834   M. Shimizu et al.

Mol. Nutr. Food Res. 2011, *55*, 832–843

decreased in human HCC samples and this is associated with poor survival of HCC patients [31–33]. In addition, an overexpression and activation of the VEGF/VEGFR axis is observed in both human CRC and HCC, and this has also been shown to correlate with poor prognosis for these malignancies [34–37]. Therefore, targeting these RTKs and their downstream pathways may be a potentially effective strategy for the prevention and, in certain cases, treatment of some types of human malignancies, including CRC and HCC [9–11]. We will mainly discuss the effects of GTCs on EGFR, IGF-1R, and VEGFR in this review.

4   Effects of GTCs on the EGF receptor family of RTKs in CRC and HCC cells

Recent studies have revealed that several phytochemicals, including GTCs, exert antitumor activity by suppressing the activation of the EGF receptor family of RTKs and their downstream effectors in cancer cells [3–5]. Liang et al. [38] demonstrated that EGCG directly blocks epidermal growth factor (EGF) binding to the EGFR and thus inhibits the phosphorylation of this receptor and DNA synthesis in human A431 epidermoid carcinoma cells. We have extended this finding and reported that EGCG inhibits the activation of EGFR, HER2, and HER3, and their multiple downstream signaling pathways in human head and neck squamous cell carcinoma (HNSCC), breast cancer, and CRC cell lines [39–43]. EGCG and Polyphenon E preferentially inhibit the growth of CRC cells, which overexpress and activate EGFR and HER2, when compared with a normal human fetal colonic epithelial cell line. Treatment with these agents inhibits the activation of EGFR and HER2, the phosphorylation of Akt and ERK, and also the transcriptional activity of the activator protein-1 (AP-1), c-fos, nuclear factor-κB (NF-κB), and cyclin D1 promoters in the HT29 human CRC cell line [42]. In SW837 human CRC cells, EGCG also inhibits the activation of EGFR, HER2, and HER3, with the subsequent inhibition of the expression of cyclooxygenase-2 (COX-2) at the level of transcription, and it reduces the production of prostaglandin E_2 (PGE_2) by these cells [43]. These findings are of interest because both the EGF receptor family of RTKs and the COX-2/PGE_2 axis are critical targets for CRC chemoprevention and treatment [9, 44].

As described above, EGCG exerts its anticancer and chemopreventive effects in part through the inhibition of the EGF family of RTKs. Therefore, there is growing interest in preventive and therapeutic strategies involving the combination of EGCG with other agents that inhibit EGFR activation because such a combination treatment targeting the same molecule might provide the potential for synergistic effects on growth inhibition in cancer cells [45]. Indeed, recent in vitro and in vivo studies with HNSCC cells revealed that the combination of EGCG and erlotinib, an EGFR-tyrosine kinase inhibitor, caused synergistic cell growth inhibition by inhibiting EGFR and Akt phosphor-

ylation, inducing apoptosis, and suppressing the NF-κB signaling pathway [46, 47]. The combination of EGCG and erlotinib also resulted in a greater inhibition of both cell proliferation and growth rate of xenografts in non-small cell lung cancer cells than either agent alone [48]. These results suggest the possibility that a combined treatment with EGCG and EGFR-targeting agents provides a promising regimen for future chemoprevention and treatment of human malignancies, owing to the synergistic effects of these compounds.

5   Effects of GTCs on the IGF/IGF-1R axis in CRC and HCC cells

In addition to the EGFR family of RTKs, increasing evidence suggests that GTCs inhibit the tyrosine kinase activities of the other members of the RTK family, such as IGF-1R and VEGFR2. We recently reported that EGCG inhibits the activation of IGF-1R in HepG2 human HCC and SW837 CRC cells that display a constitutive activation of this receptor. In these studies, the inhibition of IGF-1R activation by EGCG was associated with a decrease in the expression levels of IGF-1/2, but an increase in the expression levels of IGFBP-3, which negatively controls the function of IGF-1/2 in these cancer cells [49, 50]. EGCG inhibits the expression of matrix metalloproteinases (MMPs)-7 and -9 in CRC cells and this may play a role in upregulating the expression of IGFBP-3 [49]. Because the IGF/IGF-1R axis, which forms autocrine and paracrine loops in cancer tissues, plays an important role in the development and growth of various types of cancer [10], disruption of these loops by GTCs might be an effective strategy for the prevention and treatment of certain cancers.

