

# Hepatic Hyperplasia in Noncirrhotic Fatty Livers: Is Obesity-related Hepatic Steatosis a Premalignant Condition?

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## ABSTRACT

It is not known whether obesity increases the risk for hepatocellular carcinoma (HCC) simply because it promotes cirrhosis, a general risk factor for HCC, or via some other mechanism that operates independently of cirrhosis. If the latter occurs, then hepatocyte hyperplasia, an early event during the neoplastic process, might begin before liver cirrhosis develops. Genetically obese, leptin-deficient *ob/ob* mice are models for nonalcoholic fatty liver disease (NAFLD), a type of liver disease that is strongly associated with obesity and type 2 diabetes. Similar to obese, diabetic patients, *ob/ob* mice have an increased incidence of HCC. However, unlike humans with NAFLD, they rarely, if ever, develop cirrhosis spontaneously. To determine whether the noncirrhotic livers of *ob/ob* mice with NAFLD exhibit hepatocyte hyperplasia, parameters of proliferation and apoptosis were compared in adult *ob/ob* mice and their healthy litter mates. Adult *ob/ob* mice have an increase in liver mass relative to body mass. This hepatomegaly cannot be explained solely by lipid accumulation and is accompanied by significant increases in hepatocyte proliferative activity (as evidenced by increased Erk activation, cell-cycle related gene expression, bromodeoxyuridine incorporation, and hepatic DNA content) with concomitant inhibition of hepatocyte apoptosis (as evidenced by decreased numbers of apoptotic hepatocytes, induction of several anti-apoptotic mechanisms, and decreased activation of procaspase 3). Thus, liver hyperplasia is evident at the earliest stage of NAFLD in *ob/ob* mice, which supports the concept that obesity-related metabolic abnormalities, rather than cirrhosis, initiate the hepatic neoplastic process during obesity.

## INTRODUCTION

Obesity is associated with an increased risk for cancer of the liver and other epithelial organs including the uterus, colon, and pancreas (1–3). The mechanisms responsible for this association are unknown. Cirrhosis is generally considered to be most important risk factor for HCC,<sup>2</sup> because almost all HCCs are diagnosed after cirrhosis has developed, regardless of the original cause of liver damage (4, 5). Obesity is associated with an increased risk of cirrhosis (6, 7), and this may explain why the prevalence of liver cancer is increased in obesity. On the other hand, a growing body of epidemiological evidence from large, population-based studies is beginning to implicate obesity-related hyperinsulinemia and insulin resistance in the pathogenesis of other obesity-related malignancies, such as nonhereditary colon cancer and postmenopausal breast cancer (1–3, 8–10).

During the neoplastic process, epithelial hyperplasia and dysplasia generally precede cancer by many years. For example, systematic analyses of colonic or breast epithelia during the various stages of neoplasia demonstrate the progressive accumulation of defects that increase cellular survival, disrupting normal tissue homeostatic mech-

anisms that balance apoptosis and proliferation (11). A similar process occurs during virally mediated or toxin-related hepatic carcinogenesis (12, 13). Hence, it is reasonable to suspect that hepatic hyperplasia (*i.e.*, increased hepatocyte proliferation relative to apoptosis) also antedates the appearance of liver cancer during insulin-resistant states, such as obesity. However, to our knowledge, there is no information about whether or not hepatocyte hyperplasia occurs during obesity *per se*. If it does, then it is important to learn whether this potentially premalignant condition begins before the liver becomes fibrotic, because obesity-related hepatic steatosis (fatty liver) is very common, occurring in at least 20% of American adults (14), whereas, only a small minority of these individuals will ever develop significant hepatic fibrosis or cirrhosis (15).

To determine whether, and when, hepatic hyperplasia might occur during obesity, we assessed hepatocyte proliferative and apoptotic activities in the livers of genetically obese, *ob/ob* mice. *ob/ob* mice have a naturally occurring mutation in the *ob* gene that prevents the synthesis of leptin, an adipocyte hormone that regulates satiety and energy balance (16, 17). Consequently, these mice become obese at an early age. Similar to many other murine models of obesity, including those that result from diabetes (*db*) and fatty (*fa*) loss-of-function mutations in the *ob* (leptin) receptor, young *ob/ob* mice develop insulin resistance, hyperinsulinemia, and eventually become hyperglycemic (*i.e.*, type 2 diabetic; Refs. 18–20). An increased incidence of HCC has been demonstrated in old mice that are homozygous for the *ob* mutation, even when this defect is present in C57BL-6J strains that are generally resistant to malignancy (21). Interestingly, from a very young age, *ob/ob* C57BL-6J mice have enlarged, fatty livers (22). Although such mice are unusually vulnerable to toxic and ischemic liver injury and have been used as models for the early stages of human NAFLD (23), they do not develop cirrhosis spontaneously (24). Thus, despite the strong association between cirrhosis and HCC in humans, it is unlikely that cirrhosis accounts for the increased prevalence of HCC in *ob/ob* mice. Hence, these mice are relatively “pure” models of obesity-related hepatic insulin resistance and liver cancer that can be studied at a premalignant stage to determine whether, and when, hepatic hyperplasia develops. Our results demonstrate that, in the absence of any overt, exogenous insult, young adult male *ob/ob* mice have increased hepatocyte proliferation relative to apoptosis and suggest that this imbalance promotes increased liver mass. Given the general temporal relationship between epithelial hyperplasia and eventual neoplasia, these data support the possibility that cellular survival advantages develop in the liver during insulin-resistant states and contribute to obesity-related hepatic neoplasia.

