Fatty acid-CoA ligase 4 is overexpressed in human hepatocellular carcinoma

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Fatty acid-CoA ligase 4 (FACL4) is a central enzyme controlling the unesterified arachidonic acid (AA) level in cells. It has been shown that FACL4 blocks apoptosis and promotes colon carcinogenesis by lowering the cellular level of unesterified AA. Consistent with this, FACL4 is upregulated in colon adenocarcinoma. The status of FACL4 in other tumors including hepatocellular carcinoma (HCC) is not known. Here, we report that FACL4 is overexpressed in human HCC compared with adjacent normal liver tissues. FACL4 mRNA and protein were overexpressed in 5 out of 12 (41.7%) and 3 out of 8 (37.5%) cases of HCC, respectively. Immunohistochemical staining showed strong fine granular intracytoplasmic staining in tumor cells, whereas we observed occasional weak staining in normal liver tissues surrounding the tumors. We found that 14 out of 37 (37.8%) HCC expressed moderate to strong FACL4 immunostaining. Both normal adult and fetal liver tissues showed very weak to no detectable staining, whereas 3 out of 10 (30%) cirrhotic livers expressed weak staining. In addition, we found that 4 out of 8 (50%) human hepatoma cell lines expressed high levels of FACL4 by northern blot analysis. Our results show that FACL4 is a new molecular marker for HCC and suggest that the FACL4 pathway may be involved in liver carcinogenesis. (Cancer Sci 2003; 94: 421–424)

Unesterified arachidonic acid (also called free AA) entering cells exogenously or released endogenously is rapidly converted to AA-CoA esters by the catalytic action of fatty acid-CoA ligase (FACL), particularly by the AA-preferring FACL4.[1, 2] Several lines of evidence indicated that the level of free AA in cells regulates apoptosis.[3–7] More recently, it has been demonstrated that free AA induces apoptosis by activating the caspase-3 pathway, and that the induction of apoptosis by inhibitors of AA metabolism is a consequence of its accumulation.[8] Moreover, overexpression of FACL4 prevented AA-induced apoptosis by reducing the level of intracellular free AA.[8] These findings, together with overexpression of FACL4 in colon adenocarcinoma, strongly suggest that the FACL4 pathway may be important in colon carcinogenesis.[9] In addition, cyclooxygenase-2 (Cox-2), another AA-utilizing enzyme, is also known to be overexpressed in colon cancer.[10, 11] Therefore, AA metabolism plays an important role in colon carcinogenesis. Currently, overexpression of FACL4 and Cox-2 in colon adenocarcinoma is believed to deplete free AA, thereby removing a proapoptotic signal and promoting carcinogenesis.[8, 9]

Hepatocellular carcinoma (HCC) is one of the most common tumors worldwide and is one of the leading causes of death among cancer patients in Korea. Identification of genes that are overexpressed in HCC is of importance to understand liver tumorigenesis and to develop diagnostic and therapeutic targets. As is seen in colon cancer, Cox-2 is known to be overexpressed in human HCC,[12–14] implying that AA metabolism might also be important in liver carcinogenesis. However, it is not known whether FACL4 is also overexpressed in HCC. It is known that human placenta, brain, testis, ovary, spleen, and adrenal cortex express high levels of FACL4, whereas the gastrointestinal system, including liver, expresses a very low level.[2] In a recent paper,[15] we reported that FACL4 is frequently upregulated in HCC compared to chronic hepatitis, as revealed by high-throughput screening combining RDA and microarray. We think that assessing the status of FACL4 in HCC is of importance to check whether the FACL4 pathway is also involved in liver carcinogenesis. Therefore, in this study, we investigated FACL4 upregulation in tumor cells of HCC and in cell lines derived from hepatoma.

Materials and Methods

Tumor samples and cell lines. Twelve pairs of HCC and corresponding normal liver tissues were obtained from patients undergoing surgery in Kyungpook National University Hospital (Daegu, Korea) with the approval of the human research review committee and with patients' informed consent. A liver tissue array containing containing 30 HCC, 10 cirrhotic livers, 10 normal livers, and 10 fetal livers was obtained from SuperBioChips Laboratories (Seoul, Korea). Information on the tissue array slide is available on-line at http://www.tissue-array.com. Cell lines derived from HCC were purchased from Korea Cell Line Bank (Seoul, Korea). Hep G2 and Hep 3B cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. The SNU 354, SNU 368, SNU 423, SNU 398, SNU 449, and SNU 475 cells[16] were cultured in RPMI 1640 containing 10% FBS.

