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Increased lipogenesis, induced by AKT-mTORC1-RPS6 signaling, promotes development of human hepatocellular carcinoma

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Abstract

Background & Aims—De novo lipogenesis is believed to be involved in oncogenesis. We investigated the role of aberrant lipid biosynthesis in pathogenesis of human hepatocellular carcinoma (HCC).

Methods—We evaluated the expression of enzymes that regulate lipogenesis in human normal liver tissues and HCC and surrounding, non-tumor, liver tissues from patients using real-time reverse transcription PCR, immunoblotting, immunohistochemistry, and biochemical assays. Effects of lipogenic enzymes on human HCC cell lines were evaluated using inhibitors and overexpression experiments. The lipogenic role of the proto-oncogene *AKT* was assessed in vitro and in vivo.

Results—In human liver samples, de novo lipogenesis was progressively induced from non-tumorous liver tissue toward the HCC. The extent of aberrant lipogenesis correlated with clinical aggressiveness, activation of the AKT–mTOR signaling pathway, and suppression of AMP-activated protein kinases. In HCC cell lines, the AKT–mTORC1–RPS6 pathway promoted lipogenesis via transcriptional and post-transcriptional mechanisms that included inhibition of FASN ubiquitination by the USP2a de-ubiquitinase and disruption of the SREBP1 and SREBP2

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degradation complexes. Suppression of the genes *ACLY*, *ACAC*, *FASN*, *SCD1*, or *SREBP1*, which are involved in lipogenesis, reduced proliferation and survival of HCC cell lines and AKT-dependent cell proliferation. Overexpression of an activated form of AKT in livers of mice induced lipogenesis and tumor development.

Conclusions—De novo lipogenesis has pathogenic and prognostic significance for HCC. Inhibitors of lipogenic signaling, including those that inhibit the AKT pathway, might be useful as therapeutics for patients with liver cancer.

Keywords

AMPK; liver cancer; liver disease; lipid biosynthesis

Human hepatocellular carcinoma (HCC) is one of the most frequent tumors worldwide.^{1,2} HCC is generally a fatal disease: only few patients are amenable to surgery due to HCC late diagnosis, and alternative treatments do not significantly improve the patients' prognosis when HCC is unresectable.^{1,2} Thus, the investigation of molecular mechanisms leading to HCC development and progression is mandatory to identify new targets for its early diagnosis, chemoprevention, and treatment.

Aberrant lipogenesis has been linked to metabolic abnormalities such as diabetes, obesity, and the metabolic syndrome as well as to cancer.^{3–7} In the latter disease, unconstrained lipogenesis is necessary to maintain a constant supply of lipids and lipid precursors to fuel membrane production and lipid-based post-translational modification of proteins in a context of elevated proliferation.^{4–7}

At the molecular level, exacerbated lipogenesis is reflected by the co-ordinately increased activity and expression of lipogenic enzymes in neoplastic cells.^{4–6} ATP citrate lyase (*ACLY*), acetyl-CoA carboxylase (*ACAC*), fatty acid synthase (*FASN*), malic enzyme (*ME*), and stearoyl-CoA desaturase 1 (*SCD1*) are rate-limiting enzymes responsible for the metabolism of glucose to fatty acid (FA) at various steps, and 3-hydroxy-3-methylglutaryl-CoA-reductase (*HMGCR*), mevalonate kinase (*MVK*), and squalene synthetase (*SQS*) are involved in cholesterol and isoprenoid synthesis. These enzymes are transcriptionally activated by sterol regulatory element-binding protein (*SREBP*)1 and 2, liver X Receptor (*LXR*) α and β , and carbohydrate responsive element binding protein (*chREBP*).^{4–10}

The relevance of aberrant lipogenesis in cancer is underscored by a recent body of data showing that suppression of the main lipogenic enzymes is able to both strongly restrain the *in vitro* growth of cell lines from various tumor types and to reduce tumorigenesis *in vivo*.^{4–6,11,12} In human HCC, reports on *de novo* lipogenesis are scanty. Upregulation of *FASN*, *ACAC*, *ACLY*, and *SCD1* mRNA levels has been described in a small HCC collection,¹³ and *SREBP1* expression has been found to inversely correlate with patients' prognosis.¹⁴ In addition, an *in vitro* study suggests that suppression of *FASN* is deleterious for the growth of human HCC cell lines.¹⁵ However, the status of lipogenic proteins has not been comprehensively examined in this disease, and functional studies on the role of lipogenic enzymes on HCC cells are limited to date. Furthermore, the molecular mechanisms leading to upregulation and increased activity of lipogenic proteins in human HCC have not been clearly delineated.

