

of these conditions also suggest that such pathophysiological disorders might be critical targets for inhibiting obesity-related carcinogenesis [18]. For instance, experimental studies have revealed that improvement of chronic inflammation by inhibiting the expression of TNF- $\alpha$  and IL-6 plays a significant role in the prevention of obesity-related colorectal tumorigenesis [19–21].

The present review aims to summarize multiple pathogenic mechanisms by which obesity and related metabolic disorders influence the development of HCC, focusing on the emergence of insulin resistance and the subsequent inflammatory cascade. This article also aims to review the possibility that nutritional or pharmaceutical approaches targeting pathophysiological conditions caused by obesity might be effective in preventing obesity-related liver carcinogenesis.

### Obesity, diabetes mellitus, and HCC

HCC, which is the dominant form of primary liver carcinoma worldwide, is one of the most frequently occurring cancers in the world, accounting for 750,000 annual cases; approximately the same number of individuals (700,000) die from this malignancy each year [22]. Although HCC development is frequently associated with chronic inflammation and subsequent cirrhosis of the liver induced by a persistent infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), recent epidemiological and clinical studies have revealed that obesity and diabetes mellitus are major risk factors for the development of HCC [6–9, 12–16, 23]. In particular, a recent meta-analysis concluded that the summary relative risk of HCC was 117 % for overweight subjects (BMI 25–30 kg/m<sup>2</sup>) and 189 % for the obese individuals (BMI  $\geq$ 30 kg/m<sup>2</sup>) [14]. Obesity represents an independent HCC risk factor in patients with alcoholic and cryptogenic cirrhosis [15]. The association between HCC development and diabetes, which is characterized by hyperglycemia, insulin resistance, and hyperinsulinemia, has also been ascertained by repeated meta-analyses [10, 11]. In one population-based study, diabetes increased the risk of HCC by threefold [23]. Insulin resistance has also been shown to raise the risk for recurrence of HCC after curative radiofrequency ablation in HCV-positive patients [13].

The relationship between HCV infection and metabolic syndrome is clinically relevant because insulin resistance and subsequent diabetes and severe steatosis frequently occur in HCV-infected patients [24, 25]. Furthermore, there are synergistic effects between metabolic disorders (obesity and diabetes) and other HCC risk factors such as hepatitis virus infection and alcohol consumption [23, 26–29]. A long-term (14 years) follow-up study in Taiwan has shown that the combined presence of HCV and diabetes is

associated with a 37-fold increase in the rate of HCC development [23]. Moreover, HCC risk is increased by more than 100-fold in HBV or HCV carriers with both obesity and diabetes [23]. A recent prospective study showed that insulin resistance itself is associated with HCC in HCV-positive cirrhosis and is a strong predictor of liver-related death or transplantation [30]. Therefore, viral hepatitis patients with metabolic disorders would seem to be at high risk for the development of HCC and thus should be closely monitored for this malignancy.

### NAFLD, nonalcoholic steatohepatitis, and HCC

NAFLD is the major hepatic manifestation of obesity and its related metabolic disorders, particularly diabetes mellitus and dyslipidemia, and has become one of the most common liver disorders in developed countries [2, 3, 31, 32]. The accumulation of fat caused by excess energy intake can result in liver dysfunction as the liver synthesizes more triglycerides but fails to export them. Triglyceride deposition in hepatocytes leads to hepatic steatosis. The overlap between the prevalence of NAFLD and diabetes is equally substantial [32]. On the other hand, NAFLD is commonly associated with insulin resistance and hyperinsulinemia even in the non-obese [33], indicating that insulin resistance might be a key factor in the development of NAFLD. In addition, NAFLD that has not yet progressed to nonalcoholic steatohepatitis (NASH) can induce hepatocyte proliferation and hepatic hyperplasia, both of which initiate the hepatic neoplastic process in obesity [34].

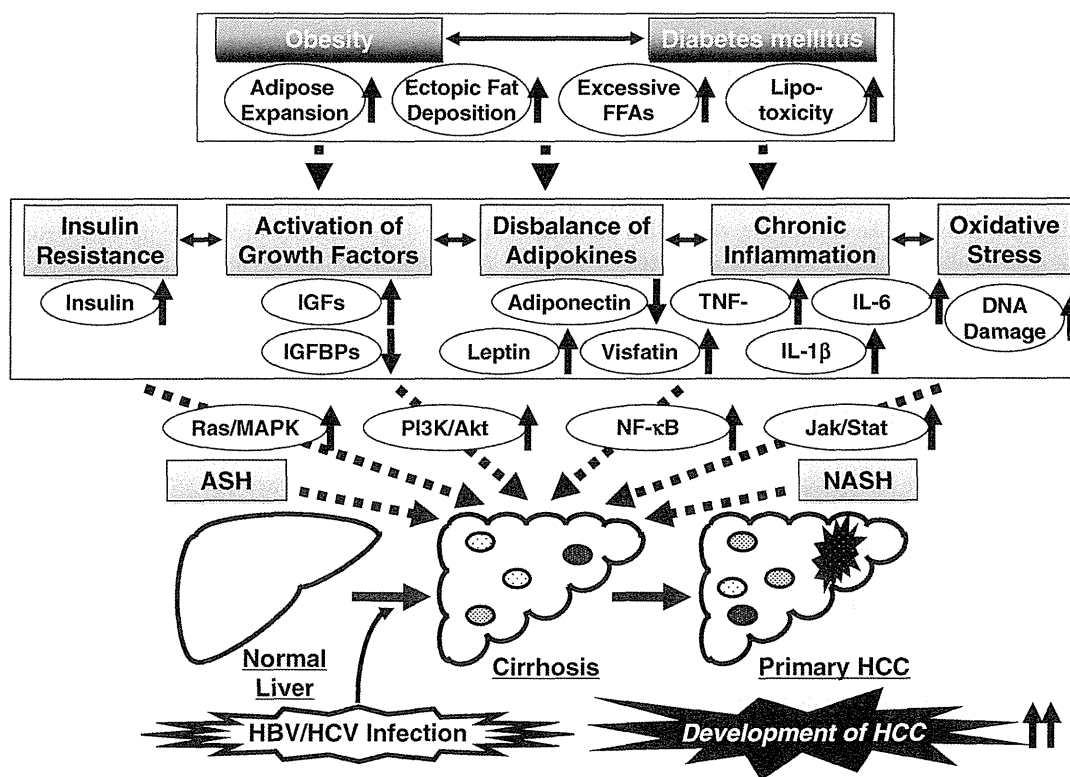
While most patients with NAFLD remain asymptomatic, 20 % progress to develop chronic hepatic inflammation or NASH, which in turn can lead to liver fibrosis, portal hypertension, cirrhosis, HCC development, and increased mortality [2, 3, 31, 32, 35]. A subsequent study of natural history in NAFLD indicates that steatohepatitis is a risk for the development of cirrhosis and HCC [36]. The exact prevalence of HCC in NASH remains unknown; however, some prospective studies found at least 2 to 3 % yearly cumulative incidence of HCC in patients with NASH [37, 38]. In 1998, Day and James proposed a “two-hit theory” to explain NAFLD/NASH pathogenesis [39]. The first hit, the flux of free fatty acids into the liver and subsequent hepatic steatosis, plays a role in lipotoxicity-induced mitochondrial abnormalities that sensitize the liver to additional proinflammatory insults, the second hit. These hits include enhanced lipid peroxidation and increased generation of reactive oxygen species. Insulin resistance is also regarded as a critical factor in the etiology of NASH [39, 40].