## MATERIALS AND METHODS

**Animals.** Eight-week-old *ob/ob* C57BL-6J mice ( $n = 12$ ) and their lean (heterozygote) litter mates ( $n = 12$ ) were purchased from Jackson laboratories (Bar Harbor, ME). The mice were maintained in a temperature- and light-controlled animal facility and permitted *ad libitum* access to water and standard pellet-type chow for 2 weeks. At the end of this period, one mouse was randomly selected from each group, anesthetized with phenobarbital and evaluated by <sup>1</sup>H-NMR-spectroscopy to quantify fat accumulation in the liver (25). Subsequently, all of the mice were weighed, given injections of BrdUrd

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<sup>2</sup> The abbreviations used are: HCC, hepatocellular carcinoma; NAFLD, nonalcoholic fatty liver disease; NMR, nuclear magnetic resonance; BrdUrd, bromodeoxyuridine; MnSOD, manganese superoxide dismutase; MAPK, mitogen-activated protein kinase; LPS, lipopolysaccharide; EMSA, electrophoretic mobility shift assay; NFκB, nuclear factor κB; Erk, extracellular regulated kinase.

i.p., and killed 2 h later by cervical dislocation. The livers were removed from the carcasses and weighed, and then a small biopsy was obtained from each organ. This tissue was placed in buffered formalin for subsequent evaluation of hepatic histology and nuclear BrdUrd incorporation. The remaining liver tissues were used immediately to isolate either fresh nuclei ( $n = 4$  mice/group) or fresh mitochondria ( $n = 4$  mice/group). Others ( $n = 4$  mice/group) were snap-frozen in liquid nitrogen, stored at  $-80^{\circ}\text{C}$  and processed subsequently to obtain whole liver extracts of RNA, DNA, and protein.

**Evaluation of Hepatocyte Proliferative Activity.** This was assessed by counting BrdUrd (+) hepatocyte nuclei on coded liver sections that were counterstained with hematoxylin. Two sections from each mouse were evaluated independently by two observers (S. Y., J. H.). In each section,  $10 \times 400$  were examined and the total number of hepatocytes with and without BrdUrd (+) nuclei were recorded. An average proliferative index (number of BrdUrd (+) hepatocytes  $\div$  total number of hepatocytes) was calculated for each mouse; then the figures from the 12 mice in each group were totaled to obtain the mean ( $\pm$  SE) proliferative index for each group. In addition, freshly isolated liver cell nuclei from four mice/group were stained with propidium iodide, and nuclear ploidy was evaluated by flow cytometry.

**Analysis of MAPKs.** Members of the MAPK family, Erk-1 and Erk-2, become phosphorylated (*i.e.*, activated) during the prereplicative phase of hepatocyte proliferation. Hence, steady-state levels of phospho-Erk-1 and -Erk-2 are useful measures of hepatocyte proliferative activity. The content of total and phosphorylated Erk-1 and Erk-2 were evaluated in whole liver homogenates from four mice/group using immunoblot analysis with specific antisera from Santa Cruz Biotechnology, Inc (Santa Cruz, CA) as described previously (26).

**RNA Analysis of Growth-related Genes.** Total hepatic RNA was isolated from the livers of four mice/group using a minor modification (27) of Chomczynski and Sacchi's method (28). RNase protection analysis of this RNA was performed using reagents from PharMingen as we have described previously (29). This assay permits the concurrent evaluation of several immediate early genes, such as *c-jun*, delayed-early genes, such as *cyclin-D1*, and S-phase-associated genes, such as *cyclin A*, as well as housekeeping genes, such as *GAPDH*, in the same RNA sample, facilitating standardization that controls for slight variability in RNA content that might occur among different samples.

**DNA Quantitation.** Hepatic DNA was extracted and quantitated as described by Blin and Stafford (30). Results are expressed as mg/g liver and also as mg/total liver.

**Evaluation of Apoptosis.** The optimal technique for assessing apoptosis in the liver remains hotly contested. Standard approaches, including TUNEL staining and evaluation of DNA fragmentation by agarose gel electrophoresis, have been criticized because these assays may also become positive in necrotic livers (31). Detection of apoptotic hepatocytes on H&E-stained tissue sections is generally acknowledged to be reliable. Thus, we used the latter approach to screen for differences in basal apoptotic rates between *ob/ob* and lean mice. Coded sections were reviewed by an experienced hepatologist (A. M. D.) who counted the numbers of apoptotic hepatocytes (characterized as shrunken, hypereosinophilic cells with pyknotic or absent nuclei) in 10 randomly selected  $\times 200$  microscope fields on each of two sections/mouse. The mean ( $\pm$  SE) number of apoptotic hepatocytes/10 microscope fields was calculated from 12 mice in each group. However, because this approach risks underestimating apoptosis in the severely fatty livers of *ob/ob* mice, alternative measures of apoptosis were also assessed.

**Caspase 3 Activation.** Whole liver homogenates from four mice/group were evaluated for evidence of caspase 3 activation. Equal amounts (40  $\mu\text{g}$ /lane) of liver protein from *ob/ob* and lean mice were separated by SDS-PAGE, transferred to nylon membranes and probed for total caspase 3 and its cleavage products using antisera from PharMingen (San Diego, CA). The cleavage of procaspase 3 to caspase 3 activates the enzyme during hepatocyte apoptosis. Moreover, caspase 3 is the major effector of apoptosis in hepatocytes. Thus, the ratio of caspase 3 cleavage products (*i.e.*, active caspase 3):procaspase 3 is a good measure of hepatocyte apoptotic activity (32). The contents of pro- and active-caspase 3 were determined in each sample of liver protein by densitometric analysis of the immunoblots and were expressed as a ratio of activated caspase 3:total caspase 3 in each sample. Mean ( $\pm$ SE) results were derived from 4 mice/group.

**Evaluation of Antiapoptotic Responses.** The basal rate of apoptosis is extremely low in normal adult livers (33), challenging the sensitivity and

reliability of assays that attempt to detect and quantitate further reductions in apoptotic activity (31). However, if basal apoptotic activity is truly inhibited, then it is reasonable to assume that antiapoptotic mechanisms might be induced. The latter include increased DNA binding activity of NF $\kappa$ B (34), accumulation of bfl-1 and bcl-xL, antiapoptotic bcl-2 family members (35), and activated antioxidant defenses, such as mitochondrial MnSOD (36). According to previously published methods (27), nuclear protein extracts (8  $\mu\text{g}$  of protein/assay) from four mice/group were analyzed individually by EMSAs using  $^{32}\text{P}$ -labeled, double-stranded oligonucleotide fragments that contain the  $\kappa$ B binding site in the immunoglobulin enhancer element (37). In some assays, 1  $\mu\text{l}$  of antisera specific for NF $\kappa$ B p65 or p50 subunits was added to verify that the complexes contained NF $\kappa$ B. In four other mice/group, the activity of a NF $\kappa$ B target gene, *MnSOD* (34), was evaluated in hepatic mitochondrial extracts and normalized per microgram of total mitochondrial protein as described previously (38). Finally, RNase protection analysis was done using a 10- $\mu\text{g}$  sample of total liver RNA from the final four mice in each group (29). Reagents from PharMingen were used to assess the expression of bfl-1, bcl-xL, and GAPDH in each sample.

