both caspase- and TG2-dependent pathways lead to silencing of Sp1 activity, which correlates with cell viability (Additional file 5 Figure S3D).

Reduced expression of growth factor receptors as the major Sp1 transcriptional targets in ACR-treated JHH-7 cells undergoing apoptosis

ACR-treated JHH-7 cells expressed decreased levels of EGFR at both mRNA (Figure 2A; 2.5-fold reduction in quantitative PCR) and protein (Figure 2B) levels. Although protein levels of c-Met and FGFR1 remained largely unaltered, mRNA levels of c-Met and FGFR1 decreased slightly following ACR-treatment. mRNA of Bcl-X_L was unchanged, but moderately altered at the protein level. ACR induced activation of caspase 3, but not its expression (Additional file 6 Figure S4A and S4B, *respectively*). While a single treatment with either a caspase inhibitor, z-DEVD (Figure 2C, *lane 4*) or overloading EGF (Figure 2C, *lane 6*) partially prevented a reduction in cell number in ACR-treated JHH-7 cells, combined treatment completely prevented this reduction (Figure 2C, *lane 8*).

To determine whether reduced expression of EGFR was due to Sp1 inactivation, transactivation of a chimeric reporter gene-construct in which expression was driven by 3 tandem functional GC box motifs derived from the EGFR promoter was monitored. ACR-treatment decreased the transactivational activity of the EGFR gene promoter (compare Figure 2D, *lanes 1 and 2*), which was partially prevented by overexpressing Sp1 (compare Figure 2D, *lanes 2 and 4*) or downregulating TG2 expression by 70% (compare Figure 2D, *lanes 2 and 6*; Additional file 2 Figure S1B). It was partially reversed by overexpression of TG2 (compare Figure 2D, *lanes 2 and 8*) and Sp1 inactivation with siRNA (compare Figure 2D, *lanes 2 and 10*). Sp1 inactivation with siRNA also reduced expression of EGFR protein (Figure 3A). In hepatocytes, treatment with Sp1 siRNA had previously decreased cell viability ([13]; *data not shown here*). siRNA knockdown of EGFR led to apoptosis (Figure 3B-3D). These results suggest that transcriptional reduction of *EGFR* due to a reduction in Sp1 activity may partially explain ACR-induced apoptosis of HCC cells.

ACR suppresses both transplant of human HCC cells in nude mice and DEN-induced rat hepatocarcinogenesis by inducing apoptosis accompanying the emergence of nuclear TG2 and CLSp1

Finally, the *in vivo* effect of ACR was examined in the 2 animal models. Using the transplant model in mice, where ACR dose-dependent reduction of serum levels of a tumor marker for HCC, α-fetoprotein (AFP) and the incidence of HCC (Additional file 7 Table S2), nuclear

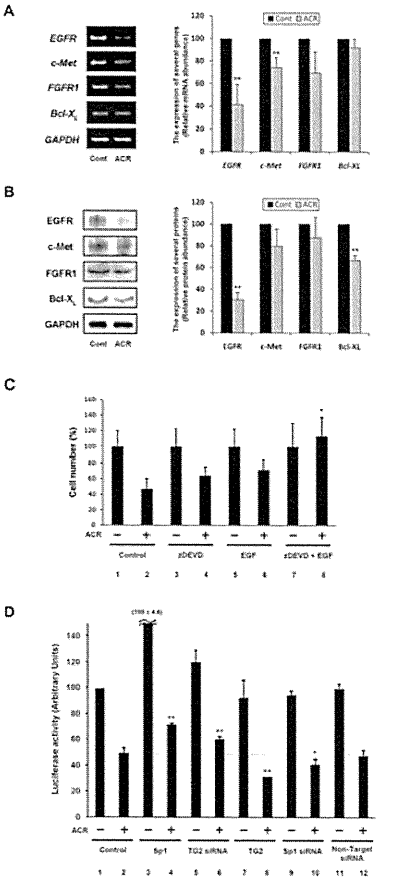

Figure 2 Induction of caspase 3- and TG2-dependent apoptosis by ACR via a reduction in the expression of EGFR due to silencing of Sp1. *A*, JHH-7 cells were treated with 10 μM ACR or vehicle for 12 h. Cells were harvested and mRNA expression of indicated genes was determined by RT-PCR. Bar graphs show densitometrically determined relative mRNA abundance normalized to *GAPDH* mRNA levels. **P < 0.01 compared to each control. *B*, JHH-7 cells were treated with 10 μM ACR or vehicle for 24 h. Cells were harvested and protein expression of indicated proteins was determined by Western blotting. The bar graph shows densitometrically determined relative protein abundance normalized to GAPDH protein levels. **P < 0.01 compared to each control. *C*, JHH-7 cells were treated with 10 μM ACR for 24 h in the presence or absence of 100 μM zDEVD, 50 ng/ml EGF or a combination of the two, and the numbers of viable cells were determined after trypsinization by Trypan Blue exclusion. Results shown are means ± SD (n = 3). *P < 0.05, compared to ACR-treated sample from control cells (*lane 2*). *D*, One day after transfection of JHH-7 cells with *EGFR* promoter GC3-Luc (1 μg/dish), cells were treated with 10 μM ACR for 24 h, co-transfected with Sp1 (*lanes 3 and 4*), TG2 shRNA (*lanes 5 and 6*), TG2 (*lanes 7 and 8*), Sp1 shRNA (*lanes 9 and 10*), and non-target siRNA (*lanes 11 and 12*) expression vector, and cell lysates were prepared. Luciferase activity of each cell lysate was determined. Results shown are means ± SD (n = 3). *P < 0.05, **P < 0.01 compared to ACR-treated control sample from control cells (*lane 2*). *Panels A-D* show representative results from three different experiments with similar results.