Similar effects of EGCG targeting the IGF/IGF-1R axis are also observed in in vivo studies. In an obesity-related colorectal carcinogenesis mice model, EGCG administration through drinking water effectively suppresses the development of premalignant CRC lesions by depressing the IGF/IGF-1R and COX-2/PGE_2 axes. In this study, EGCG caused the inhibition of the expression of COX-2 and the activation of IGF-1R on the colonic mucosa, and decreased the serum IGF-1 levels while increasing the serum IGFBP-3 levels in obese mice [13]. In accordance with this study, administration of EGCG through the drinking water also prevents obesity-related liver tumorigenesis in db/db mice by inhibiting IGF-1R, ERK, Akt, GSK-3β, Stat3, and JNK phosphorylation in the liver and decreasing the levels of insulin, IGF-1, and IGF-2 in the serum [14]. Other investigators have also demonstrated that the oral infusion of GTCs inhibits the development and progression of prostate cancer in mice by reducing the serum IGF-1 levels, inhibiting Akt and ERK activation, and increasing serum IGFBP-3 levels [51, 52]. Drinking EGCG also prevents carbon tetrachloride (CCl_4)-induced rat hepatic fibrosis by inhibiting IGF-1R expression [53]. This finding is significant when considering HCC

chemoprevention because inhibition of hepatic fibrosis, which is a precancerous condition to HCC, might be linked to the prevention of HCC development [54, 55].

6 Effects of GTCs on the VEGF/VEGFR axis in CRC and HCC cells

VEGF, which binds to and activates VEGFR, is a mitogen for endothelial cells that is often associated with pathological angiogenesis. Abnormal activation of the VEGF/VEGFR axis is therefore closely associated with tumor growth [11]. EGCG suppresses the growth of xenografts generated from the human HCC cell line Huh7 by decreasing serum VEGF levels and inhibiting the activation of VEGFR2, ERK, and Akt [16]. In CRC cell xenografts, the activation of VEGFR2, ERK, and Akt and the expression of VEGF are also inhibited by EGCG treatment and this might be associated with reduction of the expression of hypoxia-inducible factor (HIF)-1α, which strongly activates VEGF expression [17].

Several in vitro studies have also reported the inhibitory effects of GTCs on the VEGF/VEGFR axis. For instance, work from our group demonstrated that EGCG inhibits the production of VEGF in human HNSCC and breast cancer cells by blocking the activation of Stat3 and NF-κB [40]. EGCG also inhibits the phosphorylation of both VEGFR1 and VEGFR2 and induces apoptosis in chronic lymphocytic leukemia cells [56]. In addition, GTCs significantly inhibit HIF-1α protein accumulation and decrease VEGF expression in HepG2 cells by blocking both the PI3K/Akt and Ras/ERK signaling pathways [57]. EGCG inhibits ERK activation and suppresses the expression and promoter activity of VEGF in HT29 cells [58]. Similar to the findings showing the role of the IGF/IGF-1R axis in mediating the effect of GTCs, the above results suggest that the VEGF/VEGFR axis might also be a promising target of GTCs for the prevention and treatment of some types of human malignancies, including CRC and HCC.

7 Effects of GTCs on the hepatocyte growth factor (HGF)/c-Met and PDGF/PDGFR axes

In this review, we have mainly focused on a discussion of the inhibitory effects of GTCs on the activation of EGFR, IGF-1R, and VEGFR. However, it should be mentioned that GTCs also target other members of the RTK family, such as c-Met and platelet-derived growth factor receptor (PDGFR). c-Met is overexpressed in colon tumors and this is associated with poor prognosis [59, 60]. In human CRC cells, EGCG markedly suppressed the activation of c-Met in the presence of its ligand, HGF [61, 62]. In the liver of CCl4-injected rats, EGCG significantly decreased the expression of PDGFR and thus attenuated hepatic fibrosis [53]. EGCG also inhibited PDGF-induced cell proliferation and reduced the autopho-

sphorylation of the PDGFR by blocking the binding of PDGF to its receptor in human hepatic stellate cells; this might contribute to the prevention of liver fibrosis progression in patients