## RESULTS

**Hepatomegaly in Obese, *ob/ob* Mice.** *ob/ob* mice have hepatomegaly. Because *ob/ob* mice weigh about twice as much as their lean litter mates, it is not unexpected that their livers are larger than those of lean controls. However, normalization of liver weight by body weight demonstrates that the mass of *ob/ob* livers is greater than predicted solely on the basis of generalized obesity (Table 1). Given that *ob/ob* mice have fatty livers (Fig. 1a), it is conceivable that the tremendous accumulation of lipid within individual hepatocytes increases the liver:body weight ratio. However, analysis of the liver and entire body of a typical *ob/ob* mouse by  $^1\text{H}$ -NMR spectroscopy demonstrates that there is actually less accumulation of fat in the liver (about 15% of the total  $^1\text{H}$  signal) than there is in the rest of the body (in which fat accounts for almost 60% of the  $^1\text{H}$  signal); therefore, this explanation seems unlikely (Fig. 1b). In *ob/ob* mice of this age, there is no overt histological evidence for the hepatic accumulation of inflammatory cells or fibrous tissue (Fig. 1a). Hence, a comparison of the total DNA content of fatty and normal livers should provide information about hepatocyte number and should help to determine whether increases in hepatocyte number contribute to *ob/ob* hepatomegaly. Consistent with evidence that *ob/ob* hepatocytes contain more fat than normal hepatocytes, the hepatic DNA content normalized per gram of liver wet weight is about 50% lower than normal. However, *ob/ob* livers weigh almost four times more than normal. Hence, the total DNA content of an *ob/ob* liver is, on average, significantly greater than that of an age- and gender-matched lean litter mate (Table 1), which suggests that increases in hepatocyte number contribute to the increased liver mass of fatty livers.

**Increased Hepatocyte Proliferative Activity in *ob/ob* Fatty Livers.** In adults, liver mass is maintained at a relatively constant value because the rates of hepatocyte proliferation and apoptosis are similar (39, 40). Moreover, unlike cells in other epithelial tissues, hepatocytes "turn over" infrequently in uninjured livers, as evidenced by observations that at any given moment, very few hepatocytes incorporate [ $^3\text{H}$ ]thymidine or BrdUrd into nuclear DNA (33). When the normal balance between proliferation and apoptosis is disturbed to

Table 1 Results (mean  $\pm$  SE) from 12 *ob/ob* and 12 lean mice  
*ob/ob* group data are significantly greater ( $P < 0.01$ ) than lean group data for all parameters except mg DNA/g liver.

Mice	Weight			DNA	
	Body (g)	Liver (g)	Liver/body (%)	mg/g liver	mg/total liver
Lean	25 $\pm$ 5	1.1 $\pm$ 0.2	4.3 $\pm$ 0.5	8.5 $\pm$ 0.3	9.4 $\pm$ 0.3
<i>ob/ob</i>	55 $\pm$ 6	3.8 $\pm$ 0.7	6.8 $\pm$ 1.2	4.4 $\pm$ 0.6	16.5 $\pm$ 1

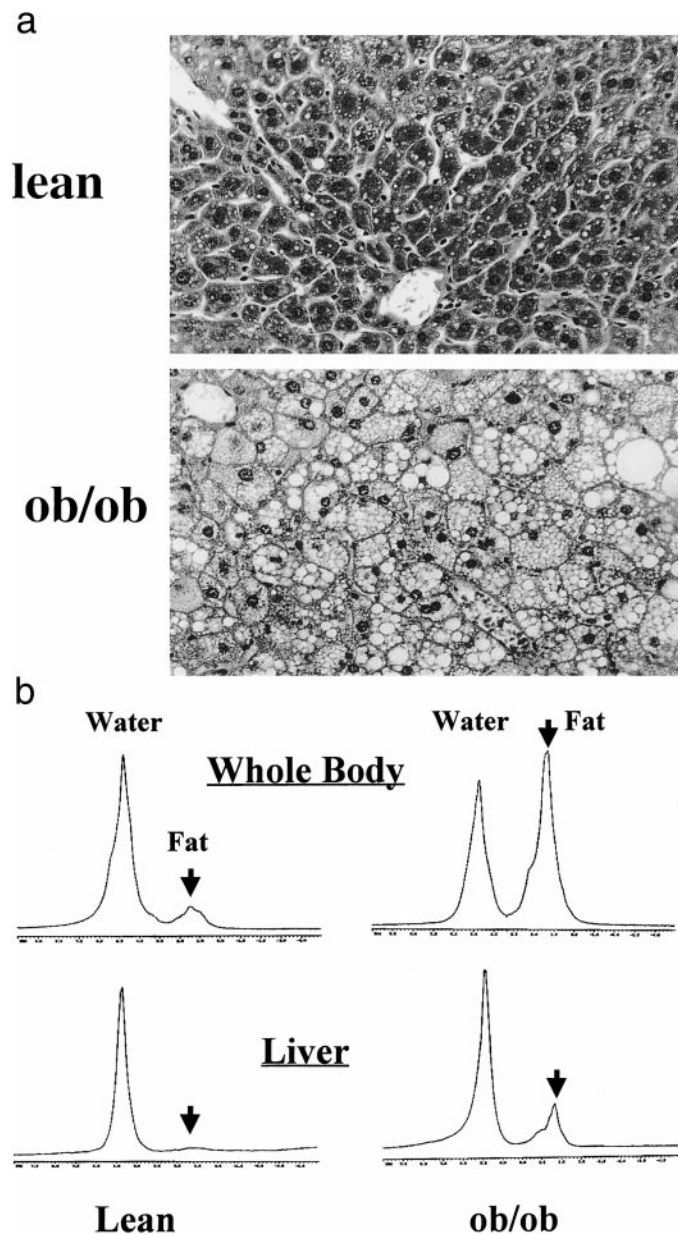


Fig. 1. Differences in hepatic fat content in lean and *ob/ob* livers. *a*, H&E-stained section of liver tissue from a representative lean (*+/ob*) and obese (*ob/ob*) mouse ( $\times 200$ ). *b*,  $^1\text{H-NMR}$  spectra derived from the livers and entire bodies of the same mice.