Here, we investigated the role of unconstrained lipogenesis in a collection of human HCC and cell lines, and in an *in vivo* mouse model. Our data indicate that aberrant lipogenesis is a pivotal mechanism contributing to liver oncogenesis and human HCC prognosis. Furthermore, we found that the v-akt murine thymoma viral oncogene homolog (AKT)

serine/threonine kinase is the major inducer of lipogenesis and its activation is oncogenic in the liver.

Materials and Methods

Human Tissue Samples

Eight normal livers, 68 HCCs and corresponding surrounding non-tumor liver tissues were used. Tumors were divided in HCC with shorter/poor (HCCP; n = 36) and longer/better (HCCB; n = 32) survival, characterized by <3 and > 3 years survival following partial liver resection, respectively.¹⁶ Patients' features are reported in Supplementary Table 1. Liver tissues were kindly provided by Dr. Snorri S. Thorgeirsson (National Cancer Institute, Bethesda, MD) and collected at the Pietro Valdoni Surgery Department (University of Rome La Sapienza, Rome, Italy). Institutional Review Board approval was obtained at participating hospitals and the National Institutes of Health.

Cell lines and Treatments

Transfection of *in vitro* growing cell lines with siRNAs, cDNAs, and treatment with specific inhibitors were performed as described in Supplementary Materials.

Hydrodynamic Injection and Histopathological Analysis

Wild-type FVB/N mice were subjected to hydrodynamic injection procedures as described (Supplementary Materials).¹⁷ Liver histopathology was assessed by two experienced pathologists (FD and ME) on tissue slides stained with H&E and the PAS reaction. To evaluate liver lipid content, frozen sections were subjected to Oil Red O staining using a standard protocol. Mice were housed, fed, and treated in accordance with protocols approved by the committee for animal research at the University of California, San Francisco.

Quantitative RT-PCR (QRT-PCR)

Primers for human and mouse *FASN*, *ACLY*, *ACAC*, *ME*, *SCD1*, *SREBP1*, *SREBP2*, *USP2a*, *chREBP*, *LXR-α*, *LXR-β*, *HMGCR*, *MVK*, *SQS*, and *RNR-18* genes were from Applied Biosystems (Foster City, CA). PCR reactions and quantitative evaluations were performed as described in Supplementary Materials.

Immunoblotting and Immunoprecipitation

Hepatic tissues were processed as reported in Supplementary Materials. Nitrocellulose membranes were probed with specific primary antibodies (Supplementary Table 2).

Immunohistochemistry

Immunohistochemical staining was performed on 10% formalin-fixed, paraffin-embedded sections as described in Supplementary Materials.

Statistical Analysis

Tukey-Kramer test was used to evaluate statistical significance. Values of $P < 0.05$ were considered significant. Data are expressed as means $\pm$ SD.

Results

Coordinated Activation of Lipogenic Proteins in Human Hepatocellular Carcinoma

Levels of the lipogenic pathway enzymes, including FASN, ACAC, ACLY, ME, SCD1, HMGCR, MVK, SQS, and those of their upstream inducers were investigated by immunoblotting and real-time reverse transcription PCR (Figure 1A,B; Supplementary Figure 1). No upregulation of LXR- α was detected in non-neoplastic surrounding livers and HCC when compared with normal livers. A progressive induction of FASN, ACLY, ACAC, ME, SCD1, HMGCR, MVK, SQS, chREBP, LXR- β , SREBP1, and SREBP2 occurred in non-tumorous surrounding livers both at protein and mRNA levels. In HCC, an additional rise in mRNA and protein levels of ACLY, ACAC, ME, SCD1, HMGCR, MVK, SQS, chREBP, and LXR- β was found, mostly in HCCP (Figure 1A,B; Supplementary Figure 1). FASN protein and mRNA levels were progressively induced in non-tumorous surrounding livers and HCC. A further upregulation of FASN was detected at protein level almost exclusively in HCCP (Figure 1A,B; Supplementary Figure 1). The latter finding suggests the existence of post-transcriptional mechanisms regulating FASN in the most biologically aggressive tumors. Previously, it has been shown that ubiquitin-specific protease 2a (USP2a) sustains FASN activity by impeding its ubiquitin-dependent degradation.¹⁸ Thus, we determined the levels of USP2a in our sample collection. Levels of USP2a (mRNA and protein) and USP2a-FASN complexes were upregulated in HCC, predominantly in HCCP (Figure 1B,C; Supplementary Figure 1). Subsequent suppression of *USP2a* by siRNA in Hep40 and SNU-182 cell lines (displaying high USP2a levels) led to FASN downregulation (Figure 1D) and decreased FA synthesis (Figure 1E), indicating that USP2a stabilizes FASN levels in HCCP. Similarly, SREBP1 and SREBP2 mRNA expression was gradually upregulated from non-neoplastic surrounding livers to HCC, with no major difference between tumor prognostic subclasses (Figure 1B). However, SREBP1 and SREBP2 protein levels were significantly higher in HCCP (Figure 1A; Supplementary Figure 1), implying the presence of mechanisms promoting their stability in this tumor subclass. Recent observations demonstrated that the AKT protooncogene reinforces SREBP1 and SREBP2 stability by impeding their degradation.¹⁹ In normal cells, SREBP1 and SREBP2 proteolysis is triggered by phosphorylation at Thr-426, Ser-430, Ser-434 (for SREBP1), Ser-432, and Ser-436 (for SREBP2) residues by glycogen synthase 3 beta (GSK-3 β), which creates a docking site for the ubiquitin ligase F-box protein FBW7 (CDC4), resulting in SREBP1 and SREBP2 degradation.¹⁹ Phosphorylation of SREBP1 residues progressively increased in non-neoplastic surrounding livers and HCCB, implying the presence of effective degradation mechanisms (Figure 1A; Supplementary Figure 1). In contrast, phosphorylation/inactivation of SREBP1 was almost ubiquitously lost in HCCP. As a causative mechanism, we investigated the levels of CDC4 and those of inactivated/phosphorylated GSK-3 β . Strikingly, both CDC4 and GSK-3 β were inactivated almost exclusively in HCCP (Figure 1C; Supplementary Figure 1). As a consequence, levels of CDC4-SREBP1 complexes were lowest in HCCP. Similarly, a strong decline in the levels of CDC4-SREBP2 complexes occurred in HCCP. Furthermore, we assessed the levels of monoacylglycerol lipase (MAGL), the enzyme responsible for hydrolysis of intracellular triglyceride to glycerol and fatty acids that has been demonstrated to contribute to cancer pathogenesis.²⁰ However, MAGL was downregulated in non-tumorous surrounding livers and HCC (Figure 1A; Supplementary Figure 1), excluding a major role played by MAGL in hepatocarcinogenesis.