**Potential pathophysiological mechanisms linking obesity and HCC development**

Figure 1 shows several pathophysiological mechanisms linking obesity and its related metabolic abnormalities to liver carcinogenesis. Substantial evidence has shown that insulin resistance, among various obesity-related metabolic disorders, significantly contributes to the development of HCC. Insulin, which is a key regulator of glucose metabolism itself, and the signal transduction network it regulates play important roles in oncogenesis [41, 42]. Insulin induces HCC cells to proliferate and resist apoptosis [43, 44], suggesting that hyperinsulinemia directly contributes to the growth of HCC cells. In addition, insulin resistance increases the biological activity of IGF-1, an important endocrine and paracrine regulator of tissue growth and metabolism. Numerous pieces of evidence indicate that the IGF-1/IGF-1R axis plays an important role in the carcinogenesis of many cancer types, including HCC [41, 42]. Insulin receptor and IGF-1R are receptor tyrosine kinases, and the binding of insulin and IGF-1 to their respective receptors on tumors and precancerous cells activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which is responsible for cellular processes like growth, proliferation, and survival [41, 42]. IGF-1R activity is also required for oncogenic transformation by a number of oncogenes,

including RAS, and can promote tumor formation in vivo [41, 45]. Activation of the IGF/IGF-1R axis is critically involved in the growth of HCC cells and in liver carcinogenesis [46–48]. For HCC, IGF-1R activation is observed in a subgroup of tumor cells but not in adjacent cirrhotic tissue [48]. We have recently reported that insulin resistance and the activation of IGF/IGF-1R axis are involved in liver carcinogen *N*-diethylnitrosamine (DEN)-induced liver tumorigenesis in obese and diabetic *C57BL/KsJ-db/db (db/db)* mice [49, 50].

An adipokine imbalance caused by excess production of storage lipids may also be related to obesity-associated liver carcinogenesis. For instance, higher levels of serum leptin, which regulates energy homeostasis and is elevated in obese individuals [51], increase the risk of HCC recurrence after curative treatment [52]. Leptin stimulates the growth of HCC cells by upregulating cyclin D1 expression [53]. Treatment with leptin also increases the proliferation of HCC-derived cells by activating several signaling pathways: signal transducer and activator of transcription-3 (Stat3), AKT, and extracellular signal-regulated kinase (ERK) [54]. In animal models, leptin has been shown to promote angiogenesis and thus could facilitate the progression of NASH to HCC [55]. In addition, lack of adiponectin, the other member of the adipokine group that is significantly reduced in obese individuals [56], enhances the progression of hepatic



**Fig. 1** Proposed mechanisms linking obesity and its related metabolic abnormalities to the development of HCC

steatosis and tumor formation in a mouse model of NASH [57]. However, this adipokine alleviates hepatic steatosis [58]. In vitro and in vivo studies show that adiponectin exerts antitumor effects in HCC cells [59]. Moreover, the induction of adiponectin plays a role in the suppression of chemically induced liver tumorigenesis in obese mice [60]. These findings suggest that obesity and its related metabolic abnormalities, such as sustained insulin resistance, activation of the IGF-1/IGF-1R axis, and adipokine imbalance, play an important role in the development of HCC and thus might be promising targets in the prevention of obesity-related liver tumorigenesis.

### Obesity-induced insulin resistance and chronic inflammation

There is substantial evidence that obesity is associated with chronic low-grade systemic inflammation, which contributes to metabolic disorders and the progression from hepatic steatosis to NASH and subsequent HCC development [4–8]. Hypertrophic adipocytes, which are associated with the deposition and accumulation of excess lipids, secrete free fatty acids (FFAs); in addition, together with various immune cells, they release various proinflammatory cytokines, including TNF- $\alpha$  and IL-6 [4–8]. In particular, macrophage infiltration into white adipose tissue, which is accompanied by TNF- $\alpha$  and IL-6 production, is an early contributing event for the development of chronic low-grade systemic inflammation [61, 62]. In 1993, Hotamisligil et al. demonstrated that adipocytes constitutively express TNF- $\alpha$  and neutralization of TNF- $\alpha$  by soluble TNF- $\alpha$  receptor decreases insulin resistance in obese mice [63]. This suggests that TNF- $\alpha$  lies at the core of the association between obesity and insulin resistance. TNF- $\alpha$  enhances obesity-related systemic insulin resistance by inhibiting the tyrosine phosphorylation of insulin receptor [64]. On the other hand, the loss of TNF- $\alpha$  and its receptor improves insulin sensitivity in obese mice [65]. TNF- $\alpha$  contributes to obesity-induced IL-6 production, which causes hepatic inflammation and activates ERK and Stat3 [66]. TNF- $\alpha$  and IL-6 expressions in the liver are strongly induced in response to a high-fat diet, but inhibition of TNF- $\alpha$  signaling or ablation of IL-6 prevents hepatosteatosis [66]. Type 2 diabetes is an inflammatory condition, as evidenced by the elevated concentrations of IL-6, which induces cellular insulin resistance in hepatocytes, observed in these patients [67–69]. The concentration of IL-6 together with IL-1 $\beta$ , which is another inflammatory cytokine that induces insulin resistance in liver-derived cells, is a more predictive risk factor for type 2 diabetes in humans than either cytokine alone [70, 71]. TNF- $\alpha$  and IL-6 increase the levels of leptin, whereas leptin influences inflammatory responses, possibly by triggering

the release of TNF- $\alpha$  and IL-6 [72, 73]. Hepatic steatosis has negative effects on liver function, which might be mediated by inflammation because the expression of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  mRNA increases in the liver with increasing adiposity [74].

### Cytokine signaling pathway associated with obesity-induced inflammation and HCC development

Several specific intracellular signaling pathways, including c-Jun N-terminal kinase (JNK) and nuclear factor (NF)- $\kappa$ B, have emerged as potential targets for many inflammatory cytokines and chemokines that promote obesity-related metabolic disorders such as insulin resistance [75]. For instance, activation of JNK inhibits normal tyrosine phosphorylation of insulin receptor substrate-1 and downstream insulin signal transduction [76]. The effects of obesity-induced activation of NF- $\kappa$ B are mediated through the synthesis of NF- $\kappa$ B target gene expression, including TNF- $\alpha$ , IL-6, and IL-1 $\beta$  [77]. Therefore, activation of JNK and NF- $\kappa$ B is associated with the induction of insulin resistance, whereas their inhibition provides glucose tolerance and protection from obesity in rodents [75]. Reactive oxygen species that are increased by adiposity have also been shown to activate JNK and NF- $\kappa$ B [78]. In addition, saturated FFAs lead to JNK activation, which can, in turn, increase the production of inflammatory cytokines capable of causing insulin resistance [79]. Saturated FFAs have also been found to enhance NF- $\kappa$ B activation in macrophages [80], suggesting that there is a potential link between elevated circulating or tissue lipid concentrations and the part of the immune system that mediates inflammation. In hepatocytes, saturated FFAs can induce time- and dose-dependent lipoapoptosis, which is the combination of lipid accumulation and induction of apoptosis in hepatocytes [81]. Experimental data have also shown that FFAs cause TNF- $\alpha$  production and subsequent NF- $\kappa$ B activation by promoting hepatic lipotoxicity [82]. These findings appear significant because lipotoxicity and lipoapoptosis play a pivotal role in the progression of NAFLD to NASH [83]. JNK1 activation also promotes the development of NASH in mice fed with methionine- and choline-deficient diets [84], which indicates that JNK and NF- $\kappa$ B are critical factors in the occurrence of NAFLD and its progression to NASH.

The role of obesity-induced inflammation in liver tumorigenesis has recently been demonstrated in several experimental models [50, 66, 85, 86]. For instance, administration of DEN was found to enhance the development of preneoplastic lesions in the livers of rats fed with high-fat diets and this was associated with elevated TNF- $\alpha$ /NF- $\kappa$ B signaling and ERK-related hepatocyte proliferation [85]. Phosphorylation of ERK, Akt, Stat3, and JNK proteins and upregulation of

TNF- $\alpha$ , IL-6, and IL-1 $\beta$  in the liver are involved in DEN-induced liver tumorigenesis in *db/db* obese mice [50]. Enhanced production of adipose-derived TNF- $\alpha$  and IL-6 and activation of Stat3 are critical in the development of obesity-related liver tumorigenesis [66]. This study [66], together with another recent study [87], clearly indicates that Stat3 activation, which is associated with TNF- $\alpha$  and IL-6 production in hepatocytes, is essential for liver carcinogenesis.

### Targeting obesity-related metabolic abnormalities for cancer prevention

As mentioned earlier, obesity and its related metabolic abnormalities, such as a state of chronic inflammation, play a critical role in the development of HCC. On the other hand, these findings may suggest the possibility that the metabolic disorders caused by obesity might be effective targets in the prevention of liver carcinogenesis [18]. For instance, ablation of IL-6 or inhibition of TNF- $\alpha$  signaling can inhibit obesity-promoted hepatocarcinogenesis by reducing hepatosteatosis and steatohepatitis [66]. Treatment with adiponectin, an anti-inflammatory adipokine, also reduces liver tumorigenesis in nude mice [59].