favor a net increase in proliferative activity, liver size increases. This can occur either as a result of factors that work predominately by increasing hepatocyte proliferation or as a consequence of factors that work predominately by decreasing hepatocyte apoptosis. The largest increases in liver mass occur when hepatocyte proliferation and apoptosis are regulated differentially (*i.e.*, when proliferation is stimulated while apoptosis is inhibited). Although tissue neoplasia typically involves increased proliferative activity, it is not clear that the latter process contributes to the hepatomegaly of *ob/ob* livers. Indeed, the possibility that basal rates of hepatocyte proliferation might be decreased in *ob/ob* livers merits consideration because it was recently reported that liver regeneration is inhibited after partial hepatectomy in obese, diabetic, *fa/fa* rats with fatty livers (41).

To compare the basal proliferative activities of normal and *ob/ob* livers, several approaches were used. First, we looked for evidence of activation of the MAPK cascade in the two groups. The content of phosphorylated MAPK isoforms (p42 Erk-1 and p44 Erk-2) is more

than 4-fold greater in fatty, *ob/ob* livers than in normal livers despite similar content of total Erk-1 and Erk-2 in the two groups (Fig. 2). MAPK activation typically occurs within minutes after hepatocytes are exposed to growth factors (33). Another early event that heralds the reentry of hepatocytes into proliferative phases of the cell cycle is the accumulation of mRNAs that encode immediate-early genes, such as *c-jun* (42, 43). RNase protection analysis of liver RNA from normal and fatty livers demonstrates that *c-jun* mRNAs are increased in fatty livers (Fig. 3). Moreover, transcripts for *cyclin D<sub>1</sub>* and *cyclin A*, genes that are up-regulated at the time of transition from G<sub>1</sub> into S-phase (44, 45), are also increased in fatty livers (Fig. 3). The induction of cell cycle-associated genes in fatty livers suggests that hepatocyte DNA synthesis might be increased in *ob/ob* mice. To evaluate that possibility more directly, liver nuclei were isolated; stained with propidium iodide, and evaluated for DNA content by flow cytometry (46). An increased proportion of nuclei with hyperdiploid DNA content were demonstrated in fatty livers (Fig. 4; Table 2). Consistent with these results, BrdUrd incorporation into hepatocyte nuclei is increased in tissue sections prepared from fatty livers (Table 2).

**Induction of Antiapoptotic Defenses in Murine Fatty Livers.** Although hepatocyte proliferative activity is increased slightly in *ob/ob* livers, this will not increase liver mass if the rate of hepatocyte apoptosis increases proportionally (39, 40). During sublethal exposure to various noxious agents, hepatocyte apoptosis increases and liver mass is preserved by an associated increase in hepatocyte proliferative activity (33). Because the accumulation of fat within hepatocytes (hepatic steatosis) occurs in the early stages of liver disease that occurs during obesity (47), it is conceivable that the increased proliferative activity of *ob/ob* hepatocytes is merely an adaptive response to increased hepatocyte apoptosis. To evaluate this possibility, we counted the numbers of apoptotic hepatocytes in H&E-stained liver sections from four *ob/ob* mice and an equal number of their lean litter mates. To our surprise, we were unable to demonstrate an increased accumulation of apoptotic hepatocytes in *ob/ob* livers. In fact, an inspection of  $10 \times 200$  microscope fields/liver section demonstrated an average of three to four apoptotic foci/section in control mice but

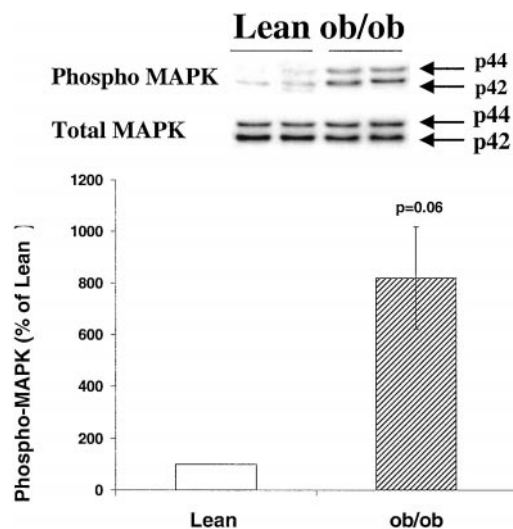


Fig. 2. Hepatic activity of the MAPKs, Erk-1 (p42) and Erk-2 (p44), in lean and *ob/ob* mice. Western blots of whole liver homogenates were probed with antisera that are specific for either activated (*i.e.*, phosphorylated) Erk-1 and Erk-2 or total (*i.e.*, phosphorylated + nonphosphorylated) Erk-1 and Erk-2. An immunoblot demonstrating the hepatic content of activated erks (*top panel*) and total erks (*bottom panel*) in two mice from each group is shown. These findings were reproduced on another immunoblot that contained liver homogenates from two other mice from each group. Both immunoblots were analyzed by densitometry and on each blot, the content of activated MAPK (p42 + p44) in *ob/ob* homogenates was normalized to that in homogenates from the lean controls. Results from all four *ob/ob* and four lean control mice are graphed.