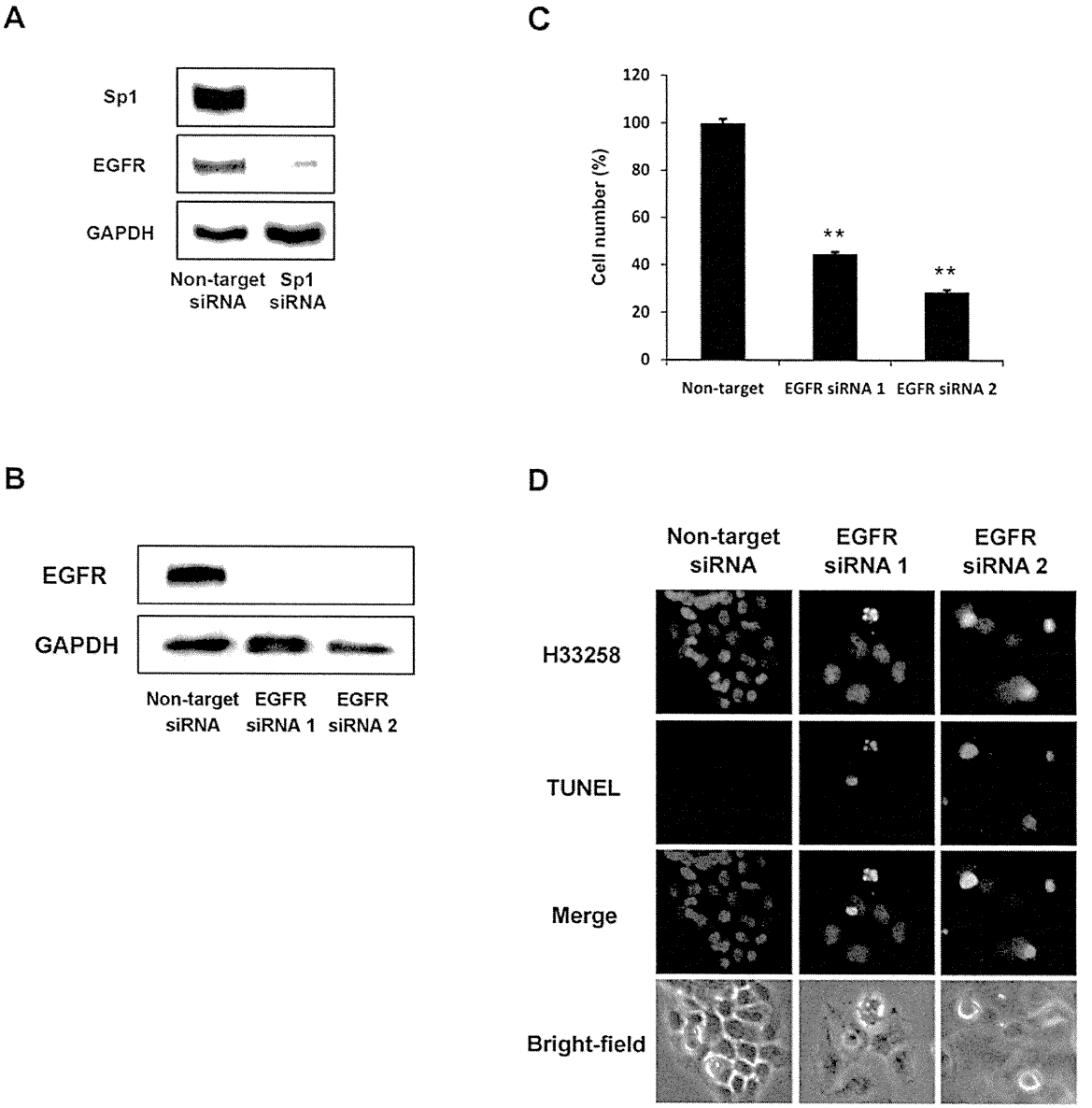

Figure 3 Induction of apoptosis in JHH-7 cells using Sp1 and EGFR siRNAs. *A*, JHH-7 cells overexpressing non-target or Sp1 siRNA were harvested and protein levels of Sp1, EGFR, and GAPDH determined by Western blotting. *B*, JHH-7 cells overexpressing non-target or 2 kinds of EGFR siRNAs were harvested and protein levels of EGFR and GAPDH determined by Western blotting. *C*, Numbers of viable cells were counted 2 days after seeding these cells on 35 mm dishes. Results shown are the number of viable cells relative to the controls, expressed as % ± S.D. **P < 0.01 compared to control cells. *D*, The seeded cells on cover slips in 35 mm dishes were fixed and stained with Hoechst (*upper panels*) and TUNEL (*second panels*). *Panels A-D* show representative results from 3 different experiments with similar results.

TG2 and CLSp1 increased in cancerous liver cells of ACR-treated nude mice transplanted with the JHH-7 cell line (Figure 4A, *panels A and B, respectively*) compared with adjacent normal liver (Figure 4A, *panels D and E*). Significant induction of TG2 and activation of caspase 3 occurred in metastatic areas in nude mice transplanted with JHH-7 cells after treatment with ACR (Figure 4A, *panels A and C, respectively*). Moreover, EGFR levels in the metastatic areas were lower than in normal areas of the same liver (compare Figure 4A, *panels G and J*). Similar results were obtained in the rat model of DEN-induced hepatocarcinogenesis, in which

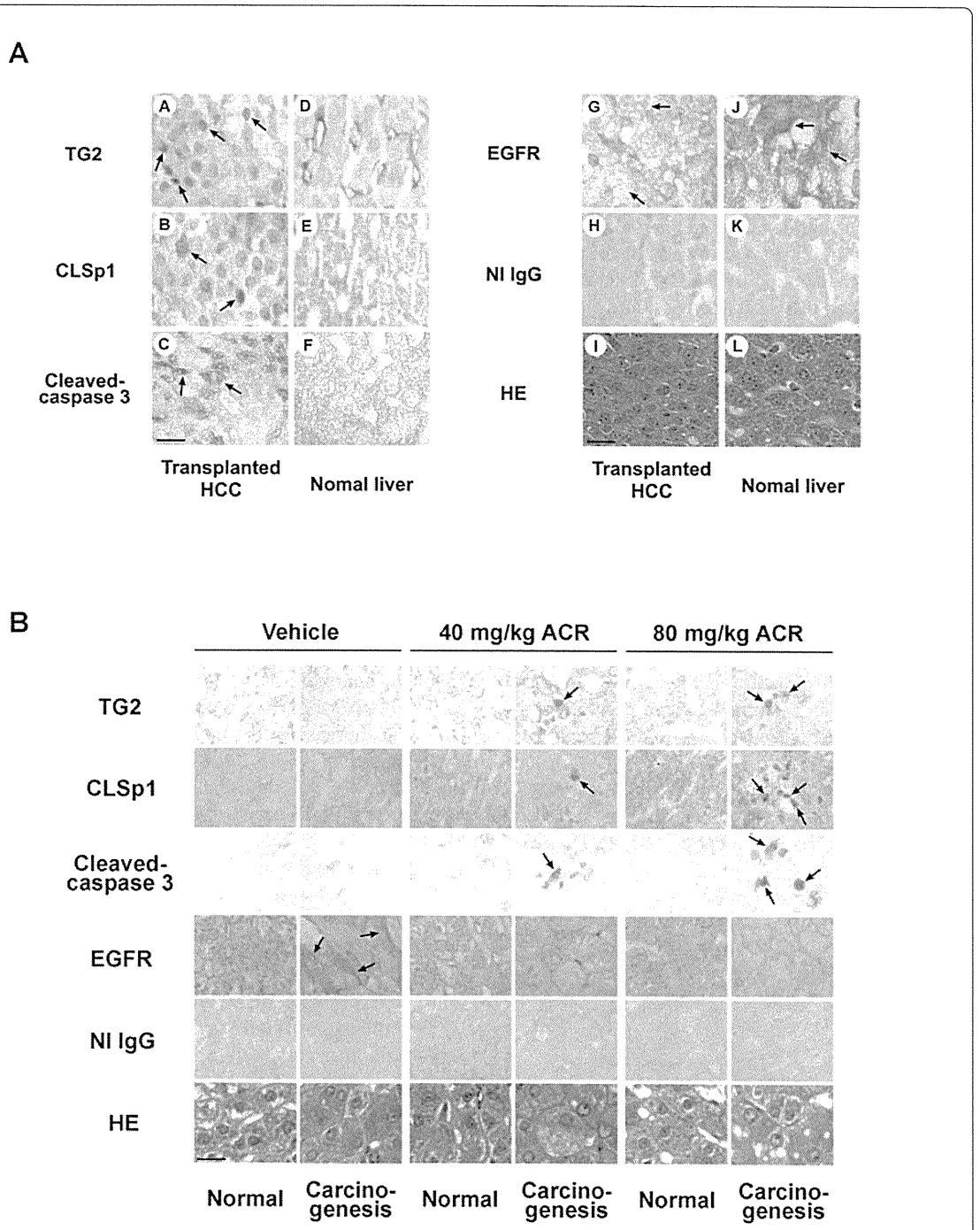

Figure 4 Nuclear accumulation of TG2 and CLSp1 observed in the liver of nude mice transplanted with JHH-7 cells, and in DEN-treated rats with liver cancer after ACR treatment. *A*, Liver sections including normal (*panels D-F and J-L*) and metastatic areas (*panels A-C and G-I*) from JHH-transplanted nude mice following treatment with ACR were stained with polyclonal anti-TG2 (30 μg/ml; *panels A, D*), anti-CLSp1 (30 μg/ml; *panels B, E*), anti-cleaved caspase 3 (10 μg/ml; *panels C, F*), anti-EGFR (10 μg/ml; *panels G, J*), and non-immune antibodies (NI IgG; 30 μg/ml; *panels H, K*). *B*, Liver sections from normal and neoplastic areas in DEN-treated rats following treatment with vehicle or ACR (at 40 and 80 mg/kg) were stained as in Figure 4A. The signals were enhanced with an ABC kit and developed with DAB substrate. Sections were counterstained with hematoxylin-eosin (HE; Figure 4A, *panels I, L*, and Figure 4B, *bottom panels*). Arrows indicate signals under the levels for each antigen. Scale bar, 50 μm.

ACR's anti-cancer effect has been reported [21]. Simultaneous induction of TG2, CLSp1, and activation of caspase 3 occurred in paralleled with a reduction in EGFR (Figure 4B).

Discussion

The data show that: (i) ACR suppresses the hyper-phosphorylation of RXRα, restored its transcriptional function, and enhanced the expression of TG2 and its nuclear accumulation, along with caspase 3 activation; (ii) Sp1 is crosslinked by TG2 and degraded by caspase 3, resulting in loss of its activity; and (iii) expression of Sp1-regulated target genes, such as EGFR (critical for cell survival), decrease, culminating in apoptosis of the cancer cells (Figure 5). The results of *in vitro* findings were confirmed by the *in vivo* models of nude mice transplanted with JHH-7 cells and DEN-induced hepatocarcinogenesis in rats (Figure 4). The recurrence of HCC in these animal models remains to be elucidated.

