidate that CSCs were increased in *fro/fro* liver tumors, we co-labeled for CD133 and pStat3. Figure 3E shows that there were pockets of CD133 (+) CSCs in *fro/fro* liver, which co-labeled exclusively with pStat3, indicating that the CD133 (+) cells are pluripotent (Fig. 3E). We could not detect CD133 (+) or pStat3 (+) cells in control (*fro/+*) liver tissue (Fig. 3E).

**Aberrent sphingolipid profile in *fro/fro* liver tumors**

To understand the mechanism of liver tumor formation related to sphingolipids, we analysed the alteration of the sphingolipid profile caused by nSMase2 deficiency. nSMase2 catalyzes SM hydrolysis to generate ceramide. Consistently, fibroblasts from *fro/fro* mice showed accumulation of SM (52). Lipidomics (LC-MS/MS) data indicated that the major SM species in the liver, C_{16}-, C_{22}-, C_{24}-, and C_{24:1}-SM, were slightly upregulated in normal adjacent *fro/fro* liver tissue when compared to age-matched *fro/+* controls (Con) (Fig.4A and not shown). Although consistent with a previous report (52), the differences were not statistically significant. In contrast, in the *fro/fro* tumors, the levels of C_{16}- and C_{18}-SM and total SM were all significantly increased when compared to...
normal adjacent liver tissues or to age-matched \textit{fro}/+ controls (Con) (Fig. 4A). In particular, the level of C\textsubscript{16}-SM was increased by more than 4-fold (Fig. 4A).

Surprisingly, total ceramide content was also significantly increased in \textit{fro}/\textit{fro} liver tumors when compared to normal adjacent liver tissues or to age-matched \textit{fro}/+ controls (Fig. 4B). Especially, the levels of C\textsubscript{16} ceramide and dhC\textsubscript{16} ceramide were increased by more than 7-fold (Fig. 4B), although they were not dominant ceramide species in the liver (not shown). In addition, the levels of dhC\textsubscript{16}- and C\textsubscript{24:1}-ceramide were significantly higher in normal adjacent tissue of \textit{fro}/\textit{fro} liver than control \textit{fro}/+ liver (Fig. 4B). Level of C\textsubscript{16}-ceramide was increased in \textit{fro}/\textit{fro} non-tumor tissue but the difference was not statistically significant when compared to \textit{fro}/+ controls (not shown).

To investigate whether accumulation of SM and ceramide are associated with CSC formation, we co-labeled liver tissues with antibodies against CD133, ceramide, and SM (pre-incubated by lysenin). CD133 reactivity was located in cells that showed strong labeling of SM (Fig. 4C) and ceramide (Fig. 4D), while cells with low SM/ceramide labeling were CD133 (-). There were no detectable CD133 (+) cells or accumulation of SM and ceramide in normal adjacent liver tissues and age-matched \textit{fro}/+ controls, suggesting that accumulation of these two sphingolipids is associated with CSC proliferation or survival in liver.

\textbf{Increased sphingolipid and neutral lipid synthesis in \textit{fro}/\textit{fro} liver tumors}

Next we set to delineate the potential mechanism of increased ceramide synthesis in \textit{fro}/\textit{fro} liver tumors by measuring the levels of ceramide synthases (CerS), especially of those that generate C\textsubscript{16}-ceramide which has been shown to exert pro-survival functions (53) and was increased the most in \textit{fro}/\textit{fro} liver tumors (Fig.4B). Two CerSs catalyze C\textsubscript{16}-ceramide generation, CerS5 and CerS6 (54). RT-PCR and qPCR showed that CerS5 expression was significantly increased when compared to normal adjacent tissues, while CerS6 was below detection limit (Fig. 5A, 5B, and not shown). These data suggest that CerS5 was distinctly upregulated in \textit{fro}/\textit{fro} liver tumors, likely to compensate for deficiency of nSMase2.
Alterations of sphingolipid metabolism are often associated with an accumulation of neutral lipids and the formation of lipid droplets (LD) in the liver (55). Recent research has found that LD formation contributes to the development of tumors and CSCs (56). To further characterize lipid alterations in the tumor, we visualized heptic LD content using Oil Red O (ORO) staining. Liver tissues from young fro/+ or fro/fro mice exhibited little ORO staining (data not shown). Some LD accumulation was observed in fro/+ liver tissues from aged mice in fro/+ liver tissues (Fig.6A), consistent with a previous report that aging increases lipogenesis in the liver (57). Notably, the fro/fro liver tumors contained substantially higher amount of LDs (Fig.6C) when compared to the adjacent normal tissue (Fig.6B) or age-matched control (Fig.6A). Interestingly, the non-tumor tissue of the fro/fro mice (Fig.6B) exhibited less ORO staining than that of aged-matched fro/+ controls (Fig.6A). Figure 6D shows additional low magnification ORO images of a fro/fro liver tumor (encircled by green arrows), which exhibit clearly the profoundly higher ORO staining in fro/fro liver tumor when compared to normal adjacent tissues (upper panel and the area outside of green arrow, Fig.6D). Collectively, these data indicate that the tumors observed in aged fro/fro mice is associated with increased lipid accumulation in contrast to the trend observed in non-tumor tissue where nSmase2 deficiency reduces LD biogenesis.