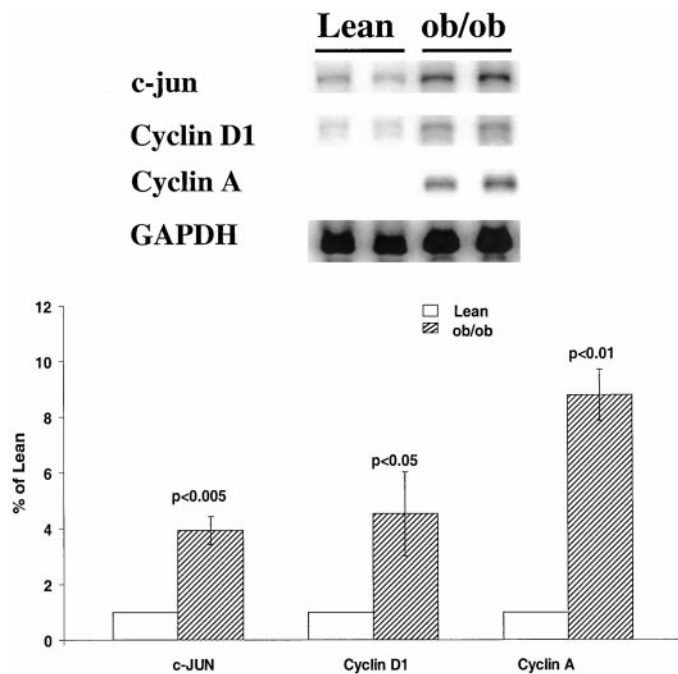


Fig. 3. Cell cycle-related gene expression in the livers of lean and *ob/ob* mice. Total hepatic RNA was analyzed by RNase protection assays using commercially available reagents that permit the concurrent evaluation of multiple cell-cycle-regulated genes and *GAPDH*, a housekeeping gene, in each of the same 10- $\mu$ g RNA samples. The autoradiographs demonstrate the expression of *c-jun*, a gene that is induced early during G<sub>1</sub>, *cyclin D-1*, a gene that is induced at the time of G<sub>1</sub>-S phase transition, *cyclin A*, a gene that is induced during S phase, and *GAPDH* in two lean and two *ob/ob* mice. This experiment was repeated with liver RNA from another two mice from each group. After phosphorimager analysis of both membranes, data (mean  $\pm$  SE) from all four lean and four *ob/ob* mice are graphed.

fewer than two apoptotic foci/section in *ob/ob* livers, which suggests that *ob/ob* livers might actually have fewer apoptotic hepatocytes than do normal livers. However, because the basal rate of hepatocyte apoptosis in adults is very low, it is difficult to detect a further reduction in the already-low number of apoptotic hepatocytes simply by inspecting tissue sections. In addition, the accumulation of fat in *ob/ob* hepatocytes might have obscured a small, but significant, increase in apoptotic cells. To overcome these technical obstacles, alternative measures of apoptotic activity were evaluated.

The cleavage of procaspase 3 activates this enzyme, which functions as the predominant terminal effector of hepatocyte apoptosis (48). Thus, the ratio of procaspase 3 cleavage products (*i.e.*, activated caspase 3) to total caspase 3 content provides a measure of apoptotic activity (32). To compare the degree of caspase 3 activation in *ob/ob*

and normal livers, equal amounts of whole liver protein from fatty and lean livers were separated by SDS-PAGE and exposed to commercially available antisera that recognize both procaspase 3 and its cleavage products. Consistent with the histological evidence for decreased apoptosis in *ob/ob* livers, the immunoblot assays demonstrated a significantly reduced ratio of activated:total caspase 3 in livers from *ob/ob* mice compared with livers from their lean litter mates (Fig. 5a). To validate this approach, a separate group of lean C57BL-6 mice were given injections *i.p.* of a small dose (10  $\mu$ g/mouse) of bacterial LPS, which is known to cause a slight increase in hepatocyte apoptosis (49). Whole liver homogenates were analyzed for procaspase 3 and its cleavage products by immunoblot (Fig. 5b). As expected, LPS treatment increases the ratio of activated caspase 3:total caspase 3 in normal mice, which confirms that this assay is a useful measure of hepatocyte apoptosis.

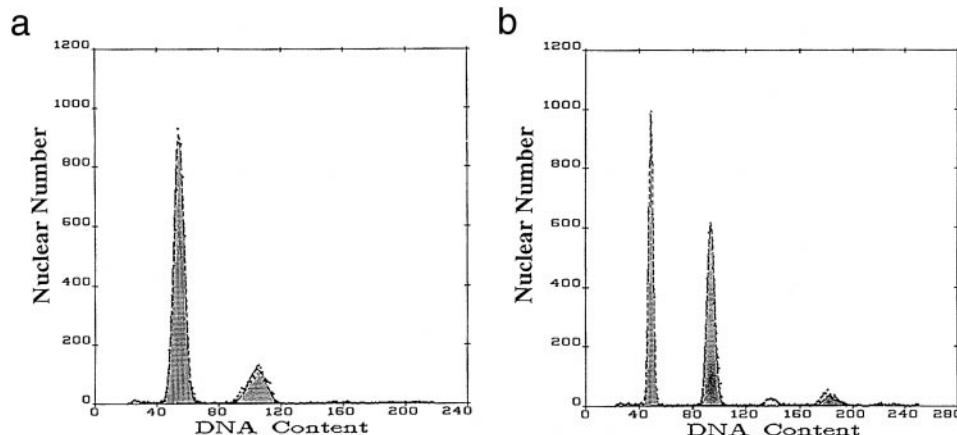
Taken together, the results suggest that basal rates of hepatocyte apoptosis are actually decreased in young adult *ob/ob* mice. If this suspicion is correct, then one or more of the cellular mechanisms that inhibit apoptosis should be induced in *ob/ob* livers. The transcription factor, NF $\kappa$ B plays a critical role in hepatocyte antiapoptotic defense (50). A comparison of NF $\kappa$ B-DNA binding activity in the *ob/ob* mice with fatty livers with that of age- and gender-matched normal mice of the same strain demonstrates increased NF $\kappa$ B-DNA binding activity in nuclear extracts from fatty livers (Fig. 6). The production of superoxide anion (O<sub>2</sub><sup>-</sup>) by mitochondria increases even before the induction of NF $\kappa$ B early during the course of apoptosis (51), and it has been shown that apoptosis is inhibited in cells that are transfected

Table 2 Proliferative parameters

Twelve lean and 12 *ob/ob* mice of the same age and gender were given injections *i.p.* of BrdUrd. Two h later, the mice were killed; a piece of each liver was fixed in formalin, paraffin embedded, sectioned, and examined for BrdUrd (+) hepatocyte nuclei. Ten randomly-selected  $\times$ 400 microscope fields were examined on each section, and the average number of labeled hepatocyte nuclei per 1000 hepatocytes was determined for each mouse. Results shown are the mean ( $\pm$ SE) data obtained from 12 mice from each group. In four mice from each group, liver cell nuclei were isolated from the remaining liver tissues and stained with propidium iodide. Flow cytometry was performed to determine the hepatocyte nuclear DNA content. The results from one representative lean and *ob/ob* mouse/group are displayed in Fig. 4. The mean cell cycle distribution data from the entire group of lean and *ob/ob* mice are displayed as % nuclei in each phase of the cell cycle. Diploid cell cycle = G<sub>0</sub>-G<sub>1</sub> (2n) and S-G<sub>2</sub>-M (4n); hyperdiploid cell cycle = G<sub>0</sub>-G<sub>1</sub> (8n) and S-G<sub>2</sub>-M (16n). All of the differences between lean and *ob/ob* groups are significant with *P* < 0.05.