To verify our hypothesis that targeting metabolic abnormalities caused by obesity might be an effective strategy for preventing cancer development in obese individuals, we have conducted several experimental studies. We initially performed chemopreventive studies using a mouse model of obesity-related colorectal carcinogenesis because increased body fat levels and BMI are associated with an increased risk of colorectal cancer [17, 88, 89]. The model used obese and diabetic *db/db* mice, which are susceptible to the colonic carcinogen azoxymethane (AOM) and thus easily develop colonic precancerous lesions [90]. We have found that pitavastatin and renin–angiotensin system inhibitors, which are drugs for hyperlipidemia and hypertension, respectively, suppress AOM-induced colonic preneoplastic lesions in *db/db* mice by inhibiting the levels of TNF- $\alpha$  and IL-6 in the serum and colonic mucosa [20, 21]. Curcumin, a component of turmeric, also exerts chemopreventive effects in the development of obesity-related colonic preneoplastic lesions in *db/db* mice, and this is associated with inhibition of NF- $\kappa$ B activity and TNF- $\alpha$  and IL-6 expression in the colonic mucosa [19]. Furthermore, branched-chain amino acids (BCAA) and (–)-epigallocatechin gallate (EGCG) prevent obesity-related colorectal carcinogenesis by improving insulin resistance and inhibiting IGF/IGF-1R axis in these mice [91, 92].

Among these agents, BCAA is considered as one of the most promising candidates to prevent obesity-related liver tumorigenesis. This is because it is widely used for the treatment of protein energy malnutrition (PEM) that frequently occurs in

patients with liver cirrhosis [93–96]. EGCG, a major biologically active component of green tea, also seems to have a considerable effect given that green tea catechins (GTCs) improve metabolic abnormalities and possess anticancer and cancer chemopreventive properties [97–100]. In the following sections, we will discuss in detail the effects of BCAA and EGCG in the prevention of obesity-related liver tumorigenesis based on our recent experimental studies. In addition, we also discuss the effects of acyclic retinoid (ACR), which is a promising agent for the chemoprevention of HCC [101–104], on the prevention of liver tumorigenesis in obese mice.

### Preventive effects of BCAA on obesity-related liver tumorigenesis

Because the liver is a critical organ for regulating metabolism, a variety of nutritional and metabolic disorders, such as PEM and insulin resistance, are frequently seen in patients with chronic liver diseases [93–96, 105, 106]. Decreased serum levels of BCAA (valine, leucine, and isoleucine) and albumin appear with a high incidence in liver cirrhosis, whereas supplementation with BCAA has been shown to improve PEM and increase the serum albumin concentration in cirrhotic patients. This subsequently improves the quality of life and prognosis in patients with liver cirrhosis by preventing complications associated with the disease [93–96]. In addition, recent clinical and experimental studies have revealed that BCAA improves insulin resistance and glucose tolerance [107–110]. In 2005, Muto et al. reported the results of a large-scale ( $n=622$ ) multicenter randomized controlled trial, the Long-Term Survival Study, which investigated the effects of supplemental BCAA therapy on event-free survival in patients with decompensated cirrhosis. In the trial, oral supplementation with a BCAA preparation significantly prevented progressive hepatic failure and improved event-free survival [95], strongly suggesting that supplementation with BCAA can serve as a first-line therapy for patients with decompensated cirrhosis.

Moreover, it should be emphasized that the results of the subset analysis from this trial demonstrated that long-term oral supplementation with BCAA was associated with a reduced frequency of HCC in obese cirrhotic patients ( $P=0.008$ ) [12]. To clarify the precise mechanisms of BCAA in the prevention of the development of HCC in obese cirrhotic patients, we performed an experimental study using the obesity-related liver carcinogenesis model in *db/db* mice [49]. In the study, BCAA supplementation significantly suppressed the development of DEN-induced hepatic preneoplastic lesions in *db/db* mice by inhibiting the expression of IGF-1, IGF-2, and IGF-1R in the liver. The development of liver neoplasms, including hepatic adenoma and HCC,

was also reduced by BCAA supplementation, and this was associated with improvement of insulin resistance, reduction of serum leptin levels, and attenuation of hepatic steatosis and fibrosis [49]. Obese cirrhotic patients generally have a particularly high incidence of hyperinsulinemia and insulin resistance [105, 106]. Therefore, our findings [49], together with the results of an *in vitro* study showing that BCAA suppresses insulin-induced proliferation of HCC cells by inhibiting the insulin-induced activation of the PI3K/Akt pathway [111], suggest that BCAA supplementation reduced the risk of developing HCC in obese cirrhotic patients. This was accomplished, at least in part, by targeting insulin resistance and its related signaling pathways (Fig. 2; Table 1). These findings are consistent with the results of an experimental study reported by Yoshiji et al. showing the chemopreventive effects of BCAA supplementation against liver tumorigenesis in obese and diabetic rats, which are also complicated with insulin resistance [112].

In addition, in our unpublished study, BCAA supplementation was shown to suppress the spontaneous development of hepatic preneoplastic lesions in *db/db* mice by inhibiting the expression of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  mRNA in the liver. BCAA supplementation also inhibited increased macrophage infiltration and the expression of TNF- $\alpha$ , IL-6, and monocyte chemoattractant protein-1 mRNA in the white adipose tissue, suggesting that chronic inflammation induced by obesity in the liver and adipose tissue could also serve as a critical target of BCAA in the inhibition of the

early phase of obesity-related liver tumorigenesis (unpublished data).

### Preventive effects of GTCs on obesity-related liver tumorigenesis

Green tea is a beverage commonly consumed worldwide. Its component polyphenols, which are known as GTCs, have received great attention for their beneficial effects, particularly their involvement in the improvement of metabolic abnormalities and prevention of certain types of malignancies [97–100]. A recent meta-analysis of clinical trials reported that GTCs help reduce body weight [98]. Supplementation with GTCs was found to decrease plasma levels of insulin, TNF- $\alpha$ , and IL-6 and improve hepatic steatosis and liver dysfunction in a rodent model of obesity and diabetes. This indicated that treatment with GTCs is effective in the prevention of the progression of obesity-related metabolic disorders such as chronic inflammation [113–115]. The anti-inflammatory properties of GTCs are also responsible for the anticancer and cancer-preventive effects of the molecules [99]. EGCG, a type of GTC, suppresses inflammation-related colon carcinogenesis in mice by decreasing the mRNA expression of TNF- $\alpha$  and IL-6 in the colonic mucosa [116]. EGCG also inhibits proliferation and induces apoptosis in HCC- and colorectal cancer-derived cells by inhibiting the activation of IGF-1R and its

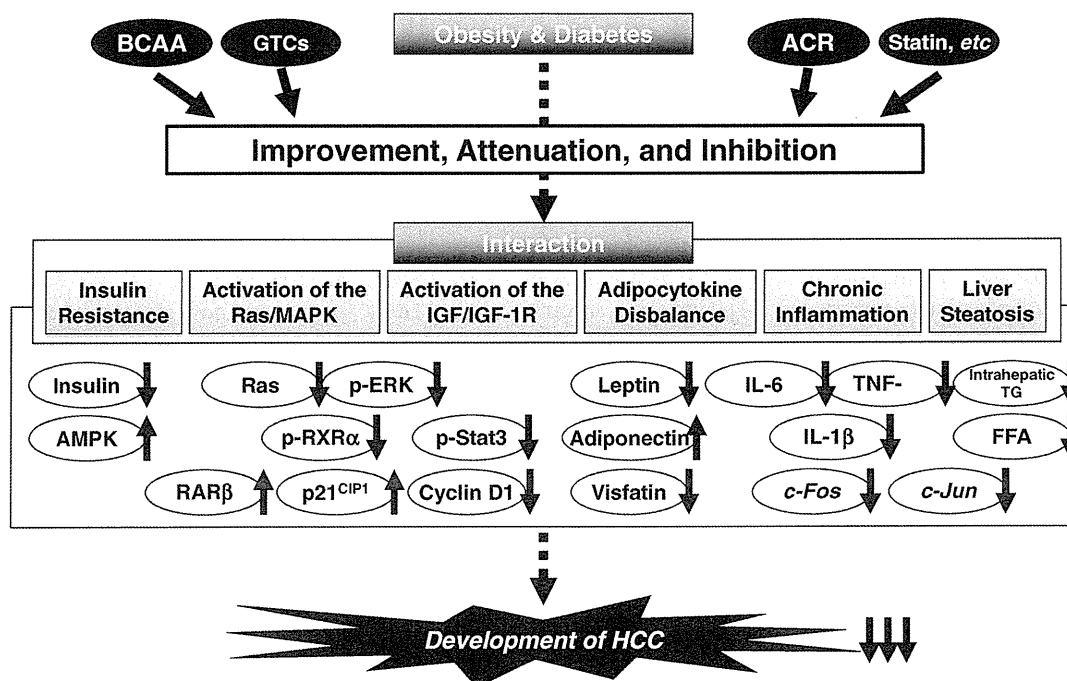


Fig. 2 Mechanisms of action of BCAA, EGCG, and ACR in the inhibition of obesity-related liver carcinogenesis