To further substantiate our data, hepatic levels of cholesterol and triglycerides, and extent of FA synthesis were assessed. Cholesterol levels, triglyceride levels, and FA biosynthesis were increased in surrounding livers when compared to normal livers [(23.8 $\pm$ 3.1 vs. 18.2 $\pm$ 1.8) nmol/mg tissue; (19.4 $\pm$ 1.2 vs. 15.2 $\pm$ 1.4) nmol/mg tissue; (20.2 $\pm$ 2.1 vs. 15.4 $\pm$ 1.6) $\times 10^3$; $P < 0.0005$]. Cholesterol amount, triglyceride levels, and FA synthesis were significantly higher in HCC than in surrounding livers [(29.8 $\pm$ 2.1 vs. 23.8 $\pm$ 3.1) nmol/mg

tissue; $(24.9 \pm 1.4$ vs. $19.4 \pm 1.2)$ nmol/mg tissue; $(35.1 \pm 2.4$ vs. $20.2 \pm 2.1) \times 10^3$; $P < 0.001$], and they were more elevated in HCCP than HCCB [$(34.8 \pm 2.4$ vs. $25.8 \pm 2.6)$ nmol/mg tissue; $(28.4 \pm 2.4$ vs. $22.4 \pm 2.0)$ nmol/mg tissue; $(41.8 \pm 3.4$ vs. $28.9 \pm 2.4) \times 10^3$; $P < 0.0001$]. Altogether, the present data indicate that increased lipogenesis is associated with HCC development and progression.

Aberrant Lipogenesis is Activated by AKT/mTORC1/RPS6 in Human Liver Cancer

Current evidence indicates that the AKT/mTOR pathway is involved in lipogenesis regulation.^{4–6} Thus, we assessed the total and activated levels of AKT, mTOR, and AMP-activated kinase (AMPK) proteins, the latter negatively modulating the lipogenic phenotype,^{4–6} in the sample collection (Figure 2A, Supplementary Figure 2). A gradual induction of activated AKT, mTOR, and the mTOR effector, ribosomal protein S6 (RPS6), was detected in non-neoplastic surrounding livers and HCC when compared with normal livers, with the highest expression being observed in HCCP. AMPK α 1 was equally expressed in normal, non-neoplastic livers, and HCC. Levels of AMPK β 1, AMPK γ , and of activated/phosphorylated AMPK α and AMPK β 1 were concomitantly downregulated in HCC, with HCCP exhibiting the lowest levels, whereas AMPK α 2 was downregulated only in HCCP (Figure 2A; Supplementary Figure 2).