ACR treatment induced apoptosis in HCC cells (JHH-7 and HuH-7), but not in normal hepatocyte cells (HC cells) (Figure 1A and 1B). As a clue to a reason for the difference, we found that both expression and phosphorylation levels of RXRα were much higher in HCC cells than in HC cells, and that ACR suppressed its phosphorylation levels without altering its expression level (Additional file 4 Figure S2A), as previously shown [5]. In further previous work, we had demonstrated that 2 amino acids in RXRα, T82 and S260, were phosphorylated in HCC, but not in HC cells [4]. Therefore, phosphorylation of RXRα observed in JHH-7 cells was referred to as "hyperphosphorylation". However, RARα and RARβ were phosphorylated not only in JHH-7 cells, but also in HC cells, and ACR downregulated their phosphorylation in both cases (Additional file 4 Figure S2A). Phosphorylation was not detected in the other 3 subtypes of RXR and RAR (Additional file 4 Figure S2A). Therefore, phosphorylation of RXRα was only specific in cancer cells, which could be a reason for the selective apoptosis of cancer cells by ACR treatment.

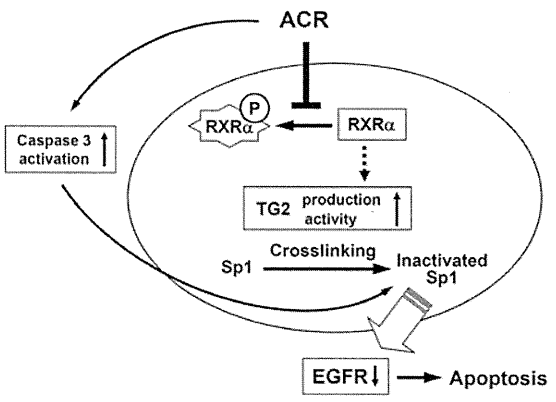

Figure 5 Schematic diagram showing the molecular mechanism by which ACR causes HCC apoptosis by restorating phospho-inactivated RXRα followed by enhanced TG2-mediated crosslinking/silencing of Sp1, thereby reducing EGFR-medicated EGF signaling.

It is noteworthy that treatment with either antisense of TG2 or inhibitors of caspase 3 only partially blocked ACR-induced apoptosis, whereas their simultaneous inhibition completely prevented apoptosis, suggesting that TG2 and caspase 3 contribute independently to the induction of apoptosis (Figure 1D and 1E). We measured the activity of caspase 3 and TG2 in the presence of an inhibitor of each other's enzyme, such as zDEVD and cystamine. When cystamine suppressed ~50% of ACR induction in TG2 activity (compare the differences between *lanes 1 and 4* with those between *lanes 2 and 5* in Additional file 6 Figure S4D), it suppressed 60% of ACR induction in caspase 3 activity (compare the differences between *lanes 1 and 4* with those between *lanes 2 and 5* in Additional file 6 Figure S4C). On the other hand, when zDEVD completely suppressed ACR-induced increase in caspase 3 activity (compare the differences between *lanes 1 and 4* with those between *lanes 3 and 6* in Additional file 6 Figure S4C), 50% of an increase in the TG2 activity remained (compare the differences with *lanes 1 and 4* with those between *lanes 3 and 6* in Additional file 6 Figure S4D). The data suggest that TG2 and caspase 3 influenced each other with a higher hierarchy of TG2 over caspase 3 in the contribution to the apoptosis of HCC induced by ACR. Synergism between inhibition in caspase and overloading of EGF in preventing apoptosis also suggests that both the caspase 3- and EGFR-dependent pathways exist (Figure 2C).

Expression of EGFR is regulated by Sp1 [19,22], and inhibition of EGFR signaling leads to growth inhibition, apoptosis, and cell cycle arrest of HCC cells [23,24]. We have linked these findings by showing that the downregulation of EGFR with siRNA induces apoptosis (Figure 3B-D), suggesting that inhibiting EGFR signaling via silencing Sp1 is a promising treatment strategy against HCC.

Induction of CLSp1 and the subsequent reduction in EGFR has been reproduced in ACR-treated HuH-7 cells (*data not shown*). In contrast, although Shao *et al.* [15] reported that ACR inhibits the cell growth through downregulation of FGFR3 expression and FGF-mediated signaling in HepG2 cells, this was not found to be the case in our ACR-treated JHH-7 cells (*data not shown*). These findings suggest that HCC cell lines differ in the way that growth factor receptors are involved in survival.

Whereas TG2 may be a substrate of caspase 3 during apoptosis of thymocytes, resulting in loss of transamidating function [25], TG2 in turn inhibits of apoptosis due to crosslinking and inactivation of caspase 3 in thapsigargin-mediated apoptosis of colon carcinoma cells [26]. In the latter article, thapsigargin treatment generated 2 additional biologically inactive species of caspase 3, viz. p40 and p64, via TG2-mediated crosslinking of caspase 3, thereby protecting cells from apoptosis. However, we failed to detect either p40 or p64 in our ACR-treated JHH-7 cells. We speculate that crosslinking of caspase 3 would be induced specifically by treatment with thapsigargin. Our data clearly shows that both caspase 3 and TG2 are functional in ACR-treated HCC cells, without apparent alteration of caspase 3 expression (Additional file 6 Figure S4A and 4B). These controversial results might be ascribed to differences in cell types and the nature of the apoptotic stimuli, although the precise mechanisms need to be elucidated.

Piedrafita and Pfahl [14] reported that caspase 3 directly cleaved and inactivated Sp1 in retinoid-treated T cells undergoing apoptosis. They showed that cleavages of PARP and Sp1 were simultaneously induced by caspase 3 and prevented with caspase inhibitors (zVAD-fmk and zDEVD-fmk). We anticipated that CLSp1 might also be partially cleaved by caspase 3; however, as molecular size differences would be too small to be recognized on the gel against a high molecular weight of CLSp1 detected at the top of the gel, we found no band shifts due to the cleavage. Hence, the possibility of simultaneous crosslinking and cleavage of Sp1 by TG2 and caspase 3, respectively, cannot be ruled out, even though we saw no truncated Sp1 with a Mw of 68 kD in ACR-treated HCC cells.

ACR-treated JHH show enhanced nuclear localization of TG2; nuclear localization of TG2 is also important for induction of TG2-dependent apoptosis. Peng *et al.* [27] reported that TG2 binds importin-α3, an important factor in nuclear translocation, and therefore we are investigating the detail mechanism of TG2 nuclear localization accompanying ACR-induced apoptosis.

Conclusions

Our new findings indicate that ACR induces both activation of caspase 3 as well as the expression and activation of TG2, which together initiate the apoptotic pathway via degrading/crosslinking and inactivation of the transcription factor, Sp1. Reduced expression of growth factor receptor genes (*e.g.* EGFR) also occurs. This dual activation of both caspase and TG-dependent apoptotic pathways could in part be central as mechanisms by which ACR inhibits tumor cell growth, resulting in the prevention of secondary tumors after treatment of primary HCCs (Figure 5). Future study should establish the possibility that regulation of TG2-dependent apoptotic pathway may help in the development of new therapies for the prevention of HCC.

Additional material

Additional file 1: Additional text. This text contains the additional "Methods" and "References"

Additional file 2: Figure S1: Efficiency of transfection with anti-sense and siRNA to TG2 in JHH-7 cells. *A*, JHH-7 cells were seeded in 60 mm dishes at 6 × 10^5/dish, and transfected with 4 μg of either empty vector (pSG5) or ASTG2-pSG5. Cells were harvested and the expression level of TG2 determined by Western blotting. Upper numbers in parentheses show the densitometrically determined relative protein abundance. *B*, JHH-7 cells were seeded in 60 mm dishes at 6 × 10^5/dish, and transfected with 4 μg of vectors expressing either non-target siRNA or TG2 siRNA. Cells were harvested and the expression level of TG2 determined by Western blotting. Upper numbers in parentheses show the densitometrically determined relative protein abundance. *Panels A and B* show representative results from 3 different experiments with similar results.

Additional file 3: Table S1: Primers for RT-PCR and quantitative-PCR experiments. The list of used specific primers for RT-PCR.

Additional file 4: Figure S2: ACR prevented phosphorylation and inactivation of RXRα, and stimulated the expression of TG2 in JHH-7 cells. *A*, JHH-7 cells (*lane 1 and 2*) and HC cells (*lane 3 and 4*) were treated with 10 μM ACR or vehicle for 12 h. Cells were harvested and nuclear extracts were prepared. Phosphoproteins affinity-purified from each nuclear extract using the Phosphoprotein Purification Kit (QIAGEN) (*left panel*) as well as whole nuclear extracts (*right panel*), were subjected to SDS-PAGE, followed by Western blotting using the indicated antibodies against 6 different RXR/RAR or GAPDH. *B*, JHH-7 cells were transfected with either an empty vector (*columns 1-4*) or vectors expressing wild-type RXRα (*columns 5-8*), its alanine mutant T82A (unphosphorylated form; *columns 9-12*), or its aspartate mutant T82 D (phosphomimic; *columns 13-16*). The next day cells were treated either with 9-*cis* RA (9cRA; 6 μM) or its vehicle, or with and/or ACR (10 μM) for 24 h. Subsequently, levels of *TG2* mRNA in cell lysates were quantified by RT-PCR (*upper panels*) and quantitative-PCR (*lower graphs*), where relative expression levels of TG2 were calculated in comparison with each control and then plotted. Treatment with 1 μM 9-cis-RA also gave basically similar results (data not shown), but the data obtained under treatment with 6 μM 9-cis-RA are shown here, giving the more significant differences. *Panels A and B* show representative results from 3 different experiments with similar results.