RT-PCR and Northern blot analysis. cDNA was synthesized from 2 μg of total RNA and small aliquots of the cDNA product were amplified with the gene-specific primer sets. The FACL4, glypican-3 (GPC3), insulin-like growth factor 2 (IGF2) and β-actin specific primers were previously reported.[15] The α-fetoprotein primers are 5′-CCCACTCCAGCATCGATCC-3′ and 5′-TTATGGCTTGGAAAGTTCGGGT-3′. PCR amplification was performed after an initial denaturation of 5 min at 94°C, followed by 32 cycles of 30 s at 94°C, 45 s at 58°C and 1 min at 72°C, and final elongation at 72°C for 10 min. For Northern blot analysis, total RNA from tissues (10 μg/lane) was separated by 1% agarose/2.2 *M* formaldehyde denaturing gel, transferred to a positively charged nylon membrane (Amersham, Buckinghamshire, United Kingdom) by capillary action using 20× SSC, and UV-cross-linked. Blots were prehybridized, hybridized with FACL4 cDNA probes, and washed according to the supplier's recommendations (QuickHyb; Clontech, Palo Alto, CA).

Western blot analysis. Anti-human FACL4 antibody was a gift from Dr. Prescott (University of Utah, USA). The antibody was raised in rabbit against a synthesized peptide (H2N-MAKRIKAKPTSDKPGC-amide) that corresponds to the deduced N-terminal sequence of human FACL4.[17] Total tissue lysate was prepared as described[18] and the protein concentration was measured using the BCA protein assay (Pierce, Rockford,

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IL). Protein samples (30 μg/lane) were separated on 10% polyacrylamide gel, and transferred to nitrocellulose membranes. The membranes were blocked with 6% milk in PBS for 1 h, and then probed with FACL4 antibody (1:2000 dilution). Horseradish peroxidase-conjugated donkey anti-rabbit Ig (Amersham) was used as a secondary antibody at a 1:5000 dilution. Bands were visualized using ECL Plus (Amersham).

Immunohistochemical staining. We used the Zymed non-biotin amplification system (Zymed, San Francisco, CA) since endogenous biotin in liver tissue gives a high background. Paraffin blocks containing both tumors and surrounding normal tissues were sectioned at 5 μm. Slides were washed twice for 5 min in xylene to remove paraffin and rehydrated through a series of graded ethanol solutions. Sections were washed twice in PBS and treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. Tissue sections were blocked for 10 min in serum blocking solution, and incubated for 30 min in PBS containing the FACL4 antibody (1:200 dilution). Slides were then rinsed with PBS, incubated with secondary antibody for 10 min, and rinsed again with PBS. Sections were incubated in tertiary antibody-HRP conjugate for 10 min, rinsed in PBS, and incubated with diaminobenzidine (DAB) for 10 min. Slides were counterstained with Mayer's hematoxylin and mounted.

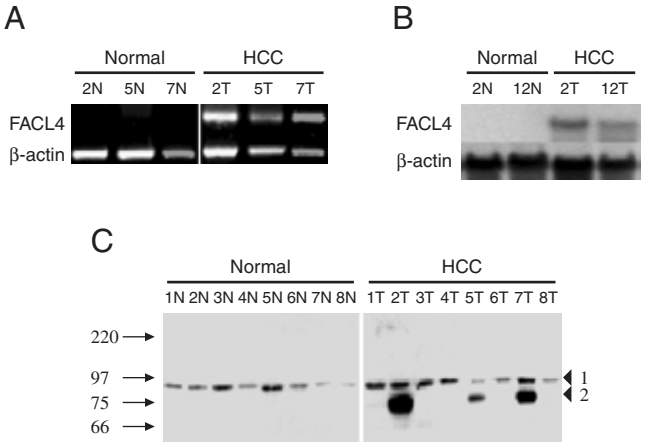

Fig. 1. FACL4 is upregulated in hepatocellular carcinoma. (A) A representative RT-PCR analysis. (B) A representative Northern blot analysis. (C) A representative Western blot. In addition to FACL4 specific bands (75 kDa; arrowhead 2), bands of apparently higher molecular weight was observed with longer exposure (arrowhead 1). In all cases, HCC (T) and paired adjacent normal tissues (N) were compared. The number on top of each panel corresponds to the case number in Table 1.