To investigate the role of the AKT/mTOR pathway in the regulation of lipogenesis in HCC cell lines, we stably transfected myristylated AKT into HLE and SNU-423 HCC cells, both expressing AKT at low levels (Figure 2B, D). Myristylated AKT consists of AKT ligated to a myristoylation sequence, resulting in an enzyme tenfold more active than the wild-type counterpart.²¹ In untreated, vector-transfected, and myristylated AKT-transfected cells, increased proliferation and apoptosis occurred 24 and 48 hours post transfection, with the highest levels of proliferation and the lowest degree of apoptosis being detected in AKT-transfected cells. As a consequence, AKT-transfected cells displayed the highest increase of overall cell growth. In parallel, AKT induced lipid synthesis and upregulation of lipogenic proteins. AKT overexpression also triggered downregulation of AMPK α 2, AMPK β 1, activated/phosphorylated AMPK α and AMPK β 1, and AMPK γ proteins (Figure 2B). Equivalent results were obtained by stable transfection of wild-type AKT1 (data not shown). An opposite effect on cell growth, lipogenesis and lipogenic and AMPK proteins was detected when HuH1 and SNU-389 HCC cells (expressing high levels of AKT) were subjected to siRNA against AKT1/2 (Figure 2C,E). Similarly, transfection with a dominant negative mutant construct of AKT or treatment with the mTOR complex 1 (mTORC1) inhibitor Rapamycin inhibited cell growth and lipogenesis in the same cell lines (Supplementary Figure 3). The role of mTORC1 in AKT-induced lipogenesis was further substantiated by the finding that suppression of both *Raptor* (a member of mTORC1) and *Rictor* (a member of mTORC2) reduced cell growth and increased apoptosis in the HLE cell line transfected with activated AKT, but only Raptor silencing resulted in decreased lipogenesis and downregulation of lipogenic proteins (Supplementary Figure 4). Also, siRNA-mediated silencing of the mTORC1 effector, ribosomal protein S6 (*RPS6*), resulted in growth restraint and suppression of lipogenesis and lipogenic proteins in HLE cells transfected with activated AKT (Supplementary Figure 5). Furthermore, immunohistochemical analysis of the human liver sample collection showed co-localization and equivalent staining patterns for activated/phosphorylated AKT, FASN, ACAC, ACLY, SCD1, USP2A, HMGCR, MVK, and activated/phosphorylated RPS6 (Supplementary Figure 6). Altogether, these data indicate that RPS6 is the major downstream target of mTORC1 stimulating lipogenesis.

Next, we determined the relevance of SREBP1 and its downstream effectors, FASN, ACAC, ACLY, SCD1 on HCC growth by modulating their levels in HCC cell lines. Forced overexpression of each of the lipogenic proteins in 7703 and Focus HCC cells (exhibiting

low levels of SREBP1 and its effectors) accelerated growth, reduced apoptosis, and increased lipogenesis, with the most striking effects being observed following *SREBP1* transfection (Figure 3A-C; Supplementary Figure 7). These effects were accompanied by increased levels of phosphorylated/activated AKT (Supplementary Figure 7). Conversely, a strong growth restraint, massive apoptosis, marked reduction of lipogenesis, and decrease in levels of phosphorylated/activated AKT accompanied the siRNA-mediated inactivation of the 5 genes, especially *SREBP1*, in HuH1 and SNU-389 cells (showing high levels of SREBP1 and its effectors; Figure 3D-F; Supplementary Figure 8).

To more directly assess the importance of lipogenic proteins on AKT/mTOR-dependent growth, HLE and SNU-423 cells stably-transfected with myristylated/activated AKT were subjected to silencing of *SREBP1*, *FASN*, *ACAC*, *ACLY*, or *SCD1* via siRNA. Strikingly, increased cell growth induced by forced overexpression of AKT was markedly reduced when one of the aforementioned lipogenic proteins was concomitantly suppressed (Figure 4A-C). Also, suppression of each of the 5 lipogenic proteins significantly restricted the upregulation of phosphorylated/activated AKT induced by gene transfection of either myristylated AKT (Figure 4A) or wild-type AKT1 (not shown), further confirming that SREBP1, FASN, ACAC, ACLY, and SCD1 positively regulate AKT activation. Furthermore, treatment of HLE and SNU-423 cells stably-transfected with myristylated AKT by using chemical inhibitors of FASN, ACAC, and SCD1 markedly reduced AKT activation (not shown) and was highly detrimental for their *in vivo* growth, whereas the same inhibitors did not affect the growth of corresponding cell lines transfected with the empty vector (Supplementary Figure 9). Altogether, these data indicate that the AKT/mTORC1/RPS6 axis is a major modulator of lipogenesis in human HCC and that lipogenic proteins contribute to AKT/mTOR-mediated HCC cell growth.

Overexpression of AKT Induces Lipogenesis and Promotes Tumor Development in the Mouse Liver

Next, we developed a mouse model to examine whether AKT overexpression contributes to aberrant lipid synthesis and hepatocarcinogenesis *in vivo*. By combining hydrodynamic injection and sleeping beauty mediated somatic integration, we stably expressed the HA-tagged myristylated Akt (Myr-Akt) into the hepatocytes of wild-type FVB/N mice.

