upregulation of the expression of type-1 IFN receptor and Stat1 [48]. ACR also acts synergistically with vitamin K_2 (VK_2) in suppressing growth and inducing apoptosis in the human HCC cells [15]. These findings are significant when considering the clinical use of ACR because both IFN and VK_2 are expected to exert preventive effects on the development and recurrence of HCC [64-67]. Therefore, in the near future, "combination chemoprevention" using ACR as the key agent, such as ACR plus IFN or VK_2, may become one of the effective strategies to prevent the development of HCC. Synergistic growth inhibition caused by ACR plus VK_2 treatment is also observed in HL-60 human leukemia cells [68].

The expected mechanisms of how the combination of ACR and other specific agents synergistically exerts chemopreventive effects in HCC cells are schematically summarized in Fig. (**5**). Among these mechanisms, dephosphorylation of RXRα by targeting the Ras-MAPK signaling pathway or its upstream RTKs seems to be one of the most crucial approaches of ACR for inducing synergistic growth inhibition in HCC cells. Indeed, ACR and VK_2 cooperatively inhibit the activation of the Ras-MAPK signaling pathway, and thus, suppress the phosphorylation of RXRα and the growth of HCC cells [15]. Trastuzumab is a humanized monoclonal antibody against human epidermal growth factor receptor-2 (HER2), a member of the RTK family, and the combination of 9cRA [69] or ACR (unpublished data) and this antibody synergistically inhibits the activation of HER2, ERK, and Akt, subsequently dephosphorylating RXRα, and thus inhibiting growth and inducing apoptosis in the HCC cells. Combined treatment with ACR plus valproic acid, a histone deacetylase (HDAC) inhibitor, also acts synergistically to induce apoptosis and G_0–G_1 cell cycle arrest in the HCC cells by inhibiting phosphorylation of RXRα, ERK, Akt, and GSK-3β proteins [16]. In addition to HCC, the combination of ACR plus gemcitabine synergistically inhibits cell growth and induces apoptosis by inhibiting Ras activation in pancreatic cancer cells [62].

In addition to targeting RXRα phosphorylation, induction of nuclear receptors that dimerize with RXR, such as RAR and PPAR [39, 70], and recruitment of their ligands may also exert synergistic growth inhibition in cancer cells when combined with ACR. For instance, both valproic acid [16] and OSI-461 [50], a potent derivative of sulindac sulfone, enhance the ability of ACR to raise the cellular levels of RARβ, thereby markedly increasing the RARE and RXRE promoter activity and inducing apoptosis in the HCC cells. These findings suggest that, among the nuclear receptors, RARβ is considered the most preferable heterodimeric partner for RXRα in ACR-based combination chemoprevention.

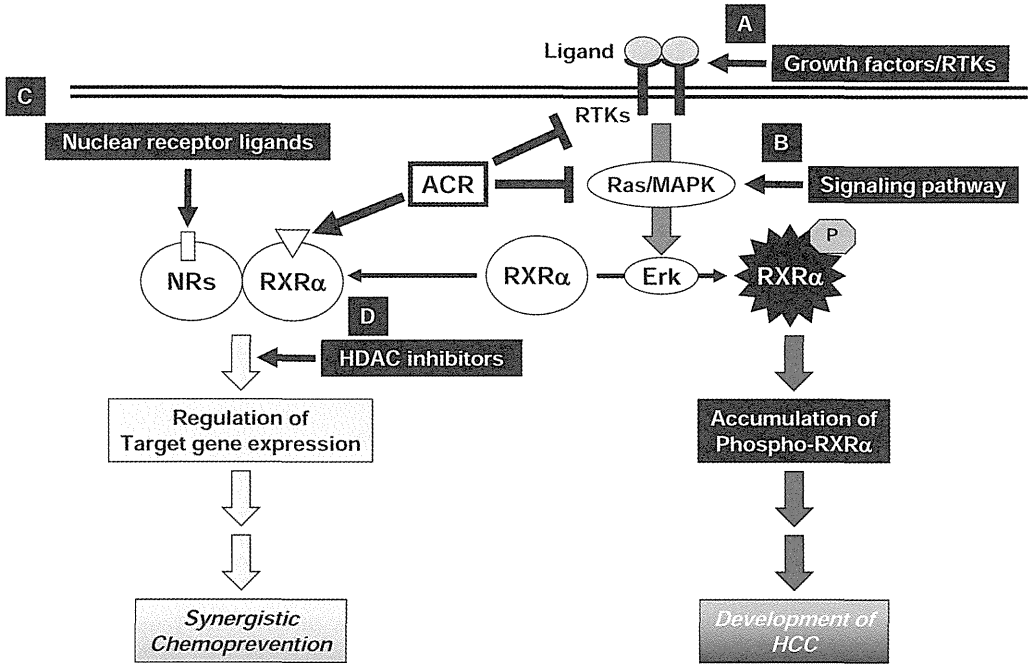

Fig. (5). "Combination chemoprevention" for HCC using ACR as the key agent. Dephosphorylation of RXRα and subsequent restoration of the function of this nuclear receptor are critical to prevent HCC development. Therefore, agents that target growth factors and their corresponding RTKs (**A**), as well as their related signaling pathways (**B**), including the Ras-MAPK that phosphorylates RXRα, may be good candidates to synergize with ACR for the chemoprevention of HCC. The ligands for the nuclear receptors (NRs) that heterodimerize with RXRs, such as RARs and PPARs (**C**), are also capable of enhancing the chemopreventive effect of ACR through the activation of target gene expression. HDAC inhibitors increase the expression of ACR-target genes by remodeling the chromatin template and increasing histone acetylation, suggesting that the combination of ACR and HDAC inhibitors may be a promising regimen for HCC chemoprevention (**D**).

FUTURE PROSPECTS OF ACR – 2: PREVENTION OF OBESITY-RELATED LIVER CARCINOGENESIS BY ACR

Recent studies have revealed that the risk of HCC is elevated in patients with metabolic syndrome, which is commonly associated with obesity and diabetes mellitus [20, 71, 72]. Several pathophysiological mechanisms linking obesity and liver carcinogenesis have been shown, including the emergence of insulin resistance and the subsequent inflammatory cascade [73]. Therefore, patients with obesity and insulin resistance comprise a high-risk group for HCC, and thus, are regarded as considerable targets for HCC chemoprevention. On the other hand, improving metabolic abnormalities such as insulin resistance and chronic inflammation by nutritional or pharmaceutical intervention might be an effective and attractive strategy to inhibit obesity-related liver carcinogenesis [74]. We have reported that the administration of branched chain amino acids (BCAA), which are used to improve protein malnutrition in patients with liver cirrhosis, and (-)-epigallocatechin-3-gallate, which is a major biologically active component of green tea, significantly prevents liver tumorigenesis in obese and diabetic *db/db* mice by targeting insulin resistance and improving chronic inflammation [75, 76]. A recent clinical trial also revealed that supplementation of food with BCAA reduced the risk of HCC in obese patients with chronic viral liver disease [72].