Mice	Liver histology		Flow cytometry of liver nuclei			
	BrdUrd (+) hepatocyte nuclei		Diploid cycle		Hyperdiploid cycle	
			G <sub>0</sub> -G <sub>1</sub>	S-G <sub>2</sub> -M	G <sub>0</sub> -G <sub>1</sub>	S-G <sub>2</sub> -M
Lean	2.3 $\pm$ 0.3		97.7	2.3	89.2	10.8
<i>ob/ob</i>	3.4 $\pm$ 0.3		96.4	3.6	83.5	16.5

Fig. 4. Cell cycle distribution of liver nuclei for lean and *ob/ob* mice. Nuclei were isolated from livers, stained with propidium iodide, and analyzed by flow cytometry. The distribution of nuclei with various DNA contents are shown for one representative lean and one typical *ob/ob* mouse. DNA content of 40 = 2n (G<sub>1</sub>), 80 = 4n (G<sub>2</sub>-M), 160 = 8n (octaploid). The cumulative flow cytometry results from four lean (a) and four *ob/ob* (b) mice are summarized in Table 2.



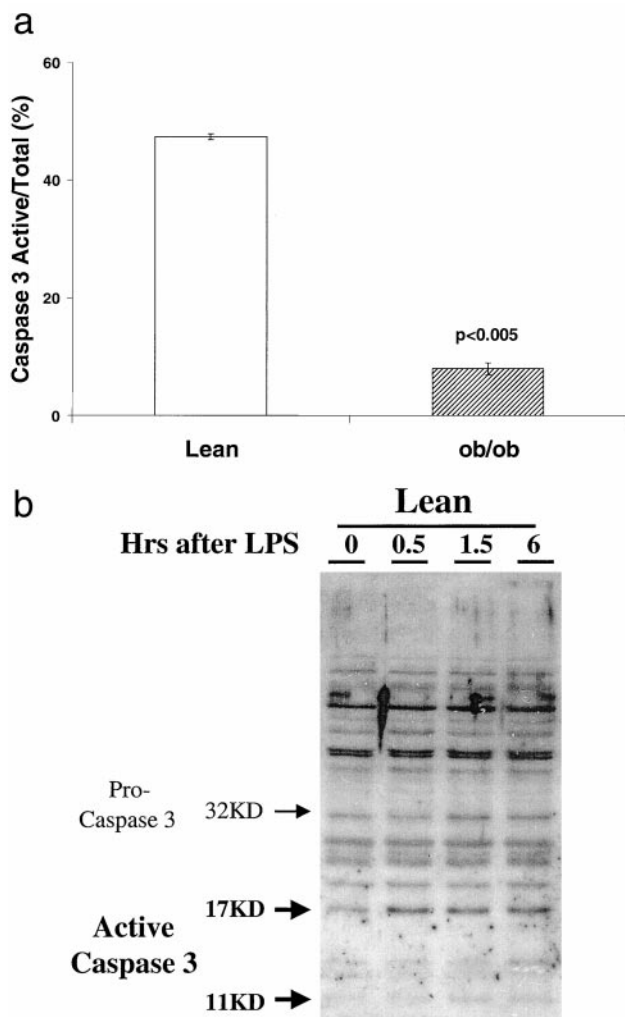


Fig. 5. Hepatic content of total and active caspase 3. Liver homogenates (40  $\mu$ g of protein/mouse) from four lean and four *ob/ob* mice were separated by SDS-PAGE and probed with antisera that recognize both procaspase 3 ( $M_r$  32,000) and its activated cleavage products ( $M_r$  17,000 and 11,000). This experiment was repeated two more times. All three blots were analyzed by densitometry. On each blot, the expression of activated caspase 3 ( $M_r$  17,000 + 11,000) was normalized to the total caspase 3 expression ( $M_r$  32,000 + 17,000 + 11,000) in each homogenate. Results are the mean ( $\pm$  SE) from four mice/group (a). A group of wild-type C57BL-6 mice ( $n = 4$ ) were given injections i.p. of 10  $\mu$ g of LPS to induce hepatocyte apoptosis. Liver homogenates were prepared from one mouse at each time point (0, 0.5, 1, or 6 h after LPS treatment). Caspase 3 activation was evaluated by immunoblot analysis as described in a. This immunoblot demonstrates the hepatic content of procaspase 3 and caspase 3 cleavage products in normal mice before and after the stimulation of apoptosis. Each lane contains 40  $\mu$ g of liver protein (b).

with mitochondrial MnSOD, the enzyme that detoxifies  $O_2^-$  (36, 52). To clarify events that might protect *ob/ob* hepatocytes from apoptosis, mitochondrial MnSOD activity was compared in *ob/ob* mice and their lean litter mates (38). Hepatic mitochondrial MnSOD activity is greater in *ob/ob* mice (Fig. 7). Activation of mitochondrial MnSOD is predicted to decrease oxidative stress (36), and, hence, this finding is difficult to reconcile with the EMSA data that demonstrate induction of NF $\kappa$ B, a redox-sensitive transcription factor (53), in *ob/ob* liver nuclei. However, overexpression of certain antiapoptotic members of the Bcl-2 family of mitochondrial membrane proteins, such as Bfl-1 or Bcl-xL, can inhibit apoptosis after cytochrome *c* release and NF $\kappa$ B activation (35, 54–56). Fatty livers from *ob/ob* mice have accumulated an increased level of bfl-1 and bcl-xL transcripts (Fig. 8). Thus, several antiapoptotic factors, including NF $\kappa$ B, MnSOD, bfl-1, and bcl-xL, have been induced in *ob/ob* livers, providing potential explanation(s) for the reduced activation of caspase 3 and decreased

accumulation of apoptotic hepatocytes in *ob/ob* mice. Although the absolute magnitude of the difference between *ob/ob* and lean livers for any one of these antiapoptotic responses is relatively small, the combination of inhibited apoptosis plus slight increases in hepatocyte proliferation cause hepatic epithelial hyperplasia and contribute to the increased mass of *ob/ob* livers.