While injection of either vector alone or other oncogenes, including Bmi or NRasV12, did not induce histological changes in the liver,²² expression of myr-AKT had strong impact on liver structure. Already 3 days after hydrodynamic injection, liver morphology was significantly altered. Indeed, single or small clusters of lesional cells, mainly located in zone 3 of the liver acinus, were visible (Figure 5B). These cells exhibited a massively enlarged clear cytoplasm, owing to increased glycogen and fat storage. Immunohistochemical detection of HA-Tag confirmed that these cells incorporated the injected construct (Figure 5B). An indication of proliferative activity was the increased PCNA expression in the cell nuclei when compared to the surrounding hepatocytes (Figure 5B). Twelve weeks after injection, livers were enlarged and the color has changed, becoming more inhomogeneous, spotty and paler than in normal controls (Figure 5A,C). Approximately 50% of the liver tissue was occupied by numerous preneoplastic lesions (Figure 5C). Individual foci consisted of more than 30 altered hepatocytes, all showing a clear-cell morphology and high lipid content (Figure 5C). Three of the five investigated animals harbored a small hepatocellular adenoma (HCA), measuring 0.7–1.8 mm in diameter. Occasionally, mitotic figures were detected in the lesions and tumors (Figure 5C). Twenty-eight weeks after injection, the continuous proliferation of preneoplastic cells led to a massive enlargement and deformation of the livers (Figure 5A). Indeed, the lesional tissue in each of the five investigated animals occupied more than 80% of the liver volume (Figure 5D). Although most of the lesions were preneoplastic, each of the animals showed numerous tumors.

Multiple HCA were detected in all mice, with the biggest measuring 13 mm in diameter. Also, 3 of 5 mice showed one HCC, measuring up to 12 mm in diameter (Figure 5E). While the fat-storing clear cell phenotype was predominant in preneoplastic foci and HCA, HCC cells showed increased cytoplasmic basophilia and loss of intracytoplasmic lipids but retained the trabecular growth and were well differentiated (Figure 5E). In HCC, mitoses, apoptotic bodies, and areas of necrosis were detected (Figure 5E). Although the hepatocellular differentiation clearly predominated during the carcinogenic process (more than 90% of the lesional tissue), a small proportion of ductular tumor-like foci, mixed hepatocellular-ductular adenomas, and, very rarely, pure cholangiocellular tumors emerged (Supplementary Figure 10), but no cholangiocarcinomas developed.

Upregulation of Lipogenic Proteins in AKT-induced Liver Lesions

Next, we investigated the levels of the genes/proteins involved in lipogenesis in uninjected and Myr-Akt-injected mice. Upregulation of AKT and mTOR mRNA and protein levels occurred in preneoplastic and neoplastic lesions from Myr-AKT mice, when compared with control livers, as detected by immunoblotting and quantitative real-time RT-PCR (Figure 6A, Supplementary Figure 11,12). AKT, mTOR, and RPS6 activation was paralleled by upregulation of lipogenic enzymes involved in fatty acid (FASN, ACLY, ACAC, ME, SCD1) and cholesterol (HMGCR, MVK, SQS) biosynthesis. Transcription factors promoting lipogenesis (chREBP, LXR- β , SREBP1 and SREBP2) were also upregulated. Importantly, FASN, SREBP1, and SREBP2 upregulation occurred only at protein level (Figure 6A, Supplementary Figure 11,12), presumably due to their impaired degradation. Indeed, high FASN protein expression was associated with upregulation of USP2a and increased levels of FASN-USP2a complexes in AKT-injected livers. Also, SREBP1 and SREBP2 upregulation correlated with inactivation/phosphorylation of GSK-3 β and, consequently, with reduced levels of CDC4-SREBP1 and CDC4-SREBP2 degradation complexes. Decreased phosphorylation of Thr-426, Ser-430, and Ser-434 residues also correlated with SREBP1 upregulation. Induction of aberrant lipogenesis was paralleled by downregulation of AMPK α 2, AMPK β 1, and AMPK γ proteins, and of activated/phosphorylated levels of AMPK α and AMPK β 1 in AKT-injected mice (Figure 6B, Supplementary Figure 11). Thus, AKT not only induces the pro-lipogenic proteins, but also suppresses anti-lipogenic signals. Overexpression of FASN, ACLY, ACAC, SCD1, HMGCR, MVK, phosphorylated/activated AKT, and USP2a proteins was confirmed by immunohistochemistry (Figure 7; Supplementary Figure 13). The overexpression did not differ much during the carcinogenic process or within the lesional cells of an individual animal, albeit the tumors were slightly stronger stained. In summary, our data indicate that overexpression of AKT leads to lipogenesis activation and hepatocarcinogenesis in the mouse.