Discussion
Liver cancer is one of the most fatal and common cancers worldwide. Although the occurrence of many cancers has decreased in recent years, the occurring rate of liver cancer has steadily increased (58, 59). Liver cancers are usually detected at a late stage and are highly resistant to conventional chemo- and radiotherapy, leading to very poor survival rates.

Genetic analyses have identified that impaired sphingolipid metabolism is common in many cancers, including liver cancer (3, 24) (60, 61). A recent genome-wide methylation analysis and epigenetic unmasking identified nSMase2 (encoded by Smpd3) as a tumor suppressor for HCC (24). Overexpression of nSMase2 reduced cell proliferation by 50% in an HCC cell line (24). Conversely, nSMase2 knockdown with small hairpin RNA promoted cell invasion and migration in vitro and increased tumor formation in vivo (24). However, the
sphingolipid metabolism affected by nSMase2 and how this altered lipid metabolism regulates tumor formation are not known.

In the current study, liver tumors were observed in over a quarter of aged male fro/fro mice (Table 1, and Fig. 1). There is no report on tumor formation in nSMase2-deficient animals, most likely because studies on older mice have not been performed. These tumors showed increased proliferation and apoptosis (Fig. 2), a common feature of many malignancies (44, 45). Interestingly, the tumors had increased number of CSCs, which was confirmed by western blot and immunofluorescence labeling of pStat3, a stemness marker (Fig. 3) (34-37). These data indicate that CSCs might be a cell source of origin of the tumor in fro/fro livers.

To study the potentially underlying mechanism, we investigated the sphingolipid metabolism affected by nSMase2 deficiency in the fro/fro mice. fro/fro tumors showed profound increase of SM and ceramide levels (Fig. 4), indicating that nSMase2 deficiency induced a sphingolipid makeover specifically in tumor cells and likely CSCs. This assumption is consistent with tumor-specific activation of de novo ceramide and sphingomyelin synthesis (62), possibly as a compensatory response to the genetic nSMase2 deficiency. Significantly higher levels of ceramides and SM have been reported in breast cancer tissue than those in normal tissue (62). SM synthase 2 (SMS2) overexpression promotes mouse liver steatosis, a major contributor for the development of HCC, whereas SMS2 deficiency has the opposite effect (63). Despite a certain level of variation, mounting evidence has demonstrated that sphingolipid metabolism, particularly elevation of SM and ceramide, has essential functions in cancer development (29, 62-67). Importantly, the CSC labeling is concurrent with strong labeling for ceramide and SM (Fig. 4C and 4D), suggesting that buildup of these two sphingolipids could be a contributing factor for CSC proliferation or survival. This is in line with previous findings that changes in SM and ceramide metabolism associate with CSC maintenance and resistance against apoptosis induction (9, 29, 65).

Expression of CerS5 was distinctly increased in the fro/fro liver tumors (Fig. 5), likely accounting for the increase of C_{16} and dhC_{16}-ceramide levels. Previous studies have shown that palmitate-induced elevation of C_{16}-ceramide
promotes LD formation in liver cells (55) and inhibition of de novo ceramide synthesis affects liver lipid metabolism and LD formation in vivo (68), suggesting that increase of ceramide levels may account for the accumulation of LDs in fro/fro tumors (Fig.6). Aberrant lipid metabolism and LD accumulation are associated with liver tumors (69). While a small side population of CSCs may already show initial alteration of sphingolipid metabolism in normal or precancerous tissue, elevation of SM, CerS5, and ceramide becomes more significant and profound once they grow into a tumor. It is also possible that elevation of SM, CerS5, C16-ceramide, accumulation of LDs, and activation of Stat3 lead to dedifferentiation of mature hepatocytes into formation and propagation of CSCs, which then causes tumor formation. These two distinct mechanisms are compatible with accumulation of LDs because of the Warburg effect, a mitochondrial dysfunction preventing fatty acid oxidation in cancer cells (70, 71). This mitochondrial damage leads to a compensatory fermentation and the initiation of dysregulated cell growth, which results in proliferation and propagation of CSCs (70, 72). We will further investigate the mechanistic connection between nSMase2 deficiency, LD formation, and liver tumor growth in future studies.