## DISCUSSION

To our knowledge, these studies are the first to demonstrate an association between obesity and hepatocyte hyperplasia. Because the work was done with genetically obese mice that were housed in a tightly monitored animal facility, it is unlikely that the findings can be explained by occult exposure to environmental toxins or hepatotro-

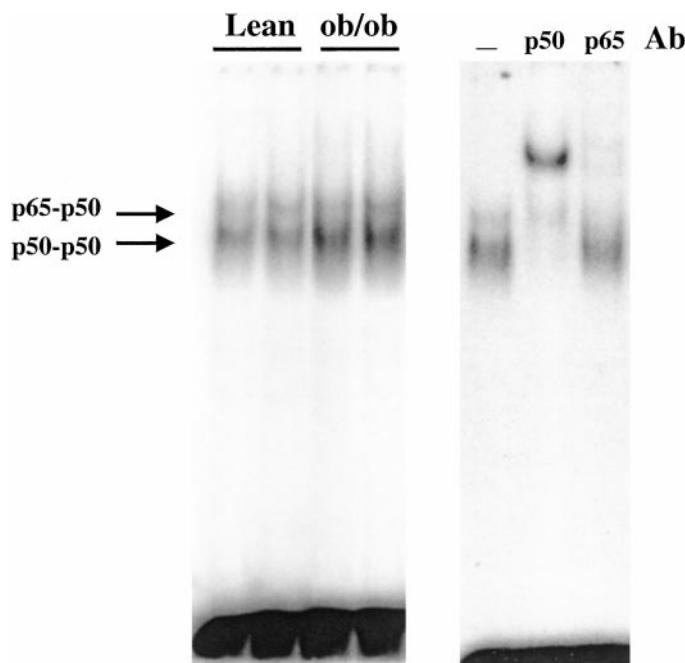


Fig. 6. NF $\kappa$ B-DNA binding activity. Hepatic nuclear extracts were prepared from the livers of two lean and two *ob/ob* mice. The binding activity of NF $\kappa$ B was evaluated by gel mobility shift assay (8  $\mu$ g of protein/lane) using  $^{32}$ P-labeled, double-stranded oligonucleotide fragments with the  $\kappa$ B binding site in the immunoglobulin enhancer element (left panel). Similar results (i.e., a 2- to 3-fold increase in NF $\kappa$ B binding activity in *ob/ob* mice) were obtained when the experiment was repeated with nuclear extracts from another two mice from each group. To demonstrate the nature of the binding complexes, the assay was repeated using 8  $\mu$ g of nuclear protein from one of the *ob/ob* mice. In this case, extract was preincubated with either preimmune serum (-) or with antisera to NF $\kappa$ B p50 or p65 (right panel). The results indicate that the upper, slower-migrating complex contains a mixture of NF $\kappa$ B p50 and p65, whereas the lower, faster-migrating complex contains predominately NF $\kappa$ B p50.

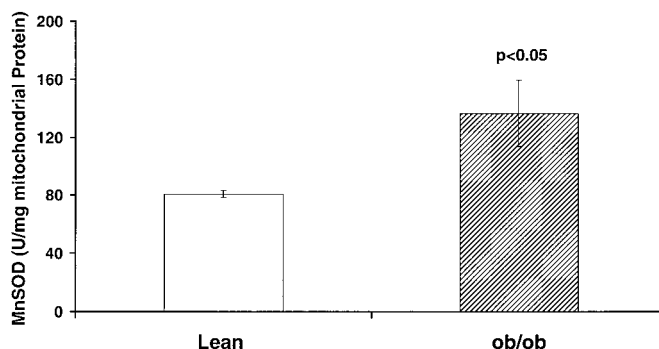


Fig. 7. Mitochondrial MnSOD activity. Mitochondria were isolated from the livers of four lean and four *ob/ob* mice immediately after sacrifice. MnSOD activity was determined and expressed per mg of mitochondrial protein. Results are shown as mean  $\pm$  SE.

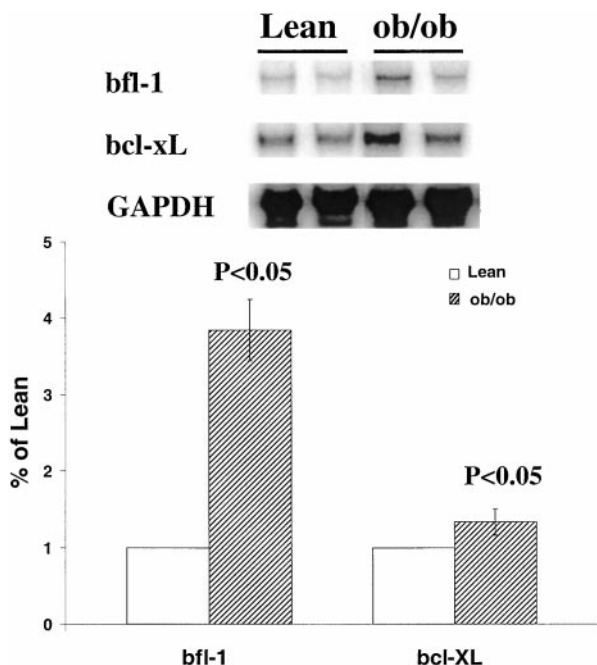


Fig. 8. Antiapoptotic gene expression. Total liver RNA from two lean and two *ob/ob* mice were evaluated by RNase protection assay as described in the legend to Fig. 3. A representative autoradiograph demonstrates the results in these four mice (10  $\mu$ g RNA/mouse). The experiment was repeated with liver RNA from another two mice from each group. Both blots were analyzed by phosphoimager and data (mean  $\pm$  SE) obtained from four *ob/ob* mice and four lean mice are graphed.

phic viruses. Moreover, the observed increases in hepatocyte proliferation and decreases in hepatocyte apoptosis occurred in the absence of overt hepatic inflammation or fibrosis and did not require exposure to tumor promoting drugs. In this regard, the fatty livers of young, adult *ob/ob* mice resemble the livers of *myc*- or *TGF $\alpha$* -transgenic mice and *myc/TGF $\alpha$* -double transgenic mice, in which increased liver mass is induced experimentally by the overexpression of a known proto-oncogene and/or hepatocyte growth factor (57, 58). Similar to these transgenic mice (58, 59), aged *ob/ob* mice also have an increased incidence of HCC (21, 60, 61), although the tumor incidence rate appears to be much greater in the former. Nevertheless, given the acknowledged association between obesity and cancer in several organs, including the liver (1, 8, 9), evidence for obesity-related increases in hepatocyte proliferative activity relative to apoptosis have direct clinical relevance.