**Table 1** Suppressive effects of BCAA, EGCG, ACR, and pitavastatin on obesity-related liver tumorigenesis in *db/db* mice

Agent	Inhibition rate (%)		Inhibition mechanisms	Reference number
	Adenoma	FCA <sup>a</sup>		
BCAA	75 <sup>b, c</sup>	50 <sup>b, c</sup>	Hepatic IGF-1, IGF-2, and IGF-1R mRNAs ↓ Hepatic steatosis ↓ Hepatic fibrosis ↓	[49]
EGCG	86 <sup>c</sup>	48 <sup>c</sup>	Hepatic pIGF-1R, pERK, and pAkt proteins ↓ Hepatic steatosis ↓ Hepatic and systemic inflammation ↓	[50]
ACR	86 <sup>c</sup>	81 <sup>c</sup>	Hepatic Ras activity ↓ Hepatic RARβ and p21 <sup>CIP1</sup> mRNAs ↑ Insulin sensitivity ↑	[86]
Pitavastatin	NE <sup>d</sup>	29 <sup>e</sup>	Pro-apoptotic effect ↑ Hepatic steatosis ↓ Serum adiponectin level ↑	[60]

<sup>a</sup>Foci of cellular alteration

<sup>b</sup>Compared to the casein supplementation mice (a nitrogen content-matched control for BCAA)

<sup>c</sup>Mice were treated with agent for 34 weeks

<sup>d</sup>Not examined

<sup>e</sup>Mice were treated with agent for 14 weeks

downstream signaling pathways, including Ras/MAPK and PI3K/Akt [46, 117]. In addition, this agent prevents carbon tetrachloride-induced hepatic fibrosis in rats by inhibiting IGF-1R expression [118], indicating that the IGF/IGF-1R axis, which is critically involved in cancer development and obesity-related metabolic disorder, might be a critical target of GTCs. Several interventional studies also provide clear evidence for the chemopreventive effects and safety of tea preparations [119–121].

Because GTCs are expected to improve metabolic disorders and exert chemopreventive properties by targeting chronic inflammation and the IGF/IGF-1R axis, we examined whether EGCG treatment inhibits obesity-associated liver tumorigenesis [50]. We found that drinking water containing EGCG significantly inhibited the development of hepatic preneoplastic lesions and adenoma [50]. EGCG consumption also improved hepatic steatosis; decreased the serum levels of insulin, IGF-1, and IGF-2; and inhibited the phosphorylation of the IGF-1R, ERK, Akt, Stat3, and JNK proteins in the liver of obese mice [50]. The serum levels of FFA and TNF-α were also decreased by drinking EGCG, which additionally lowered the expression of TNF-α, IL-6, and IL-1β mRNAs in the liver [50]. These findings suggest that EGCG prevents obesity-related liver tumorigenesis by inhibiting the IGF/IGF-1R axis, improving hyperinsulinemia, and attenuating chronic inflammation (Fig. 2; Table 1). Thus, in addition to BCAA, GTCs may also be useful in the chemoprevention of liver tumorigenesis in obese individuals.

### Preventive effects of ACR on obesity-related liver tumorigenesis

Retinoids, a group of structural and functional derivatives of vitamin A, play fundamental roles in cellular activities, including growth, differentiation, and apoptosis, as well as in morphology [122, 123]. Because of this, loss of retinoid activity or responsiveness is linked to the development of several types of human malignancies, including HCC; therefore, they might be critical targets for cancer chemoprevention and chemotherapy [103, 104, 124, 125]. Retinoids exert their biological functions primarily by regulating gene expression through two distinct nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both of which are composed of three subtypes (α, β, and γ) [122, 123]. Among the retinoid receptors, RXRα is thought to be one of the most important with respect to exerting fundamental effects on cellular activities. This is because it forms a heterodimer with other nuclear receptors and thereby acts as the master regulator of nuclear receptors [122, 123]. We have reported that abnormalities in the expression and function of RXRα are prominently involved in the development of HCC. The repression of RXRα was found to occur in the early stages of liver carcinogenesis in a rat model of chemically induced liver carcinogenesis [126]. Moreover, a malfunction of the RXRα due to phosphorylation by the Ras/MAPK signaling pathway is significantly

associated with liver carcinogenesis. That is, accumulation of phosphorylated RXR $\alpha$  protein, which is regarded as the nonfunctional form of RXR $\alpha$ , interferes with the function of normal (unphosphorylated) RXR $\alpha$  in a dominant-negative manner, thus playing a critical role in HCC development [103, 104, 127–130]. These findings therefore suggest that targeting RXR $\alpha$  phosphorylation may be an effective and important strategy for the prevention and treatment of HCC.

ACR, a synthetic retinoid that was initially developed as an agonist for RXR, is a possible candidate for this purpose because it can impede the development of HCC and it inhibits cancer cell growth by repressing the Ras/MAPK signaling pathway and subsequent RXR $\alpha$  phosphorylation [103, 104, 128, 131]. One early-phase randomized controlled clinical trial tested the chemopreventive effect of ACR on secondary HCC in patients who underwent potentially curative treatment for initial HCC. In this study, oral administration of ACR significantly reduced the incidence of recurrent or new HCC ( $P=0.04$ ) and improved the recurrence-free survival ( $P=0.002$ ) and overall survival rates ( $P=0.04$ ) [101, 102]. Moreover, a large-scale ( $n=401$ ) randomized placebo-controlled trial (phase II/III trial) also showed that ACR had a strong effect on the prevention of second primary HCC in HCV-positive patients. It showed a hazard ratio of 0.27 (95 % CI, 0.07–0.96) 2 years after the treatment, indicating that ACR reduced the recurrence of HCC, particularly after 2 years of treatment [132].

Because numerous preclinical experiments and clinical trials indicate that ACR is a promising agent for the chemoprevention of HCC, we investigated whether ACR could prevent obesity-related liver tumorigenesis [86]. In the study, treatment with ACR effectively prevented the development of obesity-related liver tumorigenesis by inhibiting the activation of Ras and the phosphorylation of ERK and RXR $\alpha$ , thus restoring RXR $\alpha$  function in the liver of DEN-treated *db/db* mice [86]. ACR administration also inhibits this tumorigenesis through attenuation of the chronic inflammation induced by excessive fatty deposits, as demonstrated by the improved liver steatosis and decreased serum TNF- $\alpha$  levels and expression levels of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  mRNA in the liver [86]. In addition, ACR administration improved insulin sensitivity, which was also associated with the prevention of obesity-related liver tumorigenesis [86] (Fig. 2; Table 1). Therefore, the results obtained from both clinical trials [101, 102, 132] and this preclinical experiment [86] encourage the clinical use of ACR for cirrhotic patients with obesity and diabetes who are at a notably higher risk of developing HCC.

## Conclusion

Obesity and its related metabolic abnormalities, including increased cancer risk, are a serious public health problem worldwide. Among all cancers, HCCs are the malignancies most frequently affected by obesity. The liver disease influenced most by obesity is NAFLD, and this disease, by itself and in synergy with other risk factors such as hepatitis virus infection, is becoming one of the most common causes of HCC in developed countries. Therefore, there is an urgent need to develop more effective therapeutic strategies to prevent the development of obesity-related HCC or halt its progression. Obesity and diabetes enhance HCC development through insulin resistance, activation of the IGF/IGF-1R axis, and lipid accumulation within hepatocytes, thereby leading to a chronic low-grade systemic inflammation. This involves abnormalities of various types of cytokines and adipokines. Among them, TNF- $\alpha$  and IL-6 play a critical role in the onset of NASH and the initiation and promotion of HCC.