Additional file 5: Figure S3: Crosslinking and silencing of Sp1 in ACR-treated JHH-7 cell cultures undergoing apoptosis and its reversion by overexpression of Sp1. *A*, JHH-7 cells were treated with 10 μM ACR for 24 h. The cells were harvested and nuclear extracts prepared. The levels of Sp1 and CLSp1 were assessed by Western blotting with an anti-Sp1 (*columns 1 and 2*) and CLSp1 (*columns 3 and 4*) antibodies, respectively. *B*, JHH-7 cells were transfected with 1.5 μg of either combination of *pClneo*, *pSG5*, *Sp1-pClneo*, or *anti-sense (AS) TG2-pSG5*. The next day they were treated with either 10 μM ACR or its vehicle in the presence or absence of 100 μM zDEVD-fmk for 24 h. Cells were harvested and nuclear extracts prepared. Sp1 DNA-binding activity of each nuclear extract (10 μg protein) was determined by gel-shift assay, using a consensus GC box as a probe (+cold; nuclear extracts + 50-fold excess of unlabeled probe, +anti-Sp1 IgG; nuclear extracts + 2 μg of anti-Sp1 antibody, +NI IgG; nuclear extracts + 2 μg of non-immune IgG). *C*, JHH-7 cells were transfected with 1.5 μg of a consensus GC3-Luc reporter and *Renilla*-Luc, plus a combination of *pClneo*, *pSG5*, *Sp1-pClneo* or *anti-sense (AS) TG2-pSG5*. The next day the cells were treated with 10 μM ACR for 24 h in the presence or absence of 100 μM zDEVD-fmk. Cell lysates were prepared and luciferase activity of each cell lysate determined. Results are means ± SD (n = 3). *D*, JHH-7 cells were

transfected with either a combination of *pCIneo, pSG5, anti-sense (AS) TG2-pSG5, Sp1-pCIneo, Sp1 C domain-pCIneo, ΔC Sp1-pCIneo.* The next day the cells were treated with 10 μM ACR for 24 h. The number of viable cells was determined. Results are means ± SD (n = 4). *Panels A-D* show representative results from 3 different experiments with similar results.

Additional file 6: Figure S4: ACR stimulated activation of caspase 3 and TG2 in JHH-7 cells and the crosstalk between these proteins. *A and B*, JHH-7 cells were treated with 10 μM ACR or the vehicle for 24 h. Cells were harvested and protein levels of activated caspase 3 and GAPDH determined by Western blots, using anti-cleaved-caspase 3 and anti-GAPDH antibodies (*A*); each of their mRNA expression was determined by RT-PCR (*B*). *C*, JHH-7 cells was seeded at 1 × 10^4 cells/96 well microplates and treated with 10 μM ACR or vehicle (0.1% ethanol) for 5 h in the presence or absence of either 100 μM zDEVD-fmk or 100 μM cystamine with 0.2 mM 5-(biotinamido)-pentylamine. Caspase 3 activity was measured using a Caspase-Glo 3/7 assay kit (Promega Corp., WI) as described in attached manual. Relative caspase 3 activity of each sample was calculated by normalization with the number of viable cells in the same sample measured with a cell counting kit-8 (Dojindo; Tokyo, Japan). *D*, JHH-7 cells seeded in 100 mm dishes at 1.6 × 10^6/dish were treated as in (C). TG2 activity was measured as described in Additional file 1. Relative TG2 activity of each sample was calculated by normalization with the number of viable cells in the same sample, measured with a cell counting kit-8 (Dojindo; Tokyo, Japan). *Panels A-D* show representative results from 3 different experiments with similar results.

Additional file 7: Table S2: Suppression by ACR of metastasis and growth of human HCC cell line, JHH-7 cells transplanted into nude mice. Nude mice that had been transplanted with JHH-7 were given orally with ACR with increasing concentrations (25, 50, and 100 mg/kg/day) as described detailed in the "Methods". Serum AFP was measured. Incidence was calculated based on level of the positive-AFP (more than 6 ng/ml). Cisplatin was used as a positive control. *p < 0.05 compared to control (Dunnett's multiple comparison test), #p < 0.05 compared to control (Fisher exact test)..

List of abbreviations

9-*cis* RA: 9-*cis* retinoic acid; ACR: acyclic retinoid; CLSp1: crosslinked Sp1; DEN: *N*-diethylnitrosamine; EGFR: epidermal growth factor receptor; FGFR3: fibroblast growth factor receptor 3; HCC: hepatocellular carcinoma; RXR: retinoid X receptor; TG2: transglutaminase 2.

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Authors' contributions

HT and TS performed the research, analyzed the data, and drafted the manuscript. YF helped with cell culture, transfection, immunostaining and Western blotting techniques. NI prepared the acyclic retinoid used in these studies. MW helped with immunostaining techniques. MO, HM and SK designed the research, interpreted the data, and revised the manuscript. All authors approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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特集I　消化器癌をめぐる栄養療法

分岐鎖アミノ酸製剤を用いた肥満関連大腸および肝発癌予防*

清水雅仁**
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はじめに
—肥満関連分子異常を標的とした発癌化学予防の可能性—

大腸癌と肝細胞癌は，わが国において癌死亡率の上位を占める癌であり，早期診断・治療のみならず，積極的な介入による発癌化学予防(cancer chemoprevention)を含めた包括的な対策が求められている．近年，糖尿病や高インスリン血症，インスリン抵抗性といった肥満に関連したさまざまな病態が，これらの発癌の重要な危険因子であることが明らかになってきているが[1][2]，このことは，栄養学的介入や薬剤投与によって肥満に関連した分子異常を改善・制御することが，大腸および肝発癌予防につながる可能性があることを示唆するものである．分岐鎖アミノ酸(branched-chain amino acids；BCAA)は，慢性肝疾患に伴う蛋白栄養障害を改善する薬剤であるが，2005年に報告された多施設無作為化比較試験(LOTUS試験)で，BCAA製剤の補充療法が肥満を合併する非代償性肝硬変患者の肝発癌を有意に抑制することが明らかになった[3][4]．また，肥満・糖尿病のモデルマウスを用いた化学発癌実験において，BCAAが肥満に関連した分子異常を制御することで，大腸および肝発癌を抑制することも確認されている[5][6]．本稿では，肥満・糖尿病と大腸および肝発癌に関する疫学研究と，それらの関連性における分子生物学的機序について述べたあと，BCAAによる肥満関連大腸および肝発癌抑制のメカニズムに関する基礎研究の結果と，BCAA製剤の補充療法が肝硬変患者の肝不全状態の悪化と肝発癌を抑制したLOTUS試験の結果について，われわれの教室の知見を踏まえ概説する．

肥満・糖尿病と大腸および肝発癌
—疫学研究—

肥満と大腸発癌に関しては以前からその関連性が指摘されていたが，2007年にWCRF(World Cancer Research Fund)/AICR(American Institute for Cancer Research)は，世界中の研究報告結果を再検討・総括し，体脂肪と内臓脂肪が大腸癌の「確実」な危険因子であることを報告した[7]．日本人を対象にした疫学検査でも，肥満は男性の大腸発癌の危険因子であること，また，高カロリーの食事や肥満，運動不足を起因とする高中性脂肪が，結腸癌の1.7倍のリスク上昇と関連していることが報告されている[8][9]．肥満と肝発癌の関連性については，米国での疫学研究において，body mass index(BMI)高値の男性では肝

* Chemoprevention of obesity-related colorectal and liver carcinogenesis by branched-chain amino acids.