Results

Upregulation of FACL4 mRNA in HCC. To verify the upregulation of FACL4 in HCC tumor samples, we first examined expression of FACL4 transcript by RT-PCR and Northern blot analysis, employing twelve pairs of tumors and normal liver tissues surrounding the tumors. A representative RT-PCR is shown in Fig. 1A. An amplified fragment of the expected size (441 bp) was detected only in tumor samples. The identity of the amplified fragment was verified by sequencing. Also, a single band of approximately 5 kb was detected only in tumor samples by Northern blot analysis (Fig. 1B). We detected overexpression of FACL4 mRNA in 5 out of 12 (41.7%) cases of HCC (Fig. 1 and Table 1).

Upregulation of FACL4 protein in HCC and in tumor cells. Next, we checked expression of FACL4 protein in HCC samples by immunoblotting using rabbit polyclonal anti-human FACL4 antibody. A major band with an approximate molecular weight of 75 kDa was detected only in the tumor samples where FACL4 transcript was detected (compare Fig. 1, A and C), suggesting that the band is FACL4-specific. The mobility of this band is the same as the reported size of FACL4 from heart or HEK 293 cells transiently transfected with a human FACL4 cDNA.[17] This result shows that FACL4 protein is indeed increased in tumors compared to adjacent normal tissues. We also detected a band of apparently higher molecular weight upon longer exposure (Fig. 1C, arrowhead 1). Whether this is another isoform of FACL that cross-reacts with the FACL4 antibody is not clear. However, the level of this band was nearly the same in both HCC and adjacent normal tissues. We also observed overall concordance between FACL4 mRNA expression and protein levels, though we performed immunoblotting with only eight out of twelve paired samples due to inadequate or absent tissues (Fig. 1 and Table 1).

Immunohistochemical staining showed strong fine granular intracytoplasmic staining in tumor cells, whereas we observed no staining in the stromal portion (Fig. 2A). We could detect occasional weak staining in normal liver tissues surrounding the tumors (Fig. 2B). Immunohistochemical analysis with the liver tissue array also showed that FACL4 staining in HCC is stronger than that of cirrhotic liver (Fig. 2E and Table 2). Alto-

Table 1. FACL4 expression in HCC patients

Case #	Age	Sex	Type (grade)[1)]	BGL[2)]	RT-PCR	Western/Immunostaining
1	48	M	P (MD)	NL	–	–
2	58	M	S (MD)	CH	+ (5)[3)]	+ (5/5)
3	63	F	S (MD)	NL	–	–
4	56	M	P (MD)	CH	–	–
5	76	M	S (MD)	CH	+ (1)	+ (1/2)
6	60	M	S (MD)	NL	–	–
7	70	M	S (PD)	CH	+ (3)	+ (3/3)
8	52	M	T (PD)	CH	–	–
9	59	M	S (MD)	CH	+ (1)	ND[4)]
10	59	M	P (PD)	CL	–	ND
11	34	M	T (PD)	CH	–	ND
12	44	F	S (PD)	CH	+ (3)	ND

1) HCC types and differentiation grade: S, solid; T, trabecular; P, pseudoglandular; WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated.
2) BGL, background liver; NL, normal liver; CH, chronic hepatitis; CL, cirrhotic liver.
3) +, expression; –, no expression; scores, relative level of expression determined by densitometry or by three blinded observers.
4) Test not done due to inadequate or absent tissue.