Although the mechanisms for the association between obesity and liver cancer are unknown, cellular survival advantages that occur during insulin resistance may contribute to hepatocarcinogenesis because obesity-related insulin resistance correlates with an increased risk for cancers in other organs, such as the breast and colon (2, 3). In general, most cases of HCC are diagnosed in patients who have had cirrhosis for many years (4, 5). However, it is not clear whether the neoplastic process begins during cirrhosis or starts at earlier stages of liver disease. If HCC begins during cirrhosis, then it is reasonable to speculate that hepatic insulin resistance might be involved, because advanced liver disease is generally associated with insulin resistance, and the association between cirrhosis and diabetes is particularly strong in obese individuals (7). On the other hand, it has been difficult to determine whether insulin resistance plays an independent role in the pathogenesis of obesity-related hepatic neoplasia or is merely an associated abnormality that, like HCC, develops during cirrhosis.

Obesity is strongly associated with a spectrum of liver diseases that have been dubbed NAFLDs because they resemble alcohol-induced fatty liver diseases (6, 62, 63). Fatty liver (hepatic steatosis) is the

earliest and most common stage of NAFLD, afflicting almost 20% of American adults (14), whereas cirrhosis, the most advanced stage of NAFLD, develops over decades in only a minority of these individuals (15). Some degree of insulin resistance is present in most NAFLD patients with hepatic steatosis (64), and, as with other types of liver disease, the severity of hyperinsulinemia and insulin resistance increase as cirrhosis becomes established (64–66). However, because insulin resistance clearly antedates cirrhosis in many patients with NAFLD, livers could be evaluated during the early stages of NAFLD to determine whether hepatocyte hyperplasia occurs during insulin resistance when the liver is not cirrhotic. Genetically obese *ob/ob* mice with fatty livers have many of the features of the insulin-resistance syndrome in humans (18, 22). Hence, the livers of these mice can be analyzed to determine whether the process of hepatocyte neoplasia begins during the early stages of obesity-related fatty liver disease, when there is insulin resistance but not cirrhosis.

The present results demonstrate clearly that hepatocyte hyperplasia occurs at the fatty liver stage of NAFLD. However, as will be discussed subsequently, whether or not insulin resistance has any direct role in this process remains uncertain. The fatty hepatocytes of *ob/ob* mice exhibit many similarities to liver cells that have been transformed experimentally with the *ras* or *raf* proto-oncogenes. For example, both fatty livers and *ras* transformation result in MAPK activation, NF $\kappa$ B induction, and the up-regulation of antiapoptotic bcl-2-related genes (67), that are transcriptional targets for NF $\kappa$ B (68). Others have demonstrated that NF $\kappa$ B is a downstream target of the MAPK cascade (69) and have shown that NF $\kappa$ B induction required for *raf*- or *ras*-neoplastic transformation of rat liver epithelial cells (67), which suggests that the induction of NF $\kappa$ B and MAPK might contribute to the hepatocyte hyperplasia and hepatomegaly that occur in obesity-related fatty livers. However, if so, then it is paradoxical that hepatic NF $\kappa$ B induction occurs in insulin-resistant *ob/ob* mice, because there is compelling evidence that insulin itself activates NF $\kappa$ B through a Raf-1-mediated pathway in other mammalian cells (70). Indeed, the ability of insulin to induce NF $\kappa$ B is required for the antiapoptotic actions of the hormone in several cell culture models (71).

Hence, it is conceivable that, rather than insulin resistance *per se*, one of the other hormonal, immunological, and metabolic abnormalities in obese, *ob/ob* mice might mediate hepatocyte hyperplasia. Given the association between type-2 diabetes, obesity, and malignancy in humans (8, 9) and experimental animals (21, 60, 61), the role of hyperinsulinemia (as opposed to insulin resistance) in the neoplastic process certainly merits investigation. In light of the recent demonstration that reactive oxygen species are required for hepatocarcinogenesis in *c-myc/TGF $\alpha$*  transgenic mice (72), the possibility that obesity-related increases in hepatic oxidative stress contribute to hepatic hyperplasia should also be considered. Hepatic mitochondrial production of reactive oxygen species is increased significantly in *ob/ob* mice (38). Thus, the possibility that oxidant stress might be one of the mechanisms driving hepatic hyperplasia in these animals is a particularly intriguing hypothesis. Although the mechanisms driving increased reactive oxygen species release from *ob/ob* liver mitochondria are unknown, leptin deficiency and secondary changes in cytokine production (29, 73, 74) might be involved. If the latter is true, then additional work is necessary before data from our studies with leptin-deficient *ob/ob* mice can be extrapolated to obese humans with fatty livers, because hyperleptinemia (rather than leptin deficiency) is the rule in human obesity (75).

Despite leaving many questions unanswered, our work is important because it demonstrates that some of the mechanisms that promote HCC in NAFLD, one of the most common types of liver disease, become operative “spontaneously,” *i.e.*, without an obvious require-

ment for comorbidity factors, such as viral infection or hepatotoxin exposure. Moreover, this hyperproliferative response begins long before cirrhosis occurs. Indeed, given evidence that *ob/ob* mice are resistant to cirrhosis (24), but eventually develop HCC (21), our observations raise the intriguing possibility that obesity-related fatty liver, itself, is a premalignant condition. If this possibility is confirmed by others, then our recent findings extend other data (7, 47, 76) that indicate that NAFLD is not always a benign process.

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