Discussion

In the present study, we show that aberrant activation of lipogenesis is a dominant oncogenic event in human HCC. Importantly, no significant differences were detected in the extent of *de novo* lipogenesis with regard to HCC etiology, suggesting that exacerbated lipogenesis is a general molecular phenomenon in hepatocarcinogenesis. Indeed, previous reports demonstrated that both hepatitis B and C viruses are able to induce FASN expression,^{23–25} and that overexpression of FASN is a typical feature of another predisposing condition for liver cancer, the alcoholic steatohepatitis.²⁶ Furthermore, strong upregulation of FASN characterizes a rat model of insulin-induced hepatocarcinogenesis,²⁷ which resembles the occurrence of HCC in people affected by type II diabetes mellitus and/or metabolic syndrome, two clinical conditions associated with an increased risk of liver cancer development.²⁸

The highest levels of lipogenic proteins were detected in HCC characterized by an aggressive phenotype, supporting an important prognostic role for *de novo* lipogenesis in HCC, as observed in many other epithelial cancer types^{4–6} and in accordance with the recent finding that SREBP1 levels correlate with HCC proliferation and patient's prognosis.¹⁴

Previous reports indicate that signaling cascades driven by AKT/mTOR, MAPK, and AMPK regulate *de novo* lipogenesis.^{4–6} Our present investigation clearly demonstrates that the AKT/mTORC1/RPS6 axis is the major regulator of the lipogenic phenotype in liver cancer. Indeed, forced overexpression of activated Akt resulted in induction of lipid biosynthesis and upregulation of lipogenic proteins in *in vitro* cultured HCC cell lines and *in vivo*, and AKT suppression by either specific siRNAs or transfection of a AKT dominant negative form was associated with decreased lipogenesis and down-regulation of lipogenic proteins. Equivalent results were obtained by treatment of AKT-transfected cells with the mTORC1 inhibitor, Rapamycin, as well as by siRNA-mediated inactivation of Raptor and RPS6. Also, we found that modulation of the MAPK cascade, another putative pathway responsible for lipogenesis,^{4–6} had no effect on either lipogenesis or levels of the lipogenic proteins (data not shown).

Significantly, the present findings indicate that the AKT/mTORC1 pathway induces lipogenesis both via transcriptional and post-transcriptional mechanisms. The pro-lipogenic transcriptional activity played by AKT is in accordance with a previous report in human retinoic pigment epithelial and osteosarcoma cell lines.²⁹ Nevertheless, we show that AKT promotes *de novo* lipogenesis by post-transcriptional mechanisms as well, namely by impeding proteasomal degradation of SREBP1, SREBP2, and FASN. Suppression of SREBP1 and SREBP2 ubiquitination was achieved by AKT through its ability of inhibiting GSK-3 β , which primes SREBP1 and SREBP2 for phosphorylation-dependent proteolysis,¹⁹ while degradation of FASN was impaired through transcriptional upregulation of the USP2a deubiquitinase. To the best of our knowledge, this is the first report showing that AKT induces USP2a in cancer.

Moreover, our results indicate that induction of lipogenic proteins is an important effector pathway of the AKT/mTORC1 axis in human HCC. On one hand, we showed that modulation of SREBP1, FASN, ACAC, ACLY, or SCD1 was able to significantly affect both AKT activation and the levels of the other lipogenic proteins, implying the presence of complex, positive feedback loops reinforcing AKT activation and sustaining overexpression of the lipogenic proteins in liver cancer (Supplementary Figure 14). Further studies are needed to define the mechanisms whereby lipogenic proteins promote each other's upregulation and AKT activation. On the other hand, we found that AKT-dependent proliferation and resistance to apoptosis were markedly reduced when AKT overexpression was accompanied by selective inactivation of SREBP1, FASN, ACLY, ACAC, or SCD1 *in vitro*. Of note, the finding that suppression of lipogenic proteins exerted a strong growth restraint only in AKT-overexpressing cells, but not in the non-transfected counterparts, envisages the possibility that treatment with inhibitors of *de novo* lipogenesis might specifically target cells characterized by activation of the AKT/mTOR pathway in liver cancer.

Finally, we demonstrated that overexpression of AKT alone is sufficient to induce hepatocarcinogenesis in the mouse, suggesting that AKT deregulation has a pivotal role in human liver cancer. Our results are consistent with published data using mice harboring a deletion of Pten, a tumor suppressor gene that negatively regulates the Akt/mTOR pathway, since liver specific inactivation of Pten leads to steatosis and, eventually, HCC development.^{30,31} Consistent with the pivotal role of Akt pathway in HCC pathogenesis, levels of