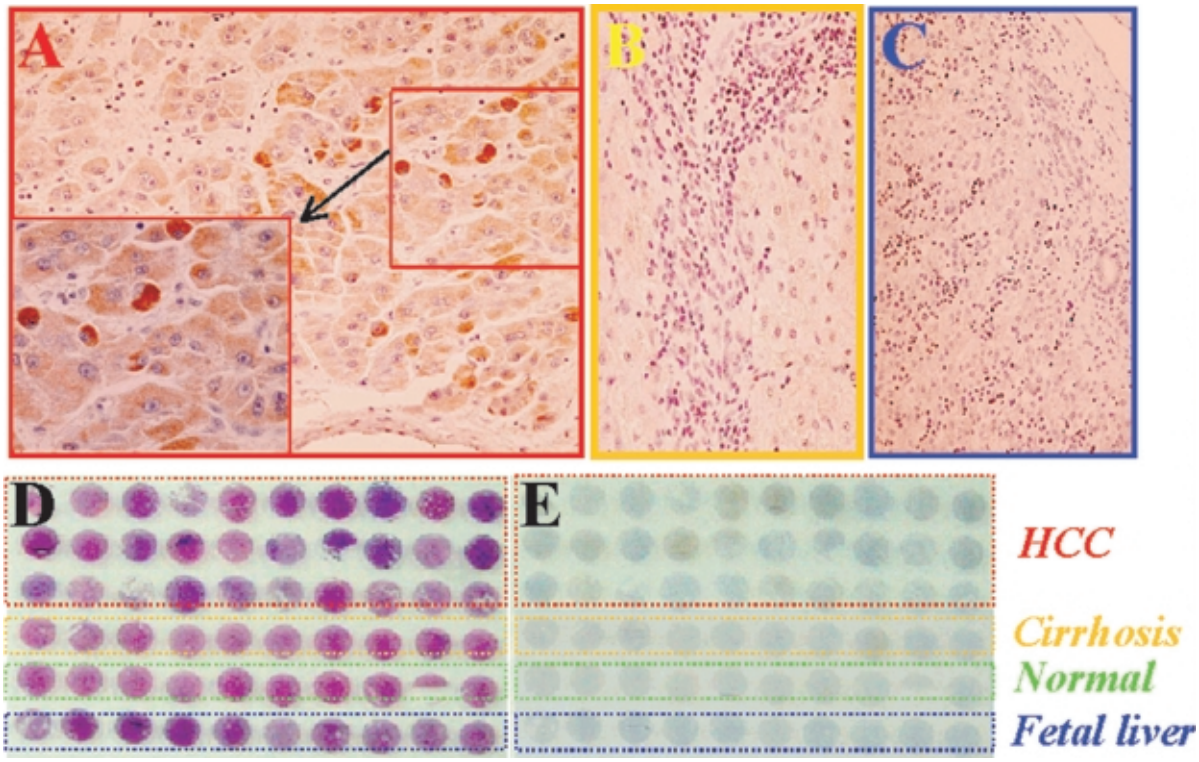

Fig. 2. FACL4 immunohistochemical staining. (A) Strong fine granular intracytoplasmic staining in tumor cells of HCC. (B) Weak staining in normal liver tissues with cirrhosis. (C) Very weak to undetectable staining in fetal liver tissue. HE (D) and FACL4 (E) staining of the tissue array containing 30 HCC, 10 cirrhotic livers, 10 normal adult livers, and 10 fetal livers.

Table 2. FACL4 immunohistochemical staining in liver tissues

Tissue[1)]	Incidence	Expression level[2)]
S-HCC	6/19 (31.5%)	3–5
T-HCC	5/11 (45.4%)	3–5
P-HCC	3/7 (42.8%)	3–5
WD-HCC	4/10 (40%)	3–5
MD-HCC	4/13 (30.7%)	3–5
PD-HCC	6/14 (42.8%)	3–5
Cirrhotic liver	3/10 (30%)	1–2
Normal liver	0/10	<1
Fetal liver	0/10	<1

1) S, solid; T, trabecular; P, pseudoglandular; WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated.
2) Relative level of expression determined independently by three blinded observers.

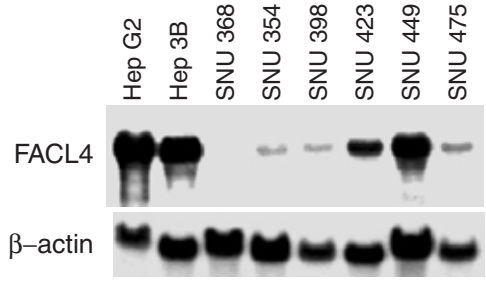

Fig. 3. FACL4 expression in hepatoma cell lines. The Northern blot was hybridized with the amplified FACL4 cDNA fragment (441 bp) as a probe. A single band of approximate 5 kb was detected in most of the cell lines (top panel). β-actin hybridization was used to control the quality and integrity of RNA samples.