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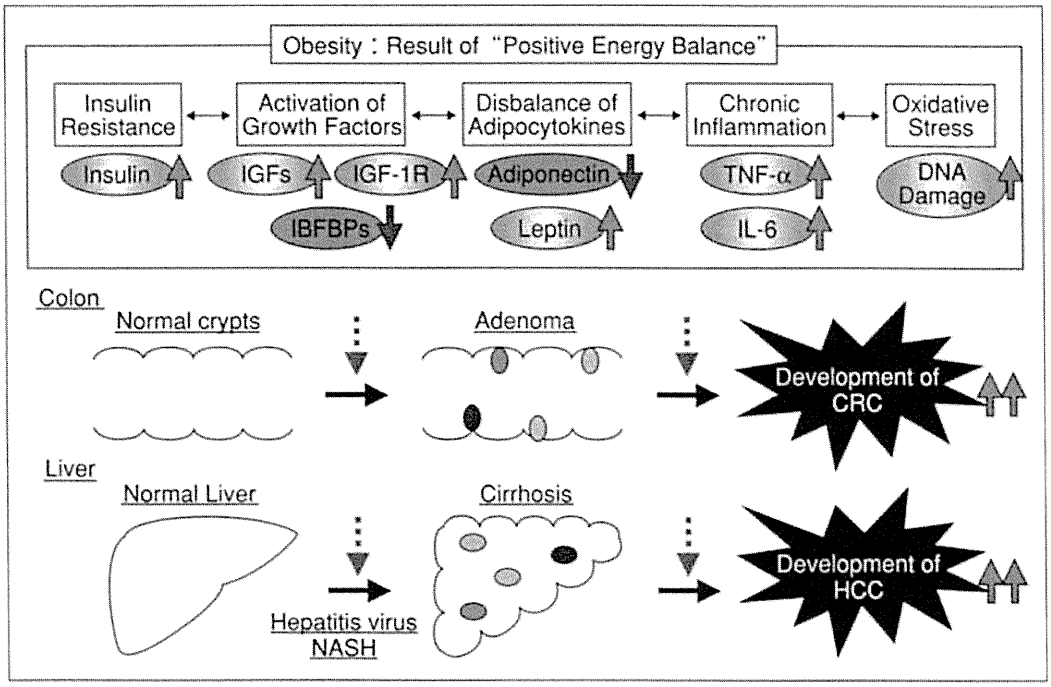

図1　肥満関連分子異常と大腸および肝発癌

細胞癌のリスクが約4.5倍に上昇することが報告されているが[10]，本邦においても，C型肝炎患者や非代償性肝硬変患者の肥満が，肝炎ウイルス感染と並んで肝発癌のリスクを有意に高めることが明らかになっている[4][11]．これらの報告に加え，糖尿病や高インスリン血症も大腸発癌や肝発癌に深く関与していることが，本邦を含めた各国の疫学研究で報告されている[12]．特に本邦の症例対照比較研究において，内臓脂肪蓄積とアディポネクチンの低下，およびインスリン抵抗性の出現が，大腸腺腫のリスク上昇に関連していることが報告されているが[13]，この研究結果は，肥満に関連した病態が大腸腫瘍形成のより初期の段階に関与していることを示唆するものである．さらに肝細胞癌に関しては，糖尿病の既往や肥満の合併，あるいは肥満に高血糖や高血圧等のメタボリックファクターの異常を重複することでその危険が有意に高まること[14]，また，糖尿病患者の主要死因として，従来注意されてきた虚血性心疾患や脳血管障害と並び，肝細胞癌や肝硬変症といった肝疾患が重要であることが明らかになっている[15]．したがって，肥満・糖尿病を合併する慢性肝疾患患者は，肝発癌の高危険群であることを念頭において診察に当たる必要がある．

肥満・糖尿病と大腸および肝発癌 —分子生物学的機序—

大腸および肝発癌に関与する肥満に伴う分子異常としては，インスリン抵抗性の出現，insulin like growth factor(IGF)/IGF-1受容体をはじめとする増殖因子シグナルの過剰活性化，アディポネクチンの低下やレプチンの上昇に代表されるアディポサイトカインの不均衡状態，内臓脂肪の増加に伴う慢性炎症状態の惹起(TNF-α，IL-6等の炎症性サイトカインの上昇)，酸化ストレスの亢進等が考えられる(図1)[1][2]．また，肝発癌においては，非アルコール性脂肪肝炎(non-alcoholic steatohepatitis；NASH)も注目すべき病態である[16]．これらの分子異常は，相互関係を保ちつつ発癌を促進するが，その中でも特に，それ自身が増殖因子でもあるインスリンの上昇(高インスリン血症)とインスリン抵抗性の出現，また，その結果ひき起こされるIGF/IGF-1受容体シグナルの過剰活性化が，大腸および肝発癌過程の「鍵」として重要であると考えられている．われわれは今までに，緑茶カテキンの一つであるEGCGが，IGF/IGF-1受容体シグナルを阻害することで大腸

表1 BCAAが*db/db*マウスのAOM誘発大腸前癌病変(ACFおよびBCAC)の発症率に及ぼす効果

群	食餌	マウス数	発生個数(mean±SD)	
			ACF(個数/マウス)	BCAC(個数/cm²)
1	基礎食	12	85.9±8.1	11.7±8.4
2	カゼイン	12	83.4±11.2	8.3±3.9
3	BCAA	12	54.5±8.6[ab]	4.2±6.7[c]

a：$P<0.001$(1群との有意差)，b：$P<0.001$(2群との有意差)，c：$P<0.05$(2群との有意差)
(文献[5]より引用改変)

表2 BCAAが*db/db*マウスのDEN誘発肝腫瘍(腺腫および肝細胞癌)の発症率に及ぼす効果

群	食餌	マウス数	発生率		発生個数(腫瘍数/マウス)(mean±SD)		
			腺腫	肝細胞癌	腺腫＋肝細胞癌	腺腫	肝細胞癌
1	基礎食	11	7/11(64%)	1/11(9%)	1.0±1.1	0.9±1.1	0.1±0.3
2	カゼイン	11	8/11(73%)	3/11(27%)	1.7±1.3	1.5±1.1	0.3±0.5
3	BCAA	11	2/11(18%)[ab]	0/11(0%)	0.2±0.4[ac]	0.2±0.4[c]	0

a：$P<0.05$(1群との有意差)，b：$P<0.05$(2群との有意差)，c：$P<0.01$(2群との有意差)(文献[6]より引用改変)

および肝細胞癌細胞株の増殖を抑制すること[17][18]，また，IGF/IGF-1受容体シグナルを阻害するとともにインスリン感受性を改善することで，肥満・糖尿病に関連したマウスの大腸および肝発癌を抑制することを報告してきた[19][20]．さらには，根治的なラジオ波焼灼療法を行ったStage Ⅰの肝細胞癌の治療後再発予測因子として，術前のインスリン抵抗性(HOMA-IR2.3以上)が有用であることを明らかにしたが[21]，これらの研究結果は，さまざまな肥満関連分子の発現・機能異常に着目した大腸および肝発癌リスクに関する新規バイオマーカーの発見や，発癌予防および治療法の開発を強く期待させるものである．

BCAAによる肥満関連大腸および肝発癌抑制—基礎実験—

BCAAは，側鎖に分岐を持つ3種類のアミノ酸(バリン，ロイシン，イソロイシン)であり，BCAA製剤は慢性肝疾患に伴う蛋白栄養障害を改善する薬剤として臨床で用いられている．近年，BCAAが骨格筋に直接作用し，グルコース輸送担体であるGLUT4の細胞膜におけるtranslocationを亢進することで糖の取り込みを促進させ，糖負荷後の血糖値を低下させることが，肝硬変モデルラットを用いた実験で明らかになっている[22]．また，2型糖尿病モデルラットを用いた肝化学発癌実験において，BCAAがVEGFの発現と血管新生を阻害することで，肝前癌病変の発生を抑制することも報告されているが[23]，これらの研究結果は，BCAAが糖代謝や肥満に伴った分子異常を標的とすることで，発癌予防効果を発揮する可能性を示唆するものである．そこでわれわれは，レプチン受容体のmutantで高度の肥満・糖尿病・高脂血症・高レプチン血症をきたす*db/db*マウスを用いて，BCAAの肥満関連大腸および肝発癌に対する抑制効果に関する以下の検討を行った．

まず，*db/db*マウスに，大腸化学発癌物質であるazoxymethaneを皮下投与して大腸前癌病変を誘発したのち，3%BCAA添加食を7週間投与したところ，BCAA投与群では，基礎食および窒素負荷対照群(casein投与群)と比較し，大腸前癌病変数の有意な低下と(表1)，大腸粘膜におけるIGF-1受容体，GSK-3β，Akt蛋白のリン酸化抑制，COX-2蛋白の発現低下，血清インスリン，IGF-1，IGF-2，中性脂肪，総コレステロール，およびレプチン値の低下が認められた[5]．また，同マウスに，肝化学発癌物質であるdiethylnitrosamineを飲水投与して肝細胞癌を誘発したのち，3%BCAA添加食を34週間投与したところ，BCAA投与群では，肝腫瘍(肝細胞癌および腺腫)の発生が有意に低下するとともに(表2)，血糖値上昇の抑制とインスリン感受性の改善，肝脂肪化と線維化の抑制，および血清レプチン値の低下が認めら