activated AKT are almost ubiquitously upregulated in human HCC.³² Therefore, the AKT overexpressing mouse might represent a valuable model both to investigate the molecular mechanisms responsible for AKT-induced hepatocarcinogenesis and to evaluate the effect of suppressing the AKT/mTOR pathway and its lipogenic effectors in human HCC treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACAC	acetyl-Coenzyme A carboxylase
ACLY	ATP citrate lyase
AKT	v-akt murine thymoma viral oncogene homolog
AMPK	AMP-activated protein kinase
CDC4	F-box protein FBW7
chREBP	carbohydrate-responsive element-binding protein
FA	fatty acid
FASN	fatty acid synthase
HCC	hepatocellular carcinoma
HCCB	HCC with better outcome
HCCP	HCC with poorer outcome
HMGCR	3-hydroxy-3-methylglutaryl-CoA-reductase
LXR	liver X receptor
ME	malic enzyme
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
MVK	mevalonate kinase
RPS6	ribosomal protein S6
SCD	stearoyl-CoA desaturase
SQS	squalene synthetase
SREBP	sterol regulatory element binding protein
USP2	ubiquitin-specific protease 2

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Figure 1.

Activation of lipogenic proteins in human liver cancer. (A) Representative immunoblotting of the major lipogenic proteins and transcription factors involved in lipogenesis. Both total (T) and nuclear (N) levels of SREBP1 were determined. (B) mRNA levels of lipogenic enzymes and pro-lipogenic transcription factor were determined by quantitative real-time PCR. NT (Number Target) = $2^{-\Delta Ct}$, $\Delta Ct = Ct \text{ RNR18} - Ct \text{ target gene}$. Tukey-Kramer test: $P < 0.0001$ *a*, vs. NL; *b* vs. SL; *c* vs. HCCB. (C) Assessment of FASN and SREBP1 regulators by immunoblotting. SREBP1 phosphorylation sites, Thr-426, Ser-430, and Ser-434, are indicated as 1, 2, and 3 in the figure. Data from SL of HCCB and HCCP did not show statistical differences, and are thus merged. (D,E) Suppression of USP2a by siRNA in Hep40 cell line leads to FASN downregulation (D), and decrease in FA synthesis (E). Equivalent results were obtained in SNU-182 cells (not shown). (E) White, gray, or black bars indicate untreated cells, treated with scramble, or with anti-USP2a siRNA, respectively. Tukey-Kramer test: $P < 0.0001$ *a*, vs. untreated cells; *b*, vs. scramble siRNA-treated cells. Abbreviations: IB, immunoblotting; IP, immunoprecipitation; NL, normal liver; SL, surrounding liver; HCCB, HCC with better outcome (longer survival); HCCP, HCC with poorer outcome (shorter survival).

Figure 2.

(A) Activation of Akt, mTOR, and loss of AMPK proteins in human HCC. Whole cell lysates were prepared from normal livers (NL), surrounding livers (SL), and HCC with better (HCCB) or poorer (HCCP) outcome and immunoblotted with indicated antibodies. Data from SL of HCCB and HCCP did not show statistical differences, and are thus merged. (B) Effect of overexpressing activated *AKT* in HLE cell lines on the levels of the major players of lipogenesis and AMPK proteins. Equivalent results were obtained in SNU-423 cells (not shown). (C) Effect of suppression of *AKT* by siRNA in HuH1 cells on the levels of lipogenesis modulators and AMPK proteins. Equivalent results were obtained in SNU-389 cells (not shown). (D,E) Effect of overexpressing (D) or silencing (E) *AKT* in HLE and HuH1 cells, respectively, on cell viability (first panel), apoptosis (second panel), and fatty acid synthesis (third panel). White bars, control (untreated cells); grey bars, transfection of vector alone (D) or scramble siRNA (E); black bars, transfection of either *AKT* cDNA (D) of siRNA against *AKT1/2* (E). Each bar represents mean $\pm$ SD. Tukey-Kramer test: $P < 0.0001$ *a*, vs. control (untreated cells); *b*, vs. empty vector or scramble siRNA.

Figure 3.

Effect of forced overexpression of *FASN*, *ACAC*, *ACLY*, *SCD1*, and *SREBP1* cDNA via transient transfection in 7703 cell line (expressing low levels of the 5 lipogenic proteins) on cell viability (A), apoptosis (B) and fatty acid synthesis (C). Equivalent results were obtained in Focus cells (not shown). Effect of *FASN*, *ACAC*, *ACLY*, *SCD1*, and *SREBP1* silencing via siRNA in HuH1 cells (expressing high levels of the 5 proteins) on cell viability (D), apoptosis (E) and fatty acid synthesis (F). Equivalent results were obtained in SNU-389 cells. Each bar represents mean $\pm$ SD. Tukey-Kramer test: $P < 0.0001$ *a*, vs. control (untreated cells); *b*, vs. empty vector or scramble siRNA; *c*, vs. lipogenic enzymes.