Table 3. FACL4 expression and some known characteristics of cell lines used in this study

Cell line	FACL4[1)]	GPC3[1)]	IGF2[1)]	AFP[1)]	*HBx*[2)]	HBsAg secretion[2)]	HBV DNA integration[2)]
Hep G2	+	+	+	+	–	No	No
Hep 3B	+	+	+	+	+	Yes	Yes
SNU 354	tr	–	–	–	+	No	Yes
SNU 368	tr	+	+	–	+	No	Yes
SNU 398	tr	tr	–	–	–	No	Yes
SNU 423	+	–	tr	–	–	No	Yes
SNU 449	+	–	tr	+	–	No	Yes
SNU 475	tr	–	–	–	–	No	Yes

+, expression; –, no expression; tr, trace amounts of transcripts detected.
Data from 3 separate RT-PCR experiments.
1) FACL4, fatty acid-CoA ligase 4; GPC3, glypican-3; IGF2, insulin-like growth factor 2; AFP, α-fetoprotein; *HBx*, *hepatitis B virus X* gene; HBsAg, hepatitis B virus surface antigen.
2) Cited from Park et al. (1995).

gether, we found that 14 out of 37 (37.8%) HCC expressed moderate to strong FACL4 immunostaining. According to differentiation grade, FACL4 staining was observed in 4 out of 10 (40%) well-differentiated, 4 out of 13 (30.7%) moderately differentiated and 6 out of 14 (42.8%) poorly differentiated HCC (Table 2). In addition, no significant difference in the incidence of FACL4 expression was observed according to the HCC types (Table 2). Both normal adult and fetal liver tissues showed very weak to undetectable staining (Fig. 2, C and E). Three out of ten (30%) cirrhotic livers showed weak to moderate levels of FACL4 (Fig. 2E and Table 2).

FACL4 expression in hepatoma cell lines. We next examined FACL4 expression in cell lines derived from hepatoma by Northern blot analysis. A single band of approximate 5 kb was detected in most of the cell lines, though the expression levels were quite various (Fig. 3). We could not find any apparent correlation between expression of FACL4 and other genes that are known to be overexpressed in HCC, such as GPC3, insulin-like growth factor 2, and α-fetoprotein, in addition to other known characteristics of these cell lines (Table 3).

Discussion

In this study, we have shown for the first time, to our knowledge, that expression of FACL4 is upregulated in HCC compared with adjacent normal liver tissues. RT-PCR, Western blot, and immunohistochemical staining analysis showed that FACL4 mRNA and protein are overexpressed in about 40% of HCCs. We found that HCC expressed moderate to strong FACL4 immunostaining, whereas cirrhotic livers and normal livers expressed undetectable to weak staining. Our data are in line with the previous report of extremely low mRNA expression of FACL4 in normal human liver.[2] Moreover, immunohistochemical staining clearly showed strong fine granular intracytoplasmic staining in tumor cells. This finding, together with high expression of FACL4 in 4 out of 8 (50%) hepatoma cell lines, suggests that FACL4 is a new molecular marker for HCC and that the FACL4 pathway may be involved in liver carcinogenesis.

FACL4 is a central enzyme controlling the unesterified AA level in cells.[2] Recently, it has been demonstrated that free AA induces apoptosis by activating the caspase-3 pathway and that overexpression of FACL4 prevented AA-induced apoptosis by reducing the level of intracellular free AA.[8] Consistent with this, FACL4 is upregulated in colon adenocarcinoma.[9] Overexpression of FACL4 in colon adenocarcinoma is believed to deplete free AA, thereby removing a proapoptotic signal.[8, 9] Overexpression of FACL4 in HCC in this study, therefore, suggests that the FACL4 pathway might be of importance in human hepatocarcinogenesis by depleting the intracellular free AA. As in colon cancer, Cox-2, another AA-utilizing enzyme, is also known to be overexpressed in HCC.[12–14] Since simultaneous activation of both the FACL4 and Cox-2 pathways had a synergistic effect in inhibition of apoptosis,[8] coordinated overexpression of FACL4 and Cox-2 might promote HCC development by inhibition of apoptosis. In addition, overexpression of FACL4, thereby increasing the level of AA-CoA esters, might promote cell proliferation and cell growth by regulating signaling molecules such as protein kinase C and binding to transcription factors such as hepatic nuclear factor-4α, since fatty acyl-CoA esters have been shown to be involved in these processes.[19, 20] If this is the case, development of selective inhibitors for FACL4 may be a novel approach for treatment and prevention of HCC, as has been suggested for colon adenocarcinoma.[9]

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