In this review, we indicate the possibility that pharmaceutical and nutraceutical approaches for targeting and restoring metabolic disorders, especially chronic low-grade inflammation involving increased levels of TNF- $\alpha$  and IL-6, may be an effective strategy for preventing the development of obesity-related HCC. We further indicate that BCAA, GTCs, and ACR are considered as some of the most promising agents for achieving this purpose. Therefore, further advanced translational research, such as pilot trials, to clarify whether active intervention using these agents can prevent the development and recurrence of HCC in patients with chronic liver disease and obesity is required. In addition, further experimental studies to determine whether specific drugs, such as antidiabetic drugs, anti-hypertensive drugs, and lipid-lowering drugs, can inhibit obesity-related liver carcinogenesis should be performed. Considering that these drugs are widely used for patients with metabolic syndrome, it would be beneficial if they could exert chemopreventive effects on obesity-associated carcinogenesis. Our recent findings that pitavastatin, a recently developed lipophilic statin, suppresses the development of chemically induced colonic and hepatic preneoplastic lesions in *db/db* mice by attenuating chronic inflammation may provide a basis for this attempt [21, 60] (Fig. 2; Table 1).

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## Special Report

# Nutritional status and quality of life in current patients with liver cirrhosis as assessed in 2007–2011

Makoto Shiraki,<sup>1</sup> Shuhei Nishiguchi,<sup>2</sup> Masaki Saito,<sup>2</sup> Yoshitaka Fukuzawa,<sup>3</sup> Toshihiko Mizuta,<sup>4</sup> Masaki Kaibori,<sup>5</sup> Tatsunori Hanai,<sup>1</sup> Kayoko Nishimura,<sup>6</sup> Masahito Shimizu,<sup>1</sup> Hisashi Tsurumi<sup>1</sup> and Hisataka Moriwaki<sup>1</sup>

<sup>1</sup>The First Department of Internal Medicine, Gifu University School of Medicine, Gifu, <sup>2</sup>Division of Hepatobiliary and Pancreatic Disease, Department of Internal Medicine, Hyogo College of Medicine, Hyogo, <sup>3</sup>Medical Education Center, Aichi Medical University School of Medicine, Aichi, <sup>4</sup>Department of Internal Medicine, Saga Medical School, Saga University, Saga, <sup>5</sup>Department of Surgery, Kansai Medical University, Osaka, and <sup>6</sup>Center for Nutrition Support and Infection Control, Gifu University Hospital, Gifu, Japan

**Aim:** Current guidelines recommended adequate nutritional support for patients with liver cirrhosis to improve clinical outcome and quality of life (QOL). However, these evidences were obtained more than 10 years ago when malnutrition prevailed. In recent years, the impact of obesity on liver damage and carcinogenesis has grown. We attempted to elucidate the nutritional state and QOL in present cirrhotics.

**Methods:** A research group supported by the Ministry of Health, Labor and Welfare of Japan recruited 294 cirrhotics between 2007 and 2011. Subjects comprised 171 males and 123 females, 158 of whom had hepatocellular carcinoma (HCC) and Child–Pugh grades A:B:C were 154:91:49. Anthropometry, blood biochemistry and indirect calorimetry were conducted, and QOL was measured using Short Form-8.

**Results:** The mean body mass index (BMI) of all patients was  $23.1 \pm 3.4$  kg/m<sup>2</sup>, and 31% showed obesity (BMI  $\geq 25.0$ ). In subjects without ascites, edema or HCC, mean BMI was

$23.6 \pm 3.6$ , and 34% had obesity. Protein malnutrition defined as serum albumin of less than 3.5 g/dL and energy malnutrition as respiratory quotient of less than 0.85 appeared in 61% and 43%, respectively, and protein-energy malnutrition (PEM) in 27% of all subjects. Among subjects without HCC, each proportion was 67%, 48% and 30%, respectively. QOL was significantly lower on all subscales than Japanese national standard values, but was similar regardless the presence or absence of HCC.

**Conclusion:** While PEM is still present in liver cirrhosis, an equal proportion has obesity in recent patients. Thus, in addition to guidelines for PEM, establishment of nutrition and exercise guidelines seems essential for obese patients with liver cirrhosis.

**Key words:** body mass index, energy malnutrition, liver cirrhosis, protein malnutrition, quality of life

## INTRODUCTION

BECAUSE THE LIVER plays the central role in nutrient and fuel metabolism, protein-energy malnutrition (PEM) is common in patients with liver cirrhosis.<sup>1,2</sup> Moreover, such malnutrition leads to poor prognosis and decline in the quality of life (QOL) of cirrhotics.<sup>3,4</sup>

Branched-chain amino acid (BCAA) administration for protein malnutrition raises the serum albumin level

and improves the QOL and survival of patients with liver cirrhosis.<sup>5–8</sup> Treatment with late-evening snack (LES) for energy malnutrition improves respiratory quotient (RQ), liver dysfunction and QOL.<sup>9,10</sup>

Therefore, the guidelines for the treatment of liver cirrhosis by Japanese Society of Gastroenterology,<sup>11</sup> American Society for Parenteral and Enteral Nutrition<sup>12</sup> and European Society for Clinical Nutrition and Metabolism<sup>13</sup> recommend such nutritional therapy.

However, these evidences were obtained in the cirrhotic patients recruited from 1995–2000, where protein or energy malnutrition prevailed in 50–87%.<sup>1–4</sup> In contrast, in the next 10 years, obesity rate in the cirrhotic patients rose to approximately 30%.<sup>14</sup> More recently, non-alcoholic steatohepatitis (NASH) or the

Correspondence: Dr Makoto Shiraki, The First Department of Internal Medicine, Gifu University School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan. Email: mshiraki-gif@umin.ac.jp  
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hepatic inflammation, fibrosis and carcinogenesis due to obesity became the topics.<sup>14–16</sup>

Therefore, it is essential to re-evaluate a nourishment state of the current cirrhotic patients to update the guidelines. In this report, we investigated comprehensive data on the nourishment state and QOL in a large group of patients with liver cirrhosis recruited in the years 2007–2011.

## METHODS

### Patients

TWO HUNDRED AND ninety-four patients with liver cirrhosis (171 men and 123 women; mean age,  $68 \pm 10$  years) undergoing treatment between 2007 and 2011 were recruited by a Research Group (Gifu University, Hyogo College of Medicine, Aichi Medical University and Saga University) supported by the Ministry of Health, Labor and Welfare of Japan. Liver cirrhosis was diagnosed by clinical and laboratory profiles and by histological examination of liver biopsy specimens. The etiology of cirrhosis was hepatitis B virus in 35 patients, hepatitis C virus in 204, alcohol in 25, NASH in six and others in 24. Child–Pugh classification of the disease severity<sup>17</sup> was A in 154 cases, B in 91 cases and C in 49 cases. One hundred and fifty-eight patients had hepatocellular carcinoma (HCC), and their clinical stage was I

in 41 patients, II in 41, III in 54 and IV in 22. Clinical profiles of the patients are presented in Table 1. The proportion of patients supplemented with BCAA or LES rose in parallel with the increasing grade of Child–Pugh classification. Patients with fever, HIV infection, overt infectious disease (septicemia, pneumonia, urinary tract infection), renal insufficiency or under immunomodulatory therapy were excluded. The study protocol was approved by the Medical Ethics Committee of Gifu University Graduate School of Medicine, and informed consent was obtained from all patients. The study protocol was in agreement with the 1975 Declaration of Helsinki as revised in 1983.

### Hematological examinations

Blood was drawn for routine laboratory examinations in the early morning after overnight fasting on the day of metabolic studies. Serum albumin, total bilirubin, alt alanine aminotransferase, prothrombin activity and urinary nitrogen (UN) were measured with a standard clinical analyzer at the central laboratory in each hospital.