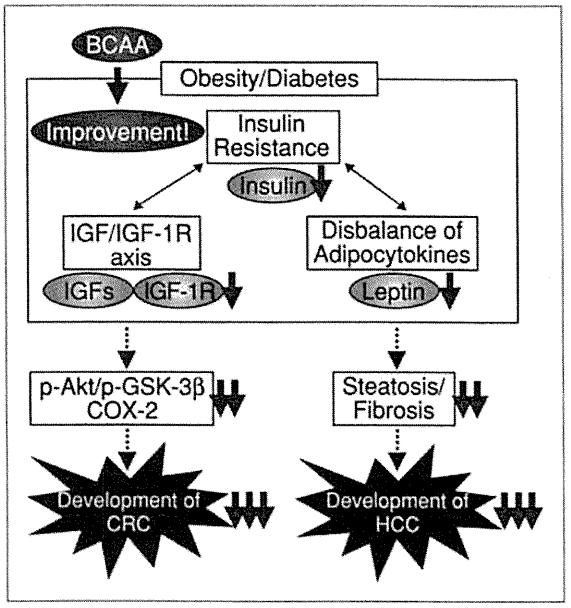

図2　BCAAによる肥満関連大腸および肝発癌抑制の作用機序

れた．また，実験食投与16週時の剖検では，BCAA投与群において，肝臓におけるIGF-1, IGF-2, およびIGF-1R遺伝子の発現低下と肝前癌病変の発生抑制が観察された[6]．これらの研究結果は，BCAAはインスリン抵抗性を改善するとともにIGF-1受容体の関連シグナルを阻害することで，肥満・糖尿病に関連した大腸および肝発癌を抑制した可能性を強く示唆するものである（図2）．

BCAA製剤による肥満関連肝発癌抑制 —臨床試験（LOTUS）—

肝硬変では肝実質細胞が減少するため，肝臓への糖の取り込みと，肝におけるグリコーゲンの合成・貯蔵能が低下し，高頻度に糖尿病や食後高血糖・高インスリン血症といった耐糖能異常を合併する．特にグリコーゲン合成の低下は，エネルギー不足を補うために蛋白質の異化を亢進させ，結果として筋肉（骨格筋）量の減少と，骨格筋における糖の取り込み減少・利用低下をひき起こす．骨格筋でエネルギー源として燃焼される基質はBCAAが主体であるため，肝硬変患者ではBCAAの血中濃度が低下する．したがって，肝硬変による蛋白質・エネルギー低栄養状態（protein-energy malnutrition；PEM）とBCAAの低下，耐糖能異常の合併は一連の病態としてとらえられる[24]．

一方，BCAA製剤の投与によって血清インスリン値やHOMA-IRの低下といった糖代謝改善作用が認められることが，いくつかの臨床研究において報告されている[25][26]．また，非代償性肝硬変患者646症例を対象とした多施設ランダム化対照臨床試験（LOTUS試験）において，BCAA製剤の補充療法が，肥満（BMI 25以上）を合併した肝硬変患者の肝発癌を有意に抑制（Estimated HR0.30, P=0.008）したことが報告されているが（図3）[4]，これらの臨床研究や基礎研究[5][6]の結果は，BCAAが肥満に関連した分子異常，特にインスリン抵抗性や高インスリン血症を標的にすることで肥満に関連した肝発癌を抑制した可能性をあらためて示唆するものである．

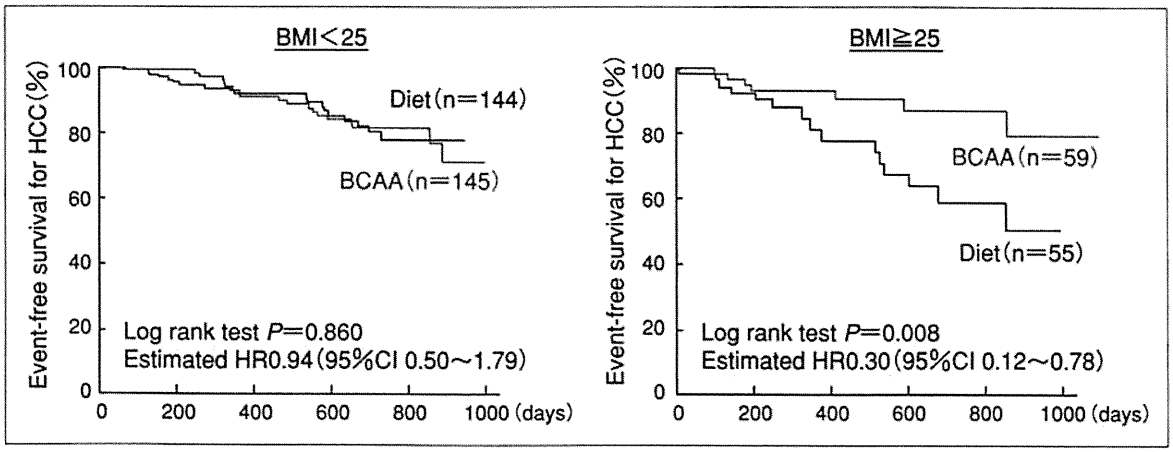

図3　肝発癌に関するイベントフリー生存率のKaplan-Meier推定曲線（文献[4]より引用改変）

おわりに

肥満や糖尿病，メタボリックシンドロームが大きな社会問題である現在，これらの病態に関連した大腸および肝細胞癌のさらなる増加が予想される．運動の励行，適正なエネルギー摂取の指導といった生活習慣の改善とともに，積極的に栄養学的介入や薬剤投与を行い，インスリン抵抗性やIGF/IGF-1受容体の過剰活性に代表される肥満に関連したさまざまな分子異常を制御することは，肥満・糖尿病合併患者の大腸および肝発癌予防を実践していく上で，重要なstrategyの一つになる可能性がある．このような肥満関連分子異常を標的とした新規発癌予防法(薬)の研究開発と臨床応用において，BCAAが重要な役割を果たすことが期待される．

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特集II 肝発癌・進展とインスリン抵抗性

インスリン抵抗性とIGF/IGF-1受容体シグナルを標的とした肥満関連肝発癌予防*

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白木　亮**
森脇久隆**

Key Words：liver cancer chemoprevention, obesity, insulin resistance, insulin like growth factor(IGF)/IGF-1R axis

はじめに

肝細胞癌は，世界の癌死亡原因の第3位を占める悪性疾患であり，有効な肝発癌予防法(薬)の開発が求められている．従来の危険因子である肝炎ウイルス感染やアルコールの大量摂取に加え，近年，糖尿病や高インスリン血症，インスリン抵抗性といった肥満に伴ったさまざまな病態が，肝細胞癌の発癌・進展に深く関与していることが明らかになってきているが[1]，これらの報告は，肥満に関連した分子異常を標的とすることが肝発癌予防につながる可能性を示唆するものである．これを裏づけるデータとして，慢性肝疾患に伴う蛋白栄養障害を改善する分岐鎖アミノ酸(branched-chain amino acids)の補充療法が，肥満を合併する非代償性肝硬変患者の肝発癌を有意に抑制した臨床試験(LOTUS試験)と[2][3]，その作用機序としてインスリン抵抗性の改善作用が重要であることを示した基礎実験の結果がある[4]．本稿では，肥満・糖尿病と肝発癌に関する疫学研究と両者の分子生物学的関連性について説明した後，分岐鎖アミノ酸による肥満関連肝発癌予防に関するこれらの研究結果について，われわれの教室の知見を踏まえ概説する．また，肝発癌予防薬として臨床応用が期待されている非環式レチノイドが，肥満に関連した肝発癌を抑制した基礎研究の結果について，併せて報告する．

肥満・糖尿病と肝発癌

肥満や糖尿病の合併が肝発癌のリスクを高めることは，欧米のみならず本邦においても明らかになっている[5]~[8]．Inoueらは，糖尿病の既往や肥満の合併，また，肥満に高血糖や高血圧等のmetabolic factorの異常を重複することで，肝発癌の危険が有意に高まることを報告している[7][8]．糖尿病患者の死因として，虚血性心疾患や脳血管障害と並び，肝細胞癌や肝硬変症といった肝疾患が重要であること[9]，また，本邦で感染者が多いC型肝炎ウイルスによる慢性肝炎および肝硬変患者が肥満を合併すると，肝発癌のリスクが有意に上昇することも明らかになっており[2][10]，肥満・糖尿病を有する慢性肝疾患患者の診察には，特に肝癌の合併に留意する必要がある．

肥満に伴う分子異常としては，インスリン抵抗性の出現，insulin like growth factor(IGF)/IGF-

* Targeting insulin resistance and the activation of IGF/IGF-1R axis for chemoprevention of obesity-related liver carcinogenesis.