Another question to be investigated is the gender-specific development of liver tumors in aged male, but not in female fro/fro mice. This gender-specificity is probably linked to the hypothalamus–pituitary–adrenal axis defect caused by nSMase2 deficiency. In terms of tumor formation, there is evidence that HCC is an androgen sensitive tumor and that sex hormones promote tumor proliferation (73-76). The pituitary gland of the smpd3 mutant mice showed reduced size, and the numbers of ACTH-positive, FSH- and LH-positive, and other hormone-positive pituicytes were all significantly reduced in the nSMase2 deficient mice (16, 17). While the previous study did not report gender-specific effects of nSMase2 deficiency, our studies on nSMse2 inhibition and deficiency on plaque formation in Alzheimer’s disease clearly showed effects in male, but not in female mice (77, 78). Hence, gender-specific hormonal effects may underlie the observation that only aged male fro/fro mice developed liver tumors. Despite these open questions, our study is the first showing that nSMase2 deficiency is associated with CSC propagation/survival and liver tumor formation in a mouse model, and will possibly spur future studies to understand the function of sphingolipid metabolism in age-related liver cancer.
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**Table 1.** Incidents of liver tumors in *fro/fro* mice

| Genotype | Gender | Age (months) | Number of mice with tumor | Total mouse number | Tumor ratio (%) |
|----------|--------|--------------|----------------------------|--------------------|-----------------|
| *fro/+*  | male   | 16-24        | 0                          | 21                 | 0               |
| *fro/fro*| female | 16-24        | 0                          | 31                 | 0               |
| *fro/fro*| male   | 16-24        | 9                          | 33                 | 27.3            |
Figure 1. Gross and Microscopic features of liver tumors in fro/fro mice. A. Tumors were observed in aged male fro/fro mouse livers. One age-matched fro/+ control (left) and two tumors are shown (right). These tumors
contained heterogeneous nodules sized from 0.1-5 mm. The color of many nodules was pale, suggestive of focal fat accumulation. Scale bar = 2 mm. B. HE staining showed two well-defined proliferation nodules within the tumor. Scale bar = 200 µm. C. Higher magnification of inset α in B. The tumor contains large and vacuolated hepatocytes (left). Liver parenchyma was pushed to the side (right). Scale bar = 10 µm. D and E. Profound cell hyperplasia (arrowheads) (D) and lymphocyte infiltration (arrows) (E) in the tumor. Scale bars = 50 µm.
Figure 2. Increased proliferation and cell death in fro/fro liver tumors. A. Proliferation was increased in fro/fro liver tumors as shown by Ki67 labeling (green). Con, fro/+ liver tissue; Tumor, fro/fro tumor. Scale bar = 20 µm. B. Apoptosis was augmented in fro/fro liver tumors as shown by TUNEL staining (red). Scale bars = 50 µm. C. Quantification of Ki67 and TUNEL positive cells in A and B. N=5. *, p<0.05
Figure 3. CSCs in fro/fro liver tumors. A and B, Levels of liver CSC markers CD133 and EpCAM were significantly increased in fro/fro liver tumors shown by RT-PCR (A) and qPCR. Con, fro/+ liver tissue; Tumor, fro/fro tumor. C and D, the level of pStat3 was significantly increased in fro/fro liver tumors as shown by western
blot (C) and densitometric quantification (D). E. CD133 (+) (green) cells were positive for pStat3 (red). Scale bar = 20 µm.
Figure 4

A. SM levels (pmol/nmol Pi)

- Con
- Normal adjacent tissue
- fro/fro tumor

B. Ceramide level (pmol/nmol Pi)

- Con
- Normal adjacent tissue
- fro/fro tumor

C. Images of SM, CD133, DAPI, and Merged for Con and Tumor conditions.

D. Images of Ceramide, CD133, and Merged for Con and Tumor conditions.
Figure 4. *Aberrent sphingolipid metabolism in fro/fro liver tumors.*

A. Lipidomics analyses showed that $C_{16}$-, $C_{18}$-, and total SM were significantly increased in $fro/fro$ liver tumors.

Control animal was $fro/+ \text{ liver}$; Normal adjacent tissue was from $fro/fro$ animals that had tumors. N=2. *, p<0.05.

B. Lipidomics analyses showed that the levels of ceramide, especially $C_{16}$-, $C_{24}$-, $C_{24:1}$-, and total ceramide were increased in $fro/fro$ liver tumors. N=2. *, p<0.05.

C. Co-immunolabeling of CD133 and SM. Scale bar = 20 µm.

D. Co-immunolabeling of CD133 and ceramide. Scale bar = 20 µm.
Figure 5. *Aberrent synthesis of sphingolipid in*/fro/fro* liver tumors.*

A. Ceramide synthase 5 (CerS5) was elevated in *fro/fro* tumors shown by RT-PCR using two sets of samples. CerS2 did not change. Controls (Con) were from normal adjacent tissues of *fro/fro* tumors (Tumor). GAPDH was used as loading control. B. CerS5 was elevated in *fro/fro* tumors shown by quantitative PCR (qPCR). N=3. *, p<0.05.
Figure 6. Aberrent lipid droplet accumulation in fro/fro liver tumors.

A-C. Oil Red O (ORO) staining of liver lipid droplets (LD) in the livers of age-matched fro/+ control (A), non-tumor fro/fro (B), and tumor of fro/fro mice (18 to 24-month-old) (C). Scale bars = 5 µm. D. Additional images of ORO staining of fro/fro liver tumors and normal adjacent tissues (18 to 24-month-old). The area encircled by green arrows is tumor. Scale bars = 20 µm. Insets were shown in the right panels.