### Nutritional assessment

Metabolic studies were carried out using an indirect calorimeter (Aeromonitor AE-300S; Minato Medical Science, Osaka, Japan) to estimate non-protein re-

**Table 1** Clinical and biochemical profiles of patients with liver cirrhosis

	Cirrhosis (n = 294)	Child A (n = 154)	Child B (n = 91)	Child C (n = 49)	P
Age (years)	$68 \pm 10$	$68 \pm 10$	$68 \pm 10$	$68 \pm 12$	n.s.
Sex (male/female)	171/123	90/64	51/40	30/19	n.s.
Height (cm)	$159 \pm 9.1$	$159 \pm 9.0$	$159 \pm 9.1$	$159 \pm 9.7$	n.s.
Weight (kg)	$59 \pm 11$	$58 \pm 9.6$	$59 \pm 11$	$60 \pm 13$	n.s.
Body mass index (kg/m <sup>2</sup> )	$23.1 \pm 3.4$	$22.9 \pm 3.0$	$23.4 \pm 3.6$	$23.6 \pm 4.0$	n.s.
Etiology (HBV/HCV/alcohol/others)	35/204/25/30	20/108/11/15	11/62/8/10	4/34/6/5	n.s.
Hepatocellular carcinoma (+/-)*	158/136	84/69	54/38	20/29	n.s.
Number of patients					
Treated with BCAA	97	35	45	17	<0.01
Supplied with LES	36	8	19	9	<0.01
Albumin (g/dL)	$3.3 \pm 0.6$	$3.6 \pm 0.5$	$3.0 \pm 0.4$	$2.6 \pm 0.4$	<0.01
Total bilirubin (mg/dL)	$1.4 \pm 1.8$	$0.9 \pm 0.4$	$1.5 \pm 1.2$	$3.2 \pm 3.8$	<0.01
Alanine aminotransferase (IU/L)	$44 \pm 31$	$43 \pm 30$	$44 \pm 29$	$45 \pm 40$	n.s.
Prothrombin time (%)	$81 \pm 30$	$91 \pm 32$	$75 \pm 23$	$66 \pm 22$	<0.01

HBV, hepatitis B virus; HCV, hepatitis C virus; BCAA, branched-chain amino acids; LES, late-evening snack; n.s., not significant.

Data are presented as number of patients or mean  $\pm$  standard deviation.

Statistical analysis was performed by one-way ANOVA or contingency table analysis for distribution among Child–Pugh grades A, B and C.

\*Clinical stage of hepatocellular carcinoma was I in 41 patients, II in 41, III in 54 and IV in 22.

spiratory quotient (npRQ) from measured oxygen consumption/min ( $\text{VO}_2$ ), carbon dioxide production/min ( $\text{VCO}_2$ ) and total urinary nitrogen using the following equation:<sup>18–20</sup>

$$\text{npRQ} = (1.44\text{Vco}_2 - 4.890\text{UN}) / (1.44\text{Vo}_2 - 6.04\text{UN}).$$

Measurements were performed between 07.00 and 09.00 hours while the patients were still lying in bed. The last meal was served at 18.00 hours on the previous day.

We measured height and bodyweight, and calculated body mass index (BMI).

### QOL questionnaire

Health-related QOL was measured using the Short Form-8 (SF-8) questionnaire.<sup>21–23</sup> The SF-8 contains eight questions that provide a quantitative evaluation on each of eight subscales: (i) physical functioning (PF); (ii) role physical (RP); (iii) bodily pain (BP); (iv) general health perception (GH); (v) vitality (VT); (vi) social functioning (SF); (vii) role emotional (RE); and (viii) mental health (MH).

### Statistical analysis

Data were expressed as the mean and standard deviation. Comparisons of measured values among Child–Pugh classification grade A, B and C were performed using one-way ANOVA. Comparisons of sex, etiology and the presence of HCC among Child–Pugh classification grades were performed using contingency table analysis. Measured QOL was analyzed by *z*-test or Student's *t*-test between each group. Data analysis was performed using JMP ver. 5.1J (SAS Institute Japan, Tokyo, Japan) and  $P < 0.05$  was considered statistically significant.

## RESULTS

### BMI of the patients with liver cirrhosis

THE MEAN BMI of all patients with liver cirrhosis was  $23.1 \pm 3.4 \text{ kg/m}^2$ .

The ratio of obese subjects with BMI of 25 or higher was 30.6% and that of less than  $18.5 \text{ kg/m}^2$  was 5.1%, respectively (Fig. 1).

We then excluded patients with ascites, edema or HCC to match the present cohort with those reported in 2002.<sup>4</sup> The number of patents in this cohort was 95, and Child–Pugh grades A, B and C were 71:22:2, respectively. Mean BMI was  $23.6 \pm 3.6 \text{ kg/m}^2$ , and BMI of

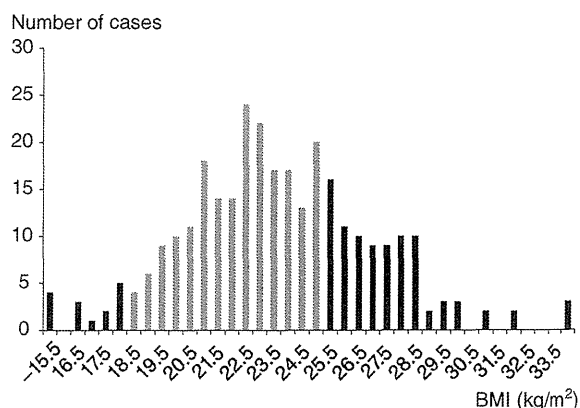


Figure 1 Distribution of body mass index (BMI) in patients with liver cirrhosis. Total number of patients = 294. Obese subjects ( $\text{BMI} \geq 25$ ) were present in 30.6%, lean ones ( $18.5 \leq \text{BMI} < 25$ ) were in 64.3% and emaciation ( $\text{BMI} < 18.5$ ) was observed in 5.1%.

less than  $18.5 \text{ kg/m}^2$  and  $25.0 \text{ kg/m}^2$  or higher were observed in 9.2% and 33.7%, respectively (Fig. 2).

### Incidence of protein malnutrition, energy malnutrition and PEM in patients with liver cirrhosis

We examined nutritional status in 181 patients with liver cirrhosis that underwent indirect calorimetry. In these patients, the male : female ratio was 112:69, HCC

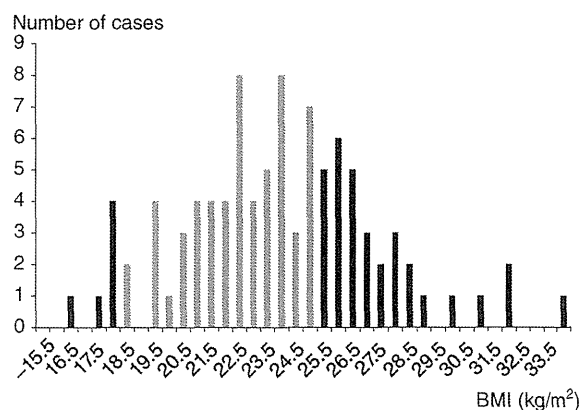


Figure 2 Distribution of body mass index (BMI) in cirrhotic patients without ascites, edema or hepatocellular carcinoma. Total number of patients = 95. Obese subjects ( $\text{BMI} \geq 25$ ) were present in 33.7%, lean ones ( $18.5 \leq \text{BMI} < 25$ ) were in 57.1% and emaciation ( $\text{BMI} < 18.5$ ) was observed in 9.2%.

**Table 2** Incidence of protein and energy malnutrition in patients with liver cirrhosis

Energy nutritional state	Protein nutritional state	
	Normal (%)	Malnourished (%)
Normal (%)	42 (23%)	62 (34%)
Malnourished (%)	28 (16%)	49 (27%)

Protein malnutrition was defined as serum albumin level of <3.5 g/dL and energy malnutrition as a respiratory quotient of <0.85.

Total number of patients = 181.

Data are presented as number of patients (%).

was present in 94, and Child–Pugh grades A : B : C were 90:58:33. When protein malnutrition was defined as serum albumin level of less than 3.5 g/dL and energy malnutrition as a non-protein respiratory quotient of less than 0.85, protein malnutrition was found in 61%, energy malnutrition in 43% and PEM in 27% (Table 2). Similarly, among 87 patients without HCC (Child–

**Table 3** Incidence of protein and energy malnutrition in cirrhotic patients without hepatocellular carcinoma

Energy nutritional state	Protein nutritional state	
	Normal (%)	Malnourished (%)
Normal (%)	13 (15%)	32 (37%)
Malnourished (%)	16 (18%)	26 (30%)

Protein malnutrition was defined as serum albumin level of <3.5 g/dL and energy malnutrition as a respiratory quotient of <0.85.

Total number of patients = 87.

Data are presented as number of patients (%).

**Table 4** Comparison of health-related quality of life between the Japanese national standard and the patients with liver cirrhosis

Subscales	Japanese national standard	Patients with liver cirrhosis	P
Physical functioning	50.1 ± 5.0	43.8 ± 5.2	<0.01
Role physical	50.2 ± 5.3	41.6 ± 6.6	<0.01
Bodily pain	51.3 ± 8.3	48.3 ± 5.3	<0.01
General health perception	50.6 ± 6.6	45.2 ± 4.4	<0.01
Vitality	52.4 ± 5.5	47.5 ± 4.3	<0.01
Social functioning	50.2 ± 6.6	46.1 ± 5.3	<0.01
Role emotional	51.3 ± 4.5	45.6 ± 5.7	<0.01
Mental health	53.3 ± 5.4	47.6 ± 4.0	<0.01

Data are presented as mean ± standard deviation.

Statistical analysis was performed by Student's *t*-test between the Japanese national standard<sup>24</sup> and the patients with liver cirrhosis.

**Table 5** Comparison of health-related quality of life in cirrhotics by the presence or absence of hepatocellular carcinoma

Subscales	Absence of hepatocellular carcinoma	Presence of hepatocellular carcinoma	P
Physical functioning	43.4 ± 4.9	44.2 ± 5.5	n.s.
Role physical	41.1 ± 6.3	42.1 ± 6.8	n.s.
Bodily pain	47.8 ± 5.3	48.7 ± 5.1	n.s.
General health perception	44.9 ± 4.5	45.4 ± 3.9	n.s.
Vitality	46.5 ± 4.3	48.4 ± 4.2	n.s.
Social functioning	45.3 ± 5.0	46.8 ± 5.4	n.s.
Role emotional	45.3 ± 5.0	45.8 ± 6.1	n.s.
Mental health	46.6 ± 3.9	48.5 ± 4.0	n.s.

n.s., not significant.

Data are presented as mean ± standard deviation.

Statistical analysis was performed by *z*-test between the presence and absence of hepatocellular carcinoma.

Pugh grades A : B : C, 36:27:24), 67% had protein malnutrition, 48% had energy malnutrition and 30% had PEM (Table 3).

### Health-related QOL of the patients with liver cirrhosis

We examined health-related QOL in 114 patients with liver cirrhosis (64 men and 50 women) using the SF-8. Sixty-two patients had HCC, and Child–Pugh grades A : B : C were 63:26:25.

Quality of life of all subjects was significantly lower on all subscales than Japanese national standard values (Table 4),<sup>24</sup> but no difference was observed between the presence and the absence of HCC (Table 5).

## DISCUSSION

**P**ROTEIN-ENERGY MALNUTRITION is a common manifestation in cirrhotic patients with reported incidences as high as 50–87%.<sup>1,2</sup> Protein nutrition is usually evaluated by serum albumin level and, for energy nutrition, indirect calorimetry is recommended for precise analysis.<sup>13</sup> Energy malnutrition typically shows reduced carbohydrate oxidation, increased fat oxidation and decline in npRQ measured by indirect calorimetry. It is reported that PEM worsens prognosis and QOL in patients with liver cirrhosis.<sup>3,4</sup> Thus, intervention for PEM is an important issue in the clinical management of liver cirrhosis.

For this purpose, BCAA administration for protein malnutrition raises the serum albumin level and improves QOL and survival of patients with liver cirrhosis.<sup>5–8</sup> LES for energy malnutrition improves npRQ, liver dysfunction and QOL.<sup>9,10</sup> Thus, many guidelines<sup>11–13</sup> recommend such nutritional therapy for liver cirrhosis.

However, these evidences were obtained in the cirrhotic patients recruited from 1995 through 2000 where malnutrition prevailed but obesity was apparently less (20%)<sup>4</sup> than the general cohort (30%).<sup>25</sup> In the next 10 years, obesity rose by approximately 1.5 times in the patients with chronic liver disease in Japan.<sup>14</sup> In addition, presence of diabetes mellitus, hyperinsulinemia or obesity is currently regarded as a significant risk factor for liver carcinogenesis.<sup>14–16</sup> Furthermore, the relationship between obesity and liver inflammation and fibrosis, including NASH has become an important issue in recent years. Therefore, it is necessary to elucidate the nourishment state of the present cirrhotic patients to update guidelines. Thus, we report in this paper a comprehensive survey of the nourishment state and QOL in the present patients with liver cirrhosis.

The etiology of the 294 cirrhotics was hepatitis B virus in 11.9%, hepatitis C virus in 69.4%, alcohol in 8.5%, NASH in 2.0% and others in 8.2% in this study. In the 44th Annual Meeting of Japan Society of Hepatology in 2008 (Matsuyama), the reported etiology of 33 379 cirrhotics was hepatitis B virus in 13.9%, hepatitis C virus in 60.9%, alcohol in 13.6%, NASH in 2.1% and others in 9.5%,<sup>26</sup> indicating similar patient composition between two studies.

Obesity is defined by BMI of 25 or higher in Japan but by 30 or higher by World Health Organization. In this study, the mean BMI excluding patients with ascites, edema or HCC was  $23.6 \pm 3.6$  kg/m<sup>2</sup> and the ratio of obese subjects with BMI of 25 or higher was 33.7% of

these patients (Fig. 2). The proportion of obese people in the general population of Japan at matched age was 30.5% in 2009.<sup>25</sup> Thus, an equal or greater proportion of patients with liver cirrhosis has obesity than the general population of Japan at present.

The increase in obesity, or excess energy nutrition status, and subsequent impaired glucose metabolism potentially bring about an unfavorable outcome in cirrhotic patients. Actually, excess energy nutrition contributed to induce carcinogenesis in liver cirrhosis,<sup>15,27,28</sup> and the number of obese subjects doubled in the candidates for liver transplantation in the previous 10 years in the USA.<sup>29–31</sup>

As to PEM exactly defined by serum albumin and npRQ, Tajika *et al.* reported that protein malnutrition was identified in 75%, energy malnutrition in 62% and PEM in 50% of 109 patients with liver cirrhosis in 1995.<sup>4</sup> In our study, 87 patients without HCC composed a group to show comparable backgrounds to those by Tajika *et al.*<sup>4</sup> Among them, 67% had protein malnutrition, 48% had energy malnutrition and 30% had PEM (Table 3). Taken together, the protein malnutrition remains almost similar in liver cirrhosis, but the patients with energy malnutrition, particularly PEM, substantially decreased.

The above-mentioned results urge that two concerns are addressed. The first is the effect of altered nutritional state of cirrhotics on their QOL, and the second is a question if exercise should be prescribed for obese cirrhotics. Regarding QOL, reduction in bodyweight achieved by chronic liver disease patients with obesity was associated with improved liver dysfunction, histology or QOL.<sup>32,33</sup>

In this study, basal QOL was estimated by the SF-8, and was significantly lower on all subscales than Japanese national standard values. However, no difference was observed by the presence or absence of HCC. In contrast, QOL of cirrhotic patients significantly correlated with the grade of disease severity as defined by the Child–Pugh classification (data not shown). It was thus suggested that the degree of the hepatic functional reserve contributed to a greater extent than the progression of cancer as for QOL of cirrhotic patients.

In conclusion, while PEM is still present in liver cirrhosis, a greater proportion shows obesity in Japanese patients at present. Because exacerbated inflammation, fibrosis and carcinogenesis has been reported in obese patients with liver cirrhosis, the present findings urge revision of nutritional and, possibly, establishment of exercise guidelines for obese patients with liver cirrhosis, in addition to the current PEM guidelines.



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**Short Communication**

# Combination of acyclic retinoid with branched-chain amino acids inhibits xenograft growth of human hepatoma cells in nude mice

Masahito Shimizu, Yohei Shirakami, Hiroyasu Sakai, Junpei Iwasa, Makoto Shiraki, Koji Takai, Takafumi Naiki and Hisataka Moriwaki

Department of Medicine, Gifu University Graduate School of Medicine, Gifu, Japan

**Aim:** Combination chemoprevention is a promising strategy to improve the prognosis of hepatocellular carcinoma (HCC). A malfunction of retinoid X receptor- $\alpha$  (RXR- $\alpha$ ) due to phosphorylation by Ras/mitogen-activated protein kinase is closely associated with liver carcinogenesis and acyclic retinoid (ACR) can prevent HCC development by inhibiting RXR- $\alpha$  phosphorylation. The present study examined the possible combined effects of ACR plus branched-chain amino acids (BCAA), which can also prevent the development of HCC in obese patients with liver cirrhosis, in human HCC xenografts in nude mice.

**Methods:** This study examined the effects of the combination of ACR plus BCAA on the growth of Huh7 human HCC xenografts in nude mice. The effects of the combination on the phosphorylation of RXR- $\alpha$ , extracellular signal-regulated kinase (ERK), Akt and insulin-like growth factor-1 receptor (IGF-1R) proteins, and on the expression levels of retinoic acid receptor- $\beta$  (RAR- $\beta$ ) and p21<sup>CIP1</sup> mRNA, were also examined by western blot and real-time reverse transcription polymerase chain reaction analyses, respectively.

**Results:** The combined treatment with ACR plus BCAA significantly inhibited the growth of Huh7 xenografts. The combination of these agents caused a marked inhibition of the phosphorylation of RXR- $\alpha$ , ERK, Akt and IGF-1R proteins in the xenografts. In addition, the expression levels of RAR- $\beta$  and p21<sup>CIP1</sup> mRNA significantly increased by these agents.

**Conclusion:** The combination of ACR and BCAA restores the function of RXR- $\alpha$  by inhibiting its phosphorylation and increasing the level of RAR- $\beta$ , a heterodimeric partner for RXR- $\alpha$ , and thus suppresses the growth of HCC xenografts. Therefore, this combination might be an effective regimen for the treatment and, probably, chemoprevention of HCC.

**Key words:** acyclic retinoid, branched-chain amino acids, hepatocellular carcinoma, phosphorylated retinoid X receptor- $\alpha$ , retinoic acid receptor- $\beta$

## INTRODUCTION

THE POOR PROGNOSIS for patients with hepatocellular carcinoma (HCC) has created an urgent need to develop more effective strategies for prevention of this malignancy. Retinoids, which have tumor-suppressive and chemopreventive properties in various organs, are considered to be promising agents for improving outcomes in individuals with HCC.<sup>1,2</sup> A clinical trial demonstrated that the administration of acyclic

retinoid (ACR), a synthetic retinoid that targets retinoid X receptor- $\alpha$  (RXR- $\alpha$ ), significantly reduced the incidence of post-therapeutic recurrence of HCC.<sup>3</sup> ACR inhibits growth in human HCC cells by inducing apoptosis and arrest of the cell cycle in G<sub>0</sub>–G<sub>1</sub>.<sup>4,5</sup> The inhibition of growth in cancer cells by ACR is also associated with induction of cellular levels of retinoic acid receptor- $\beta$  (RAR- $\beta$ ), an important retinoid receptor for regulation of apoptosis, and the inhibition of RXR- $\alpha$  phosphorylation.<sup>5–8</sup> The latter effect is more significant because the accumulation of phosphorylated (i.e. inactivated) RXR- $\alpha$  (p-RXR- $\alpha$ ) interferes with the function of normal RXR- $\alpha$  in a dominant-negative manner, and therefore plays a critical role in the development of HCC.<sup>2,9,10</sup>

In addition, ACR acts synergistically with various agents (e.g.  $\beta$ -interferon, OSI-461, trastuzumab, valproic

Correspondence: Dr Masahito Shimizu, Department of Medicine, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan. Email: shimim-gif@umin.ac.jp

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acid and vitamin K<sub>2</sub>) that target other signaling pathways in suppressing growth and inducing apoptosis in human HCC cells.<sup>5,11–14</sup> These findings have clinical significance for the treatment and chemoprevention of HCC because the combined use of two or more agents can diminish drug toxicity while exerting synergistic effects. Branched-chain amino acids (BCAA; leucine, isoleucine and valine), which improve protein malnutrition in patients with liver cirrhosis, are candidate partners in ACR-based combination chemoprevention because a recent clinical trial showed that oral supplementation with these agents reduced the risk of HCC in obese patients with chronic viral liver disease.<sup>15</sup> Treatment with BCAA also prevents the development of liver tumorigenesis in a rodent model, while also inhibiting the growth of HCC cells.<sup>16–18</sup> The purpose of this study is to investigate whether the combination of ACR plus BCAA significantly inhibits the growth of human HCC xenografts and to examine the possible mechanisms of this action.

## METHODS

### Materials

**A**N ACYCLIC RETINOID (peretinoin) was supplied by Kowa Pharmaceutical (Tokyo, Japan). BCAA was obtained from Ajinomoto (Tokyo, Japan). The BCAA composition (2:1:1.2 = leucine : isoleucine : valine) was set at the clinical dose used for the treatment of decompensated liver cirrhosis in Japan.<sup>15</sup>

### Experimental procedure

Thirty-two male BALB/c nude mice (5 weeks of age) were obtained from Charles River Japan (Tokyo, Japan). Xenograft tumors were made by the s.c. injection of Huh7 human HCC cells (Japanese Cancer Research Resources Bank, Tokyo, Japan) into the flanks of the mice at a concentration of  $5 \times 10^6$  cells per 200  $\mu$ L.<sup>19</sup> The mice were randomly divided into four groups (eight mice per group) 1 week after tumor cell injection. The mice in group 2 (ACR alone) were given the basal diet, CRF-1 (Oriental Yeast, Tokyo, Japan), containing 0.03% ACR with free access to feeding for 5 weeks. Group 3 (BCAA alone) was given the basal diet supplemented with 3.0% BCAA (w/w). The mice in group 4 (combination group) received a diet containing 0.03% ACR and 3.0% BCAA. Group 1 was given the basal diet and served as an untreated control. The tumor volume was calculated at the termination of the experiment using the formula: largest diameter  $\times$  (smaller diameter)<sup>2</sup>  $\times$  0.5.

### Protein extraction and western blot analysis

Total protein was extracted from the xenografts of Huh7 cells and equivalent amounts of protein (20 mg/lane) were examined by a western blot analysis.<sup>19</sup> The primary antibodies for RXR- $\alpha$  ( $\Delta$ N-197 and D-20), extracellular signal-regulated kinase (ERK), phosphorylated ERK (p-ERK), Akt, phosphorylated Akt (p-Akt), insulin-like growth factor-1 receptor (IGF-1R), phosphorylated IGF-1R (p-IGF-1R) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) have been previously described.<sup>5,10,19,20</sup> The  $\Delta$ N-197 antibody is regarded as a specific antibody for the phosphorylated form of RXR- $\alpha$  protein.<sup>8,10</sup> The intensities of the blots were quantified with NIH Image software ver. 1.62.

### RNA extraction and quantitative real-time reverse transcription polymerase chain reaction analysis

Total RNA was isolated from the xenografts of Huh7 cells using the RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX, USA). The cDNA was amplified from 0.2  $\mu$ g of total RNA using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The primers used for the amplification of RAR- $\beta$ , p21<sup>CIP1</sup> and GAPDH-specific genes have been previously described.<sup>6,19</sup> A quantitative real-time RT-PCR analysis was performed in a LightCycler (Roche Diagnostics, Mannheim, Germany) with SYBR Premix Ex Taq (TaKaRa Bio, Shiga, Japan).<sup>16</sup> The gene expression levels were normalized to the GAPDH expression levels using a standard curve.

### Statistical analysis

The data are expressed as the mean  $\pm$  standard deviation. Statistical significance of the difference in mean values was assessed by one-way ANOVA, followed by Scheffé's *t*-test.

## RESULTS

### Combined treatment with ACR plus BCAA significantly inhibits growth of HCC xenografts

**A**S SHOWN IN Figure 1, neither treatment with 0.03% ACR alone nor 3.0% BCAA alone inhibited the growth of Huh7 xenografts. These findings suggest that such doses of ACR and BCAA are insufficient to suppress the tumor growth of HCC in the present study, although similar concentrations of these agents have had a significant effect on preventing the development