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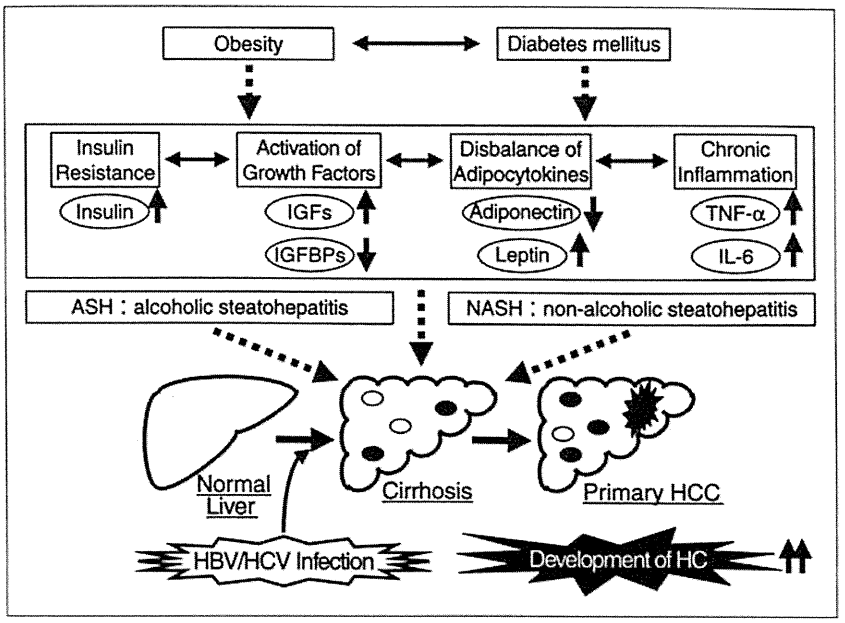

図1　肥満関連分子異常と肝発癌

1受容体をはじめとする増殖因子シグナルの過剰活性化，アディポネクチンの低下，レプチンの上昇に代表されるアディポサイトカインの不均衡状態，内臓脂肪の増加に伴う慢性炎症状態の惹起(TNF-α，IL-6等の炎症性サイトカインの上昇)，酸化ストレスの亢進等があげられる．また，アルコール性肝炎や非アルコール性脂肪肝炎(non-alcoholic steatohepatitis；NASH)による脂肪肝炎も，肝発癌を考える上で注意すべき病態である．hepatitis C virus(HCV)感染も肝に脂肪化を起こすが，HCV感染患者における内臓脂肪蓄積型肥満の合併が増加することも危惧されている．肝炎ウイルス感染と肥満に伴うこれらの分子異常は，相互関係を保ちつつ肝細胞癌の発癌・進展に深く関与している(図1)[11][12]．

これらの分子異常のなかでも，特に肥満・糖尿病に関連した肝細胞癌の発癌・進展に重要であると考えられている因子は，インスリン抵抗性とIGF/IGF-1受容体シグナルの過剰活性化である．インスリンは強力な細胞増殖因子であり，高インスリン血症は肝細胞癌患者において癌の増殖速度を速める[13]．基礎研究において，IGF/IGF-1受容体シグナルの活性亢進が肝癌細胞の増殖を促進すること，また，同シグナルを阻害することで，その増殖が抑制されることが報告されている[14]．また，われわれは今までに根治的治療を行ったStage Ⅰの肝細胞癌の治療後再発予測因子として，術前のインスリン抵抗性(HOMA-IR 2.3以上)が有用であることを報告している[15]．これらの研究結果は，さまざまな肥満関連分子の発現・機能異常が，肝発癌予測の有用なバイオマーカーである可能性を示唆するとともに，これらの分子異常を標的とした新規肝発癌予防および治療法の可能性を期待させるものである．

分岐鎖アミノ酸による肥満関連肝発癌抑制：臨床試験(LOTUS)

肝硬変では肝実質細胞が減少するため，肝臓への糖の取り込みと，肝におけるグリコーゲンの合成・貯蔵能が低下する．特にグリコーゲン合成の低下は，エネルギー不足を補うために蛋白質の異化を亢進させ，全身の筋肉量の減少や，骨格筋における糖の取り込みの減少とグルコース分解の低下をひき起こす．このため肝硬変患者は，肝および骨格筋の糖利用の低下が著明となり，糖尿病や食後高血糖，高インスリン血症といった耐糖能異常を合併する．また，骨格筋でエネルギー源として燃焼される基質は分岐鎖アミノ酸(バリン，ロイシン，イソロイシン)が主体であり，肝硬変患者ではその血中濃度が低下することが知られている．すなわち，肝硬変患者にみられる耐糖能異常の合併，蛋白質・エ

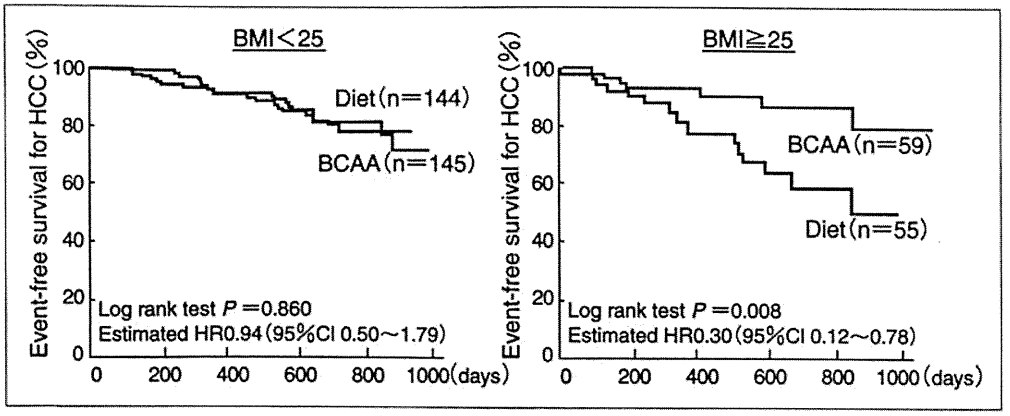

図2　肝発癌に関するイベントフリー生存率のKaplan-Meier推定曲線(文献[2]より引用改変)

ネルギー低栄養状態(protein-energy malnutrition；PEM)，および分岐鎖アミノ酸の低下は，相互に関連しあった一連の病態と考えられる[16][17]．

慢性肝疾患にみられるこのような病態において，減少した分岐鎖アミノ酸の補充療法が患者の生命予後を改善することを証明したのが，LOTUS試験である．646症例の非代償性肝硬変患者を対象とした同試験(多施設ランダム化対照臨床試験)において，分岐鎖アミノ酸製剤(リーバクト®)の経口投与は，患者の血清アルブミン濃度を上昇させるとともに，肝硬変の進行に伴って出現する有害事象(肝不全状態の悪化，静脈瘤破裂，肝癌の発生，死亡)の発症を遅らせ，患者のQOLと生命予後を改善したが，特に層別解析において，分岐鎖アミノ酸製剤の補充療法が，肥満(BMI 25以上)を合併した肝硬変患者の肝発癌を有意に抑制したことは注目すべき結果である(estimated HR0.30, P=0.008, 図2)[2][3]．本試験の結果を踏まえ，平成22年ウイルス性肝硬変に対する包括的治療のガイドライン(2010年3月改訂，厚生労働省科学研究費補助金肝炎等克服緊急対策事業，熊田班)に，「肝機能を維持(AST・ALT値，アルブミン値を改善)し肝発癌の抑制を目指す」ために，栄養療法として分岐鎖アミノ酸製剤(リーバクト®)の使用が盛り込まれていることを特記する．

分岐鎖アミノ酸による肥満関連肝発癌抑制：基礎実験

分岐鎖アミノ酸による，血清インスリン値やHOMA-IRの低下といった糖代謝改善作用については，いくつかの臨床研究において明らかになっているが[18][19]，基礎研究としては，肝硬変モデルラットにおいて，ロイシン，イソロイシンが骨格筋に直接作用し，グルコース輸送担体であるGLUT4の骨格筋細胞膜におけるtranslocationを亢進することで糖の取り込みを促進し，糖負荷後の血糖値を有意に低下させること[20]，また，2型糖尿病モデルラットを用いた肝化学発癌実験において，分岐鎖アミノ酸の投与がVEGFの発現と血管新生を阻害することで，肝前癌病変の発生を抑制することが報告されている[21]．これらの研究結果は，分岐鎖アミノ酸が糖代謝を調節するとともに，肥満に関連した分子異常，特にインスリン抵抗性を標的にすることで肥満関連肝発癌を抑制した可能性を示唆するものである．