Figure 4. Effect of inactivating *FASN*, *ACAC*, *ACLY*, *SCD1*, and *SREBP1* expression via siRNA in HLE cells stably transfected with myristylated/activated AKT. (A) Representative immunoblotting on the levels of AKT, FASN, ACAC, ACLY, SCD1, and SREBP1 following overexpression of myristylated AKT by stable transfection (+ AKT cDNA) and silencing of the 5 lipogenic proteins in HLE cells. Effect of *FASN*, *ACAC*, *ACLY*, *SCD1*, and *SREBP1* silencing via siRNA on the growth of HLE cells stably transfected with myristylated AKT (+ AKT cDNA) on cell viability (B) and apoptosis (C). Each bar represents mean $\pm$ SD of 3 independent experiments conducted in triplicate. Tukey-Kramer test: $P < 0.0001$ *a*, vs. control (untreated cells); *b*, vs. vector alone; *c*, vs. AKT-transfected-only cells; *d*, vs. lipogenic enzymes.

Figure 5.

Stepwise development of hepatocarcinogenesis in AKT-overexpressing mice by hydrodynamic gene delivery. (A) Macroscopic appearance of wild-type and AKT-injected livers (the latter analyzed 12 and 28 weeks after hydrodynamic injection). The livers of mice injected with myristylated AKT 12 weeks before (12w) showed a spotty and paler appearance than of normal controls (wild-type mice; WT) and were considerably enlarged, as indicated by the scale bar above the livers. The changes were much more prominent and several large tumors emerged 28 weeks after injection (28w; the arrow indicates a large HCC). (B) Histological features of AKT overexpressing livers 3 days after hydrodynamic injection. At this early time point, the first alterations in the liver tissue were noted: several altered hepatocytes emerged in the liver (some marked by arrows), characterized by a massively enlarged clear cytoplasm. Lesional cells were mostly located in the acinar zone 3 (v, the hepatic vein) while the periportal hepatocytes (acinar zone 1; p, portal tract) remain unaffected (B; main panel). Immunohistochemically, these cells were proven to harbor HA-Tag (brown staining; B, top right panel), and the increased nuclear expression of PCNA (indicated by arrows; B, bottom right panel) indicated their proliferative activity. (C) Twelve weeks after hydrodynamic injection, as a consequence of the proliferation of AKT overexpressing preneoplastic cells, approximately 50% of the liver was occupied by clear-cell preneoplastic foci, consisting of large clusters of cells (indicated by asterisks; C, main panel). Owing to their size, the lesions more often expanded into the periportal areas (acinar zones 1 and 2), although they were still mainly located in the acinar zone 3 (v: hepatic vein;

p: portal tract). The cytoplasm of altered cells was rich in lipids (Oil red O staining; C top right panel). In larger lesions, occasional mitotic figures (indicated by an arrow) were visible (C, bottom right panel). (D) Twenty-eight weeks after hydrodynamic injection, approximately 80% of the liver was occupied by lesional tissue (indicated by arrows; the asterisk marks an area of normal tissue), and hepatocellular carcinoma developed (E). These tumors displayed a macrotrabecular growth, considerably less intracytoplasmic lipid but increased cytoplasmic basophilia, significant nuclear atypia, and multiple mitotic figures (indicated by an arrow). Bottom edge of the pictures of the panels represents: 0.4 mm (B, main and bottom right panel; C, main and top right panel); 0.2 mm (B, top right panel; E); 0.1 mm (C; bottom right panel); 2.5 mm (D).

Figure 6.

Activation of lipogenic proteins in liver lesions from mice injected with myristylated Akt. (A) Immunoblot analysis of AKT, mTOR, and their downstream lipogenic effectors in uninjected wild-type livers and preneoplastic livers and tumors (hepatocellular adenomas and carcinomas) from AKT-injected mice. (B) Expression of USP2a, CDC4, phosphorylated/inactivated GSK-3β and SREBP1, and AMPK proteins in liver lesions from mice injected with AKT. Six to eight samples per each group were used for the analysis, and representative images are shown. β-Actin was used as loading control. Abbreviations: IB, immunoblotting; IP, immunoprecipitation.

Figure 7.

Activation of AKT, USP2a, and members of the lipogenic pathway in mice overexpressing myristylated AKT, as assessed by immunohistochemistry. The first row shows overexpression of phosphorylated/activated AKT, ACLY (as one example for lipogenic enzymes) and USP2a in preneoplastic hepatocytes, 3 days after injection. The middle block of 4 panels demonstrates the combined overexpression of ACAC, ACLY, SCD1, and FASN lipogenic enzymes in preneoplastic livers, 12 weeks after injection. Identical results were observed in hepatocellular tumors. In the bottom row, an example of a large hepatocellular adenoma and surrounding admixed preneoplastic and normal liver tissue is depicted (serial sections), showing AKT overexpression. Note the stronger AKT immunoreactivity in the tumor when compared with neighbouring preneoplastic cells (28 weeks after injection). Bottom edge of the pictures of the panels represents 0.25 mm in the upper row, 0.5 mm in the middle block, and 5 mm in the bottom row.