ACR effectively prevents the development of obesity-related liver carcinogenesis by inhibiting the activation of Ras and phosphorylation of ERK and RXRα in the liver of DEN-treated *db/db* mice. In this study, ACR administration also improved liver steatosis and insulin sensitivity, while attenuating the chronic inflammation induced by excessive fatty deposits [17]. Obesity and metabolic syndrome are the major healthcare problems in the present society, and the influences of metabolic abnormalities, in particular, such as promotion of cancers including HCC, are critical issues awaiting resolution. The results of this preclinical experiment [17] may encourage the clinical use of ACR for cirrhotic patients with obesity and diabetes, who are at a notably higher risk of developing HCC [20, 71, 72].

CONCLUSION

HCC prevention is an urgent issue demanding attention across the globe. One of the most practical approaches to prevent HCC is realization of the concept of "clonal deletion," and ACR is a promising candidate for this purpose because it may accomplish this concept. Experimental studies strongly suggest that RXRα phosphorylation is profoundly involved in liver carcinogenesis, and ACR mainly exerts chemopreventive effects on HCC *via* inhibition of RXRα phosphorylation. ACR-based combination chemoprevention, which is expected to exert synergism, holds a great possibility to perform a central role in HCC chemoprevention. Obese individuals, who are at an increased risk for HCC, might also be critical targets of ACR to prevent the development of this malignancy.

CONFLICT OF INTEREST

The authors declare that no conflicts of interest exist.

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ABBREVIATIONS

ACR	=	acyclic retinoid
AFP-L3	=	lectin-reactive α-fetoprotein factor 3
BCAA	=	branched chain amino acids
DEN	=	diethylnitrosamine
ERK	=	extracellular signal-regulated kinase
GSK-3β	=	glycogen synthase kinase-3β
HBV	=	hepatitis B virus
HCC	=	hepatocellular carcinoma
HCV	=	hepatitis C virus
HDAC	=	histone deacetylase
HER2	=	human epidermal growth factor receptor-2
IFN	=	interferon
MAPK	=	mitogen-activated protein kinase
PIVKA-II	=	protein induced by vitamin K absence or antagonist-II
PPAR	=	peroxisome proliferator-activated receptor
RA	=	retinoic acid
RAR	=	retinoic acid receptor
RARE	=	retinoic acid receptor responsive element
RTK	=	receptor tyrosine kinase
RXR	=	retinoid X receptor
RXRE	=	retinoid X response element
VK_2	=	vitamin K_2

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Review Article

Acyclic retinoid in chemoprevention of hepatocellular carcinoma: Targeting phosphorylated retinoid X receptor-α for prevention of liver carcinogenesis

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Abstract

One of the key features of hepatocellular carcinoma (HCC) is the high rate of intrahepatic recurrence that correlates with poor prognosis. Therefore, in order to improve the clinical outcome for patients with HCC, development of a chemopreventive agent that can decrease or delay the incidence of recurrence is a critical issue for urgent investigation. Acyclic retinoid (ACR), a synthetic retinoid, successfully improves HCC patient survival by preventing recurrence and the formation of secondary tumors. A malfunction of the retinoid X receptor-α (RXRα) due to phosphorylation by the Ras-MAPK signaling pathway plays a critical role in liver carcinogenesis, and ACR exerts chemopreventive effects on HCC development by inhibiting RXRα phosphorylation. Here, we review the relationship between retinoid signaling abnormalities and liver disease, the mechanisms of how RXRα phosphorylation contributes to liver carcinogenesis, and the detailed effects of ACR on preventing HCC development, especially based on the results of our basic and clinical research. We also outline the concept of "clonal deletion and inhibition" therapy, which is defined as the removal and inhibition of latent malignant clones from the liver before they expand into clinically detectable HCC, because ACR prevents the development of HCC by implementing this concept. Looking toward the future, we discuss "combination chemoprevention" using ACR as a key drug since it can generate a synergistic effect, and may thus be an effective new strategy for the prevention of HCC.

Keywords: Acyclic retinoid, chemoprevention, clonal deletion and inhibition, combination therapy, HCC, RXRα phosphorylation

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequently occurring cancers in the world. The number of new cases per year is estimated to be 750,000; approximately the same number (700,000) of people die from this malignancy each year.[1,2] Chronic inflammation and subsequent cirrhosis of the liver, most cases of which are induced by persistent infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), contribute to the development of HCC.[2,3] In order to improve the prognosis of HCC, several effective strategies to prevent the development of primary HCC and intrahepatic recurrence of this malignancy have been demonstrated in clinical trials. A meta-analysis reported that antiviral treatment reduces the risk of HBV-related HCC recurrence and decreases liver-related mortality as well as overall mortality.[4]

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For patients with HCV-related HCC, the effectiveness of interferon therapy for preventing the recurrence of HCC has been shown by meta-analyses.[5,6]

In addition to studies of antiviral treatment, other important trials using specific agents have been conducted to seek ways for preventing HCC development. For instance, supplementation with branched-chain amino acids reduces the risk of HCC in cirrhotic patients who are obese.[7] We reported the results of our prospective randomized study,[8-10] in which the oral administration of acyclic retinoid (ACR), a synthetic retinoid, inhibited the development of a second primary HCC and thus improved patient survival. The pleiotropic effects of ACR in the prevention of HCC and suppression of cancer cell growth have also been revealed in many experimental studies.[11-13] The aim of this article is to review the detailed effects of ACR in preventing the development of HCC, based on our clinical and basic research. In particular, we focus on the effects of ACR in targeting the phosphorylation of retinoid X receptor-α (RXRα), because malfunction of this nuclear receptor due to aberrant phosphorylation is closely involved in liver carcinogenesis.[14] We also review the concept of "clonal deletion and inhibition" therapy, a practical approach to preventing HCC development. Finally, we discuss the possibility of "combination chemoprevention," which uses ACR as a key drug and is expected to be an effective strategy that takes advantage of pharmacologic synergism to prevent the formation of HCC.

Retinoids and their receptors

Retinoids, a group of structural and functional derivatives of vitamin A, have fundamental effects on cellular activities, including growth, differentiation, and apoptosis, as well as on morphology.[15-17] Retinoids exert their biologic functions primarily by regulating gene expression through two distinct nuclear receptors, the retinoic acid receptors (RARs) and RXRs, which are ligand-dependent transcription factors. Both RARs and RXRs are composed of three subtypes (α, β, and γ), which are characterized by a modular domain structure.[15-17] RARs can be activated by both all-*trans*-retinoic acid and 9-*cis*-retinoic acid with similar affinities, whereas RXRs are exclusively activated by 9-*cis*-retinoic acid.

After ligand binding, RXRs form homodimers as well as heterodimers with RARs, which interact with the retinoid X response element (RXRE) or the retinoic acid receptor responsive element (RARE), located in the promoter region of target genes, thereby modulating gene expression.[15-17] In addition to forming a heterodimer with RARs, RXRs can also form heterodimers with several other nuclear receptors, indicating that RXRs act as common heterodimerization partners for various types of nuclear receptors.[16] For instance, RXRs interact with peroxisome proliferator-activated receptors (PPARs), which are receptors for fatty acids, thus regulating PPAR-mediated pathways and controlling energy homeostasis.[18] Therefore, RXRs function as auxiliary factors, determining the effects of other hormones, and acting as master regulators of nuclear receptors.[16]

Retinoid abnormalities and liver disease

The liver is one of the most important target organs for retinoid actions. Hepatocytes play central roles in the uptake and processing of dietary retinol in the liver and secrete retinol-binding protein into the plasma. Hepatic stellate cells (HSCs) are critically involved in the storage of retinoids in the liver, suggesting that the development of hepatic disease is highly correlated with impaired hepatic retinoid metabolism and storage.[19,20] In alcoholic patients, diminished hepatic retinoid storage is associated with progressively worsening stages of hepatic disease.[21] The loss of hepatic retinyl ester stores from the lipid droplets within HSCs leads to HSC activation and the development of liver fibrosis.[22,23] A progressive decrease in serum retinol levels has also been observed in patients diagnosed with liver cirrhosis compared with healthy subjects, and those patients with both cirrhosis and HCC had significantly lower levels than patients with cirrhosis alone.[24,25]

Several experimental studies using genetically engineered mice have revealed the pivotal effects of retinoids on fat metabolism in the liver. Yanagitani *et al.*[26] reported that RARα dominant-negative form transgenic mice developed steatohepatitis through the downregulation of hepatic mitochondrial β-oxidation activity of fatty acids. Studies in hepatocyte RXRα-deficient mice also demonstrated that RXRα plays vital roles in fatty acid and cholesterol metabolism in the liver.[27,28] These observations suggest that retinoids and their receptors are involved in the mediation of normal hepatic lipid metabolism.

Retinoid abnormalities and liver carcinogenesis

As previously noted, retinoids and their receptors, especially RXRs, play an essential role in controlling normal cell proliferation, differentiation, metabolism, and death (regulation of apoptosis).[15-17] On the other hand, these facts also suggest that the loss of retinoid activity or responsiveness is linked to deviation from normal cell proliferation and death, which are key factors for cancer development.[29,30] Indeed, it is well established that abnormalities in retinoid signaling are prominently involved in carcinogenesis in several organs, including the liver. In a rodent model of liver carcinogenesis,[31] retinol was observed to be locally deficient

in HCC lesions, but not in the adjacent normal liver tissues. Functional loss of retinoic acid leads to the occurrence of cellular dysplasia and cancer in the liver.[26] Overexpression of cellular retinoic acid-binding protein-II, which shows a high affinity for all-*trans*-retinoic acid, is associated with induction of retinoic acid resistance in HCC cells.[32] On the other hand, lecithin:retinol acyltransferase knockout mice, which possess increased retinoid signaling in the liver, are less susceptible to diethylnitrosamine (DEN)-induced hepatocarcinogenesis.[33] These findings suggest that supplementation with additional retinoids and improvement of retinoid signaling may be a promising strategy for the prevention and/or treatment of HCC.

In addition to retinoid depression, abnormalities in retinoid receptors are also associated with liver carcinogenesis and the growth of HCC cells. The *RARβ* gene, a tumor suppressor gene, is an HBV integration site, and the expression of this gene is markedly decreased in human HCC.[34,35] RARβ expression is also suppressed in liver cancer cell lines.[36,37] We have previously reported that the levels of RARβ protein and mRNA were decreased in HCC lesions in a rat model of chemically induced liver carcinogenesis.[38] On the other hand, RARγ is overexpressed in HCC tissues and cells, which is associated with the growth of HCC cells.[39] It has been reported that RARγ often resides in the cytoplasm of HCC cells and interacts with the p85α regulatory subunit of phosphatidylinositol 3-kinase, resulting in the activation of Akt and nuclear factor-κB, which are critical regulators of the growth and survival of cancer cells.[39,40] These reports support an oncogenic potential for RARγ in liver carcinogenesis.

RXRα phosphorylation and HCC

Among retinoid receptors, RXRα is most abundant in the liver; therefore, its alterations are particularly implicated in the development and progression of HCC. RXRα is reported to bind to the enhancer element of HBV and modulate viral replication.[41] The expression of RXRα is decreased not only in HCC and liver cell adenoma, but also in glutathione *S*-transferase placental form-positive foci, a precancerous HCC lesion, as seen in a rat model of chemically induced liver carcinogenesis.[38] These findings indicate that repression of RXRα occurs even in the early stage of liver carcinogenesis.

Moreover, we have shown that aberrant phosphorylation of RXRα is critically involved in liver carcinogenesis. Initially, we revealed that the RXRα protein is anomalously phosphorylated at the serine and threonine residues and that it was seen to accumulate in both surgically resected human HCC tissues and HCC cell lines, whereas in normal hepatocytes, RXRα is unphosphorylated and is broken into smaller peptides.[14,42] We previously reported that the phosphorylated form of RXRα protein was present in higher concentrations in HCC tissues than in noncancerous surrounding and normal liver tissues among all 10 cases examined.[14] Activated extracellular signal-regulated kinase (ERK) is highly expressed in HCC cells, and constitutive phosphorylation at the serine at position 260 of RXRα, a mitogen-activated protein kinase (MAPK)-ERK consensus site, is closely associated with its retarded degradation, low transcriptional activity, and promotion of cancer cell growth. In turn, the abrogation of phosphorylation by an MAPK inhibitor or transfection with unphosphomimic mutant RXRα restores the degradation of RXRα in a ligand-dependent manner.[14,43] Phosphorylated RXRα abolishes its ability to form heterodimers with RARβ, and this is associated with uncontrolled cell growth and resistance to retinoids.[44] Moreover, phosphorylated RXRα is resistant to proteolytic degradation via the ubiquitination/proteasome-mediated pathway in human HCC cells, resulting in an accumulation of this phosphorylated protein within the HCC tissues.[45] Therefore, in HCC tissues and cells, the accumulation of phosphorylated RXRα, which is regarded as the nonfunctional form of RXRα, may interfere with the function of normal (unphosphorylated) RXRα in a dominant-negative manner. Our observations suggest that not only retinoid depletion, but also malfunction of retinoid receptors, especially phosphorylation of RXRα, may play a critical role in HCC development [Figure 1].

Mechanisms of ACR in HCC chemoprevention

ACR, which is the same substance as NIK-333 and Peretinoin (Kowa Pharmaceutical Co., Tokyo, Japan), inhibits both chemically induced liver carcinogenesis in rats and mice and spontaneously occurring HCC in mice.[31,46-49] ACR was initially developed as an agonist for both RXR and RAR.[50,51] Therefore, this agent activates the promoter activity of RXRE and RARE and controls the expression of target genes, including RARβ, $p21^{CIP1}$, and cyclin D1, which results in induction of apoptosis, cell cycle arrest in G_0–G_1, and growth inhibition in human HCC-derived cells.[43,52-62] These findings suggest that ACR suppresses HCC, at least in part, by working as a ligand for retinoid receptors and controlling their target genes, especially RARβ and $p21^{CIP1}$.

On the other hand, many experimental studies have shown that ACR exerts chemopreventive effects in HCC cells by inhibiting RXRα phosphorylation. In human HCC cells, ACR restores RXRα function by inactivating the Ras–MAPK signaling system, leading to dephosphorylation of RXRα.[43] ACR also suppresses cancer cell growth by inhibiting the activation and expression of several

types of growth factors and their corresponding receptor tyrosine kinases (RTKs).[47,48,56,63-65] These findings are significant because RTKs play a role in the activation of Ras–MAPK signaling and the subsequent phosphorylation of RXRα, which may contribute to liver carcinogenesis. In addition, we have recently reported that ACR inhibits the activation of Ras and the phosphorylation of ERK and RXRα proteins in the liver of DEN-treated obese mice.[49] The inhibitory effects of ACR on Ras activation are also observed in human HCC and pancreatic cancer cells.[52,66] These findings may indicate that activation of the RTK–Ras–MAPK signaling pathway and subsequent RXRα phosphorylation are critical targets of ACR for the inhibition of HCC development and cancer cell growth [Figure 1].

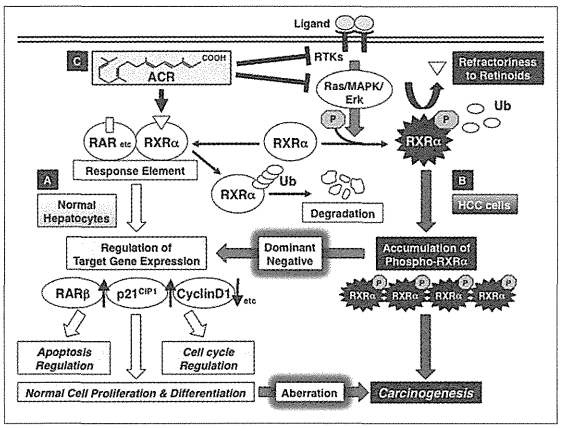

Figure 1: Retinoid refractoriness due to phosphorylation of retinoid X receptor alpha (RXRα), and its restoration by acyclic retinoid (ACR) in liver carcinogenesis. (a) In normal hepatocytes, when ACR binds to and activates RXRα, it forms homo- and/or heterodimers with other nuclear receptors, including retinoic acid receptors (RARs). This results in expression of the target genes, such as RARβ, $p21^{CIP1}$, and cyclin D1, which regulate normal cell proliferation and differentiation, as well as controlling the induction of apoptosis and cell cycle progression. Thereafter, RXRα is rapidly ubiquitinated (Ub) and degraded via the proteasome pathway. (b) In hepatocellular carcinoma (HCC) cells, the Ras–mitogen-activated protein kinase (MAPK) pathway is highly activated and phosphorylates RXRα at serine residues, impairing dimer formation and the subsequent transactivation functions of the receptor (refractoriness to retinoids). Furthermore, nonfunctional phosphorylated RXRα is sequestered from ubiquitin/proteasome-mediated degradation and accumulates in liver cells. This interferes with the physiologic function of the remaining unphosphorylated (ie, functional) RXRα in a dominant-negative manner, causing a deviation from normal cell proliferation and differentiation, thereby playing a critical role in liver carcinogenesis. (c) ACR is not only a ligand for RXRα, but also a suppressor of the Ras–MAPK signaling pathway; it inhibits RXRα phosphorylation, thereby restoring the function of the receptor and activating the transcriptional activity of the responsive element. ACR also inhibits, directly or indirectly, the ligand (growth factor)-dependent RTK activities, which contribute to the inhibition of ERK and RXRα phosphorylation and suppression of growth in HCC cells

Chemoprevention of HCC by ACR

In order to test whether ACR can reduce the incidence of recurrent and second primary HCC, an early phase randomized controlled clinical trial was conducted in patients who underwent potentially curative treatment for initial HCC.[8-10] In this trial, treatment with ACR (administered to 44 patients, 600 mg/day) for 12 months significantly reduced the incidence of recurrent or new HCCs compared with placebo (administered to 45 patients) after a median follow-up period of 38 months; 12 patients (27%) in the ACR group developed HCC compared with 22 patients (49%) in the placebo group (P = 0.04).[8] After further follow-up to 62 months, ACR was also found to improve both recurrence-free survival (P = 0.002) and overall survival (P = 0.04). The estimated 6-year overall survival was 74% in the ACR group and 46% in the placebo group.[9] The relative risks for the development of secondary HCC and death were 0.31 [95% confidence interval (CI), 0.12 – 0.78] and 0.33 (95% CI, 0.11–0.79), respectively.[8,9] Moreover, the preventive effects of ACR lasted up to 50 months after randomization, or 38 months after completion of the drug.[10] The results of these reports suggest that administration of ACR for only 12 months exerts a long-term effect on the prevention of second primary HCC without causing severe adverse effects from retinoid.

The preventive effect of ACR on the development of second primary HCC in HCV-positive patients, who underwent curative therapy for initial HCC or first recurrence, has also been confirmed by a multicenter large-scale (n = 401) randomized placebo-controlled trial with a median follow-up of 2.5 years. In this trial, oral administration of ACR (600 mg/day) exerted a strong effect on the prevention of second primary HCC with a hazard ratio of 0.27 (95% CI, 0.07–0.96) at 2 years after treatment. Cumulative recurrence-free survival rates in the ACR-treated group were higher than those in the placebo group (after the first year: ACR, 71.9%; placebo, 66.0% and at 3 years: ACR, 43.7%; placebo, 29.3%), indicating that ACR reduced the recurrence of HCC, especially after 2 years of treatment.[67] In addition, subgroup analysis of this study showed the significant result that ACR powerfully prevented the development of a second primary HCC, with a hazard ratio of 0.38 (95% CI, 0.20–0.71) in patients who were Child–Pugh A and had small tumors (size < 20 mm).[68] The results of these clinical trials suggest that ACR inhibits *de novo* carcinogenesis and is therefore a novel first-line therapy to reduce the development of second primary HCC, especially for patients with well-preserved liver function who have undergone curative therapy for a small tumor. Common treatment-related adverse events observed in this trial were albuminuria, hypertension, and headache; however, these adverse events were tolerated. The safety of ACR was also

determined in a phase I pharmacokinetics clinical trial. In that trial, doses of 300 and 600 mg/day did not result in any adverse effects or dose-limiting toxicities, whereas a dose of 900 mg/day resulted in grade 3 hypertension as a dose-limiting toxicity.[69]

Concept of "clonal deletion and inhibition" therapy

The annual incidence of HCC reaches approximately 3% in HBV- and 7% in HCV-infected cirrhotic patients. More serious is that the frequency of HCC recurrence after curative treatment is very high in cirrhotic patients; the annual incidence rises to approximately 20%–25% and the recurrence rate at 5 years after definitive therapy exceeds 70%.[70-73] This typical clinical course of patients with HCC is associated with the clinical characteristic mode of liver carcinogenesis, multicentric carcinogenesis, which is also expressed by the term "field cancerization." Once a liver is exposed to a continuous carcinogenic insult, such as hepatitis virus infection, the whole liver is regarded as a precancerous field that possesses multiple as well as independent premalignant or latent malignant clones. Based on this characteristic, a curative treatment for HCC is difficult once this malignancy has developed; therefore, we believe that one of the most promising and practical strategies for HCC treatment is the removal and inhibition of latent malignant clones from the chronically damaged liver that is in a hypercarcinogenic state before the latent malignant clones expand into a clinically detectable tumor. We have proposed this new concept of "clonal deletion and inhibition" therapy for HCC chemoprevention.[74] We believe that ACR prevents the development of HCC through implementation of this concept for the following reasons.

First, the serum levels of lectin-reactive a-fetoprotein factor 3 (AFP-L3) and protein induced by vitamin K absence or antagonist-II (PIVKA-II), both of which indicate the presence of latent (ie, invisible) malignant clones in the remnant liver, were significantly reduced after ACR administration for 12 months in an early-phase clinical trial.[8-10] Next, ACR was found to prevent the appearance of serum AFP-L3 in patients whose AFP-L3 levels were negative at trial enrolment, whereas the number of patients whose serum AFP-L3 appeared *de novo* was significantly increased in the placebo group, and these patients had a significantly higher risk of developing secondary HCC.[74] These observations are explained by the implementation of "clonal deletion and inhibition" therapy with ACR; namely, ACR eliminates the AFP-L3- and PIVKA-II-producing premalignant clones from the remnant liver before they expand into clinically detectable tumors ("clonal deletion"). At the same time, ACR inhibits the development of such clones, which have the potential to become HCC, in the liver ("clonal inhibition").

Once the malignant clones are eliminated or inhibited from the remnant liver by ACR, it takes several years for *de novo* HCC to develop in the cirrhotic liver. Therefore, as demonstrated in an early-phase clinical trial,[10] short-term administration (12 months) of ACR could exert a long-term (ie, several years) preventive effect on HCC development even after termination of treatment. The roles of ACR in the implementation of "clonal deletion and inhibition" therapy are schematically represented in Figure 2.

"Combination chemoprevention" of HCC with ACR

In order to establish more effective strategies to prevent HCC development, we have conducted a study of "combination chemoprevention" using ACR as a key agent. Combination chemoprevention with ACR provides an opportunity to take advantage of the synergistic effects of ACR on growth inhibition in HCC cells. We have initially found that the combination of ACR and interferon-β synergistically inhibits cell growth and induces apoptosis in HCC cells.[58] This finding is significant when considering the clinical use of ACR in the near future because interferon exerts a chemopreventive effect against the recurrence of HCC.[5,6]

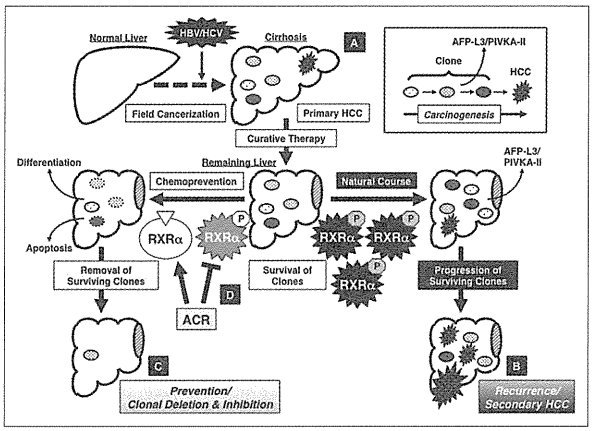

Figure 2: Concept of "clonal deletion and inhibition" therapy for hepatocellular carcinoma (HCC) chemoprevention and the effects of acyclic retinoid (ACR) on implementation of this concept. (a) Persistent inflammation caused by hepatitis viral infection transforms the liver into a precancerous field ("field cancerization"), which contains of multiple latent malignant clones that can, at some point, develop into HCC. (b) Even after early detection and removal of the primary HCC, the remaining clones survive in the remaining liver and grow into secondary HCC lesions (natural course), which is a major cause of the poor prognosis for patients with this malignancy. (c) Therefore, one of the most promising strategies to prevent secondary HCC is the deletion and inhibition of such transformed clones by inducing cell differentiation or apoptosis before the clones expand into clinically detectable tumors. This is the concept of "clonal deletion and inhibition" therapy for HCC chemoprevention. (d) ACR, which binds to RXRα and inhibits phosphorylation of this nuclear receptor, prevents the recurrence and development of secondary HCC via the mechanism described by this concept

In addition to interferon, other agents, particularly those that target RXRα phosphorylation, are also anticipated to be potential partners of ACR for inducing synergistic growth inhibition in HCC cells. For instance, ACR acts synergistically with vitamin K_2 in suppressing growth and inducing apoptosis in human HCC cells by inhibiting Ras–MAPK signaling activation and RXRα phosphorylation.[52] Dephosphorylation of RXRα by targeting the Ras–MAPK signaling pathway and its upstream human epidermal growth factor receptor-2 (HER2) using trastuzumab, a humanized monoclonal antibody against HER2, also enhances the effect of retinoids, including ACR, on inhibiting growth and inducing apoptosis in human HCC cells.[75] Combined treatment with ACR plus valproic acid, a histone deacetylase inhibitor, also acts synergistically to induce apoptosis and G_0–G_1 cell cycle arrest in HCC cells by inhibiting phosphorylation of RXRα, ERK, Akt, and glycogen synthase kinase-3β proteins.[53] In addition to HCC, in both human pancreatic cancer and leukemia cells,[66,76] the combination of ACR plus gemcitabine or vitamin K_2 synergistically inhibits cell growth and induces apoptosis by inhibiting Ras activation and RXRα phosphorylation. Moreover, induction of nuclear receptors that dimerize with RXR, such as RAR and PPAR,[77,78] and recruitment of their ligands also exert synergistic growth inhibition in cancer cells when combined with ACR.[53,60] In particular, upregulation of cellular levels of RARβ and the subsequent increase of the RARE promoter activity are critical to enhance the ability of ACR to induce apoptosis in the HCC cells.[53,60]

CONCLUSION

Finally, we should mention the results of our recent rodent experiment, which showed that ACR has the potential to inhibit obesity-related HCC.[49] In this study, ACR was found to effectively prevent the development of obesity-related liver carcinogenesis by inhibiting the activation of Ras and the phosphorylation of ERK and RXRα in the liver of DEN-treated *db/db* obese and diabetic mice.[49] This finding is significant because obesity and diabetes mellitus, both of which are major health care problems in the current society, are critical risk factors for HCC development.[2,79] Therefore, the results of this study may encourage the clinical use of ACR for cirrhotic patients with obesity and diabetes, who are at a notably higher risk for developing HCC.

Retinoids have been used as potential chemotherapeutic or chemopreventive agents because of their differentiation, antiproliferative, and proapoptotic properties.[29,30] For instance, all-*trans*-retinoic acid is an effective first-line therapy for the treatment of acute promyelocytic leukemia.[80] We expect that ACR will also become an effective first-line therapy for the prevention of HCC in the near future.

In conclusion, in order to improve the therapeutic outcome for patients with HCC, there is an urgent need to develop more effective strategies for chemoprevention of this malignancy. Realization of the concept of "clonal deletion and inhibition" therapy is one of the most promising and practical approaches for preventing HCC, and the use of ACR is expected to accomplish this goal. Liver carcinogenesis is accompanied by phosphorylation of RXRα, which is a critical target on which ACR can exert its chemopreventive effects against HCC. ACR-based combination chemoprevention, which is based on synergism, also holds great potential for becoming an important strategy for HCC chemoprevention. The clinical application of ACR as an "HCC chemopreventive drug" in patients with liver cirrhosis is awaited with great anticipation.

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Review

Nutraceutical Approach for Preventing Obesity-Related Colorectal and Liver Carcinogenesis

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Abstract: Obesity and its related metabolic abnormalities, including insulin resistance, alterations in the insulin-like growth factor-1 (IGF-1)/IGF-1 receptor (IGF-1R) axis, and the state of chronic inflammation, increase the risk of colorectal cancer (CRC) and hepatocellular carcinoma (HCC). However, these findings also indicate that the metabolic disorders caused by obesity might be effective targets to prevent the development of CRC and HCC in obese individuals. Green tea catechins (GTCs) possess anticancer and chemopreventive properties against cancer in various organs, including the colorectum and liver. GTCs have also been known to exert anti-obesity, antidiabetic, and anti-inflammatory effects, indicating that GTCs might be useful for the prevention of obesity-associated colorectal and liver carcinogenesis. Further, branched-chain amino acids (BCAA), which improve protein malnutrition and prevent progressive hepatic failure in patients with chronic liver diseases, might be also effective for the suppression of obesity-related carcinogenesis because oral supplementation with BCAA reduces the risk of HCC in obese cirrhotic patients. BCAA shows these beneficial effects because they can improve insulin resistance. Here, we review the detailed relationship between metabolic abnormalities and the development of CRC and HCC. We also review evidence, especially that based on our basic and clinical research using GTCs and BCAA, which indicates that targeting metabolic abnormalities by either pharmaceutical or nutritional intervention may be an effective strategy to prevent the development of CRC and HCC in obese individuals.

Keywords: obesity; colorectal cancer; hepatocellular carcinoma; chemoprevention; green tea catechins; branched-chain amino acids

1. Introduction

Obesity, which is the result of a positive energy balance, is a serious health problem throughout the world. The World Health Organization (WHO) estimates that currently, more than 1.5 billion adults worldwide are overweight, of which at least 500 million are obese [1]. Obesity is linked to several health disorders such as cardiovascular disease, hypertension, diabetes mellitus, and hyperlipidemia, which are collectively known as "metabolic syndrome". In addition, mounting evidence indicates that obesity and its related metabolic abnormalities, especially diabetes mellitus, are associated with the development of certain types of human epithelial malignancies, including colorectal cancer (CRC) and hepatocellular carcinoma (HCC) [2–8]. On the basis of systematic reviews of epidemiological evidence as well as mechanistic interpretations and data from animal experimental models, the World Cancer Research Fund and American Institute for Cancer Research released a report in 2007 on the causal relationship between high body fatness and an increased risk of CRC [9]. A large-scale meta-analysis (221 datasets on 282,000 incidence cases) also revealed that the magnitude of risk for CRC was greater among obese men than non-obese men [10]. In a prospectively studied population of more than 900,000 American adults, the body mass index (BMI) was found to be significantly associated with higher rates of death from cancer, especially HCC, because the relative risk of death from HCC was significantly higher (4.52 times) among men with a BMI of at least 35.0 than those who had normal weight (95% confidence interval, 2.94–6.94) [11].

Several pathophysiological mechanisms that link obesity and colorectal and liver carcinogenesis have been shown, including the emergence of insulin resistance, alterations in the insulin-like growth factor-1 (IGF-1)/IGF-1 receptor (IGF-1R) axis, the state of chronic inflammation, induction of oxidative stress, and occurrence of adipocytokine imbalance [2–6]. On the other hand, these findings also suggest that targeting these pathophysiological disorders via nutritional or pharmaceutical intervention might be an effective and promising strategy to inhibit obesity-related carcinogenesis. For instance, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor pitavastatin, which is widely used to treat hyperlipidemia, prevents obesity-related colorectal and liver carcinogenesis by attenuating chronic inflammation [12,13]. Captopril and telmisartan, which are anti-hypertensive drugs, also suppress the development of colonic preneoplastic lesions in obese and diabetic mice, and this suppression is associated with the reduction of oxidative stress and chronic inflammation [14].

In recent years, green tea catechins (GTCs) have received considerable attention because of their beneficial effects: they improve metabolic abnormalities and prevent cancer development [15–19]. Dietary supplementation with branched-chain amino acids (BCAA; leucine, isoleucine, and valine), which can prevent progressive hepatic failure in patients with chronic liver disease by improving insulin resistance [20–22], also reduces the risk of HCC in such patients who are obese [8]. In this article, we review the many mechanisms by which obesity and the related metabolic abnormalities influence the development of CRC and HCC while especially focusing on the emergence of insulin

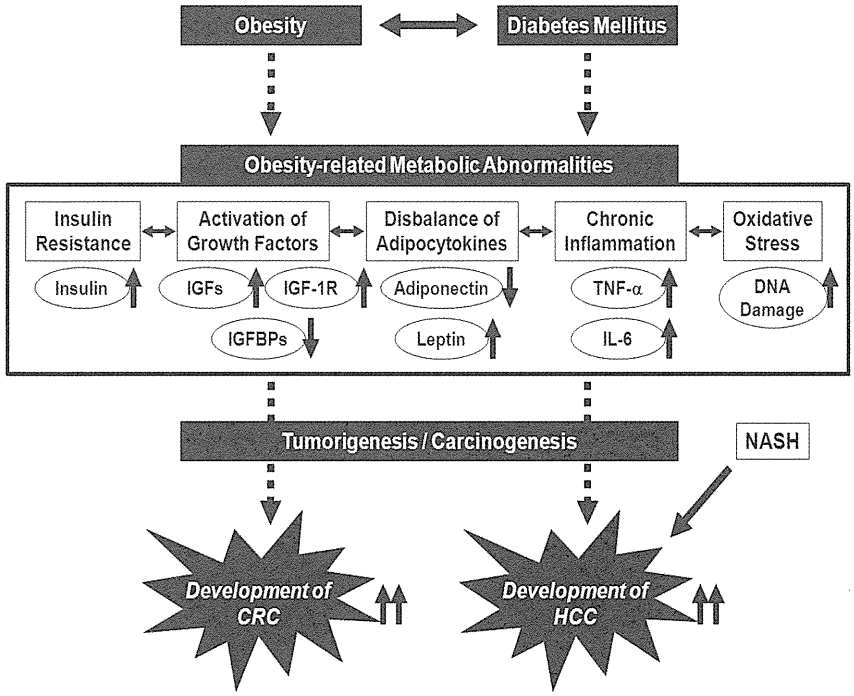
resistance and the subsequent inflammatory cascade. We also prove that the nutraceutical approach using GTCs and BCAA might be effective in preventing obesity-related carcinogenesis in both the colorectum and liver.

2. Potential Pathophysiological Mechanisms Linking Obesity and the Development of CRC

Obesity is the main determinant of insulin resistance and hyperinsulinemia, which is a risk factor for CRC [23]. Insulin itself and the signal transduction network it regulates have important roles in oncogenesis [24,25]. In animal models, insulin stimulates the growth of CRC cells while also promoting CRC tumor growth [26,27]. In addition, insulin resistance increases the biological activity of IGF-1, an important endocrine and paracrine regulator of tissue growth and metabolism. The binding of insulin and IGF-1 to the cell-surface receptors, insulin receptor and IGF-1R, respectively, on tumors and precancerous cells activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which is responsible for cellular processes like growth, proliferation, and survival [24,25]. Alterations in the IGF/IGF-1R axis caused by insulin resistance contribute to the development of CRC [28]. IGF-1 is positively correlated with body fat and waist circumference [29]. Moreover, insulin resistance and increased adipose mass create an oxidative environment in the tissues that upregulates the expression of various pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which stimulate tumor growth and progression [30–34]. Increased oxidative stress promotes damage to cell structures, including DNA, and activates the PI3K/Akt pathway, and both these processes play a key role in cancer development [35,36]. Therefore, insulin resistance and the subsequent inflammatory cascade involving increased oxidative stress are regarded as important factors in the development of obesity-associated CRC.

Excess production of storage lipids causes an adipocytokine imbalance, which entails increased levels of leptin and decreased levels of adiponectin in the serum, for example. This imbalance may also be related to obesity-associated carcinogenesis [37,38]. Leptin stimulates cell growth in CRC [39]. An epidemiologic study by Stattin *et al.* [40] suggested an association between circulating leptin levels and the development of CRC. TNF-α and IL-6 increase the levels of leptin, while leptin influences inflammatory responses, possibly by triggering the release of TNF-α and IL-6 [41–43]. These findings suggest that the pathophysiological abnormalities caused by obesity cooperatively aggravate the risk of cancers, including CRC, in obese individuals (Figure 1).

Figure 1. Proposed mechanisms linking obesity and its related metabolic abnormalities to the development of colorectal cancer (CRC) and hepatocellular carcinoma (HCC).



3. Potential Pathophysiological Mechanisms Linking Obesity, Non-Alcoholic Fatty Liver Disease/Non-Alcoholic Steatohepatitis, and the Development of HCC

Several pathophysiological mechanisms linking obesity, steatosis, and liver carcinogenesis have been shown, including insulin resistance and the subsequent inflammatory cascade. Insulin induces HCC cells to proliferate and resist apoptosis [44,45]. Insulin resistance raises the risk for recurrence of HCC after curative radiofrequency ablation in hepatitis C virus-positive patients [46]. Insulin resistance also leads to an increased expression of TNF-α and its dysregulation is associated with the development of steatosis and inflammation within the liver [47]. Activation of the IGF/IGF-1R axis is involved with liver carcinogenesis [48,49]. High levels of serum leptin, which stimulates the growth of HCC cells [50], increase the risk of HCC recurrence after curative treatment [51]. These findings suggest that in addition to colorectal carcinogenesis, obesity and its related metabolic abnormalities also play an important role in the development of HCC (Figure 1).

Non-alcoholic fatty liver disease (NAFLD), which is known to be a hepatic manifestation of metabolic syndrome, is the most common form of chronic liver disease in developed countries [52,53]. It covers a spectrum of disorders ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), which can progress to cirrhosis and thus HCC (Figure 1) [52,53]. Retrospective data suggest that in as many as 4–27% of cases, NASH progresses to HCC after cirrhosis develops [53,54]. Insulin resistance is considered a critical factor in the etiology of NASH [55]. The flux of free fatty acids to the liver and insulin resistance lead to hepatic fat accumulation, which causes inflammatory changes in the liver [56,57]. Enhanced TNF-α expression and increased leptin levels are also found in

patients with NASH [58,59]. In addition, Wong *et al.* [60] recently reported interesting results from a cross-sectional study, indicating that NASH is associated with a high prevalence of colorectal adenomas and advanced neoplasms. This finding may suggest that in addition to HCC, NASH may be associated with an increased risk of CRC.

4. Preventive Effects of GTCs on the Metabolic Abnormalities and Cancer Development

Numerous studies have indicated that tea catechins, especially GTCs, are beneficial for various reasons, such as their anti-obesity effects [15]. A recent meta-analysis of clinical trials reported that GTCs help reduce body weight [61]. The underlying mechanisms include an increase in energy expenditure, stimulation of fatty acid oxidation, and reduction of nutrient absorption [62]. The effects of GTCs whereby they suppress metabolic syndrome have also been investigated in laboratory, epidemiological, and intervention studies [63,64]. In a rodent model of obesity and diabetes, treatment with green tea or its constituents was found to result in significantly reduced body weight and, therefore, improved hyperglycemia, hyperinsulinemia, hyperleptinemia, hepatic steatosis, and liver dysfunction [65–67]. GTCs supplementation was also found to decrease plasma levels of insulin, TNF-α, and IL-6 in a rat insulin resistance model [68]. These reports suggest that long-term treatment with GTCs may be effective for preventing the progression of obesity-related metabolic disorders.

In addition to the anti-obesity effects, GTCs possess anti-cancer and cancer-preventive properties [16–19]. Intervention studies provide clear evidence of the chemopreventive effects of tea preparations [69,70]. A pilot study also showed that GTCs successfully prevent colorectal adenomas, the precancerous lesions of CRC, after polypectomy [71]. Several properties of GTCs are responsible for their anti-cancer and cancer-preventive effects, including their antioxidant and anti-inflammatory properties [16,72]. An increasing number of studies have reported that GTCs, especially the major biologically active component in green tea (−)-epigallocatechin gallate (EGCG), inhibit proliferation of and induce apoptosis among cancer cells by modulating the activities of different receptor tyrosine kinases (RTKs) and their downstream signaling pathways, including the Ras/extracellular signal-regulated kinase (ERK) and PI3K/Akt signaling pathways [17–19,73,74]. EGCG suppresses cell growth by inhibiting the activation of IGF-1R, a member of the RTK family, in human CRC and HCC cells, and this inhibition is associated with a decrease in the expression of IGF-1/2, but an increase in the expression of IGF-binding protein-3 (IGFBP-3), which negatively controls the function of the IGF/IGF-1R axis [49,75]. EGCG also prevents carbon tetrachloride-induced hepatic fibrosis in rats by inhibiting IGF-1R expression [76]. These reports indicate that the IGF/IGF-1R axis, which plays a critical role in both cancer development and obesity-induced pathological events [24,25], might be a critical target of GTCs.

5. Preventive Effects of BCAA on Metabolic Abnormalities and HCC in Obese, Cirrhotic Patients: Results Form the LOTUS Study

Because the liver, an important target organ of insulin, plays a critical role in regulating metabolism, patients with chronic liver diseases often suffer from several nutritional and metabolic disorders, such as protein-energy malnutrition and insulin resistance [77–80]. Decreased serum levels of BCAA and albumin are associated with a high incidence of liver cirrhosis, while supplementation with BCAA has

been shown to improve protein malnutrition and increase the serum albumin concentration in cirrhotic patients [20,77,78]. In addition, recent experimental studies have revealed that BCAA improves insulin resistance and glucose tolerance [81–83]. She *et al.* [81] reported that mitochondrial branched-chain aminotransferase knock out mice, which show a significant elevation in the serum BCAA level, exhibit decreased adiposity and remarkable improvements in glucose and insulin tolerance. BCAA has favorable effects on glucose metabolism not just in the liver but also in skeletal muscle and adipose tissue [84–86]. In the liver, BCAA activates liver-type glucokinase and glucose transporter (GLUT)-2, while suppressing the expression of glucose-6-phosphatase, which catalyzes the final steps of gluconeogenesis [84]. On the other hand, BCAA promotes glucose uptake through activation of PI3K and subsequent translocation of GLUT1 and GLUT4 to the plasma membrane in the skeletal muscle [86]. Moreover, in mice fed a high-fat diet, BCAA supplementation ameliorated insulin resistance by improving adipocytokine imbalance, inhibiting lipid accumulation in the liver, and increasing the hepatic levels of peroxisome proliferator-activated receptor-α [87,88]. Several clinical trials have also reported that oral BCAA supplementation improves glucose tolerance and insulin resistance in patients with chronic liver disease [22,89,90].

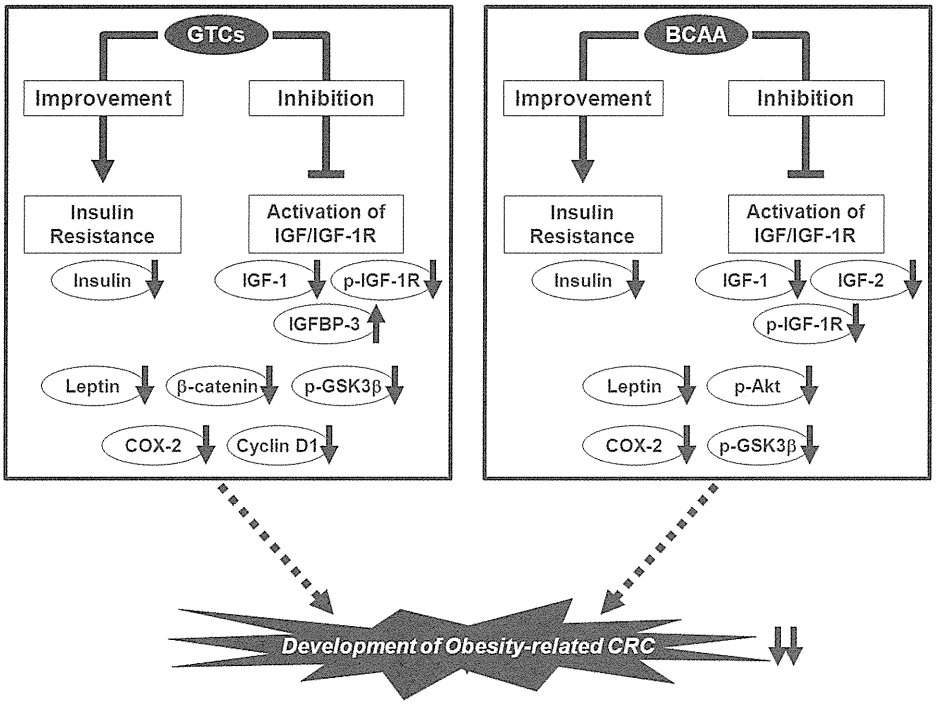
The Long-Term Survival Study (LOTUS) was a large-scale (n = 622) multicenter randomized controlled trial conducted from 1997 to 2003 in Japan to investigate the effects of supplemental BCAA therapy on event-free survival in patients with decompensated cirrhosis. In this trial, oral supplementation with a BCAA preparation was found to significantly prevent progressive hepatic failure and improve event-free survival [20]. Moreover, subset analysis from this trial demonstrated that long-term oral supplementation with BCAA is associated with a reduced frequency of HCC in obese patients (BMI score $\geq$ 25, P = 0.008) with decompensated cirrhosis [8]. What could the mechanisms of action of BCAA in the prevention of HCC have been? It seems reasonable to consider that the improvement of glucose metabolism by BCAA contributes to a decrease in the HCC incidence in obese cirrhotic patients because these patients generally have a particularly high incidence of hyperinsulinemia and insulin resistance [79,80]. In addition, Hagiwara *et al.* [91] recently reported significant findings that BCAA suppresses insulin-induced proliferation of HCC cells by inhibiting the insulin-induced activation of the PI3K/Akt pathway and the subsequent anti-apoptotic pathway. The precise mechanisms of action of BCAA in relation to carcinogenesis are explained in detail in the following sections.

6. Prevention of Obesity-Related CRC via the Nutraceutical Approach—GTCs and BCAA Effectively Prevent Obesity-Related Colorectal Carcinogenesis

Recent evidence indicates that increased body fatness and BMI are associated with an increased risk of CRC [4,5,9–11]. In contrast, studies have provided convincing evidence that dietary habits, especially high fruit and vegetable consumption, may reduce the risk of this malignancy [92]. Hirose *et al.* [93] established a useful preclinical model to determine the underlying mechanisms of how specific agents prevent the development of obesity-related CRC. The model used was C57BL/KsJ-*db/db* (*db/db*) mice, which are a genetically altered animal model with phenotypes of obesity and diabetes mellitus [94]. These mice have hyperlipidemia, hyperinsulinemia, and hyperleptinemia and are susceptible to the colonic carcinogen azoxymethane (AOM) because AOM-induced colonic precancerous lesions,

aberrant crypt foci (ACF) and β-catenin accumulated crypts (BCAC), develop to a significantly greater extent in these mice than in the genetic control mice [93]. The colonic mucosa of *db/db* mice expresses high levels of IGF-1R, the phosphorylated (activated) form of IGF-1R (*p*-IGF-1R), β-catenin, and cyclooxygenase-2 (COX-2) [95]. Dietary supplementation with certain types of flavonoids, such as citrus compounds, suppresses the development of these putative lesions for CRC in the *db/db* mice [96–98].

Figure 2. Mechanisms of action of green tea catechins (GTCs) and branched-chain amino acids (BCAA) in the inhibition of obesity-related colorectal carcinogenesis.



We used this experimental model to investigate in detail the effects of EGCG and BCAA on the prevention of obesity-related colorectal carcinogenesis. We found that drinking water with EGCG significantly decreased the number of ACF and BCAC, which accumulate the IGF-1R protein, and this decrease was associated with inhibited expression of IGF-1R, *p*-IGF-1R, the phosphorylated form of glycogen synthase kinase-3β (GSK-3β), β-catenin, COX-2, and cyclin D1 on the colonic mucosa [95]. EGCG also increased the serum level of IGFBP-3 while decreasing the serum levels of IGF-1, insulin, triglycerides, total cholesterol, and leptin [95]. In accordance with this study, supplementation with BCAA also caused a significant reduction in the number of ACF and BCAC compared with the control diet-fed groups by inhibiting the phosphorylation of IGF-1R, GSK-3β, and Akt on the colonic mucosa [99]. The serum levels of insulin, IGF-1, IGF-2, triglycerides, total cholesterol, and leptin were also decreased [99]. These findings suggest that both EGCG and BCAA effectively suppress the development of premalignant CRC lesions by suppressing the IGF/IGF-1R axis; improving hyperlipidemia, hyperinsulinemia, and hyperleptinemia; and inhibiting the expression of COX-2,