われわれは，分岐鎖アミノ酸による肥満関連肝発癌予防のメカニズムを解析するために，以下の実験を行った[4]．レプチン受容体のmutantで高度の肥満・糖尿病をきたす*db/db*マウスに，肝化学発癌物質であるdiethylnitrosamine(DEN)を2週間飲水投与して肝腫瘍を誘発したのち，3％分岐鎖アミノ酸添加食を34週間混餌投与したところ，分岐鎖アミノ酸投与群では，基礎食および窒素負荷対照群(カゼイン投与群)と比較し，肝腫瘍(肝細胞癌および腺腫)の発生が有意に低下した(表1)．分岐鎖アミノ酸投与群の血清および肝組織を解析したところ，血糖値上昇の抑制およびインスリン感受性の改善と，血清ALTおよびレプチン値の低下を認めるとともに，肝の脂肪化および線維化の抑制が認められた．また，

表1 *db/db*マウスの肝腫瘍(adenoma・HCC)の発症率に及ぼす分岐鎖アミノ酸の効果

群	食餌	マウス数	発生率		発生個数 (腫瘍数/マウス) (mean±SD)		
			Adenoma	HCC	Adenoma+HCC	Adenoma	HCC
1	基礎食	11	7/11(64%)	1/11(9%)	1.0±1.1	0.9±1.1	0.1±0.3
2	カゼイン	11	8/11(73%)	3/11(27%)	1.7±1.3	1.5±1.1	0.3±0.5
3	BCAA	11	2/11(18%)[ab]	0/11(0%)	0.2±0.4[ac]	0.2±0.4[c]	0

a：$P<0.05$(1群との有意差)，b：$P<0.05$(2群との有意差)，c：$P<0.01$(2群との有意差)．

(文献[4]より引用改変)

実験食投与開始後16週時に剖検したところ，分岐鎖アミノ酸投与群において，肝臓におけるIGF-1, IGF-2, IGF-1R遺伝子の発現低下と，肝前癌病変の発生抑制が観察された．これらの結果は，分岐鎖アミノ酸による肥満関連肝腫瘍抑制の機序として，インスリン抵抗性の改善とIGF受容体関連シグナルの活性化の阻害，および肝の線維化と脂肪化の抑制が重要であることを示している(図3)．

非環式レチノイドによる肥満関連肝腫瘍形成の抑制

ビタミンAおよびその誘導体・類縁化合物であるレチノイド(retinoid)は，細胞の正常な分化・増殖・死(アポトーシス)といった基本的活動を制御することで，発癌予防効果や腫瘍細胞の増殖抑制・分化誘導作用を発揮することが知られている[22]．われわれは，初発肝癌根治治療後の二次肝発癌を予防し生存率を改善することが報告されている非環式レチノイドが[23][24]，肥満に関連した肝腫瘍形成を抑制するか検討した．*db/db*マウスとDENを用いた肥満関連マウス化学肝発癌モデルにおいて，非環式レチノイドは血清インスリンを低下させるとともにインスリン感受性を回復し，肝腫瘍の形成を有意に抑制した．また，非環式レチノイド投与群において，肝脂肪化の改善と肝臓におけるAMPK蛋白の活性化，および血清TNF-α値の低下と肝臓におけるTNF-α, IL-6, IL-1β遺伝子の発現低下が認められた[25]．これらの結果は，肥満に伴ったインスリン抵抗性と全身および肝臓における炎症状態の改善が，肥満関連肝発癌抑制につながる可能性をあらためて示唆するものである(図3)．

おわりに

肥満や糖尿病，メタボリックシンドロームが大きな社会問題である今日の状況を考えれば，これらの病態を合併した慢性肝疾患患者，ある

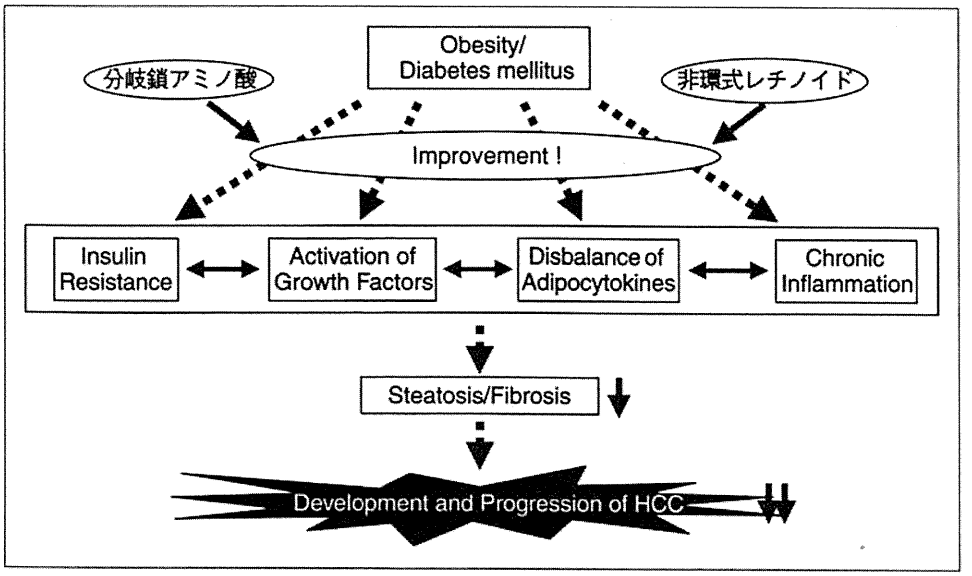

図3 分岐鎖アミノ酸と非環式レチノイドによる肥満関連肝発癌抑制の作用機序

いはNASH由来の肝硬変，肝細胞癌患者のさらなる増加が危惧される．日常診療ですでに用いられている分岐鎖アミノ酸製剤や，肝発癌予防薬として臨床応用が期待されている非環式レチノイドが，肥満に伴ったさまざまな分子異常を改善し，肥満関連肝発癌を抑制したことは，今後の肝癌診療を考える上で大変興味深い結果と考えられた．特に分岐鎖アミノ酸製剤に関しては，血清のインスリン，IGF-1, IGF-2, レプチン値を低下させ，大腸粘膜のIGF-1受容体の活性化を抑制することで，肝発癌同様に肥満・糖尿病・インスリン抵抗性が重要な危険因子である大腸発癌を抑制することが報告されていることより[26]，同剤は，肝細胞癌の発癌・再発予防のみならず，大腸癌やその他の肥満やインスリン抵抗性が危険因子である癌腫や疾患の予防および治療に対して，有効な薬剤である可能性を含んでいる．今後，分岐鎖アミノ酸製剤や非環式レチノイドを用いた，積極的介入に基づく癌化学予防に関する研究が進展し臨床応用が展開され，慢性肝疾患患者の予後が改善されることが期待される．

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Primary Squamous Cell Carcinoma of the Liver: An Uncommon Finding in Contrast-Enhanced Ultrasonography Imaging

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Abstract
Primary squamous cell carcinoma (SCC) of the liver is rare tumor with an unfavorable prognosis. We report a case of advanced primary SCC of the liver arising adjacent to a nonparasitic liver cyst, invading into the right diaphragm and the right lung tissue. Contrast-enhanced ultrasonography (CE-US) demonstrated unique enhancement in the late vascular phase, which was incompatible with those observed in hepatocellular carcinoma, cholangiocellular carcinoma, or metastatic adenocarcinoma. The patient underwent surgical resection of the tumor followed by systemic chemotherapy with 5-fluorouracil (5-FU) and cisplatin (CDDP), while radiation chemotherapy was not applied because of relatively poor performance status. Although postoperative image analysis revealed no recurrence 4 months later, the patient died 13 months after the operation from recurrence. Immunohistological analysis of the resected specimen revealed that this SCC contained many capillary endothelial vessels expressing CD31 or CD34, possibly reflecting the unique imaging pattern in the late vascular phase of CE-US, which has been reported in choangiolocellular carcinoma. In addition, we reviewed which kind of treatment would be suitable for advanced hepatic primary SCC in the literature. From the review, it could be proposed that a combination of radiation therapy, systemic chemotherapy (5-FU and CDDP) and surgical resection, if possible, is appropriate for advanced primary SCC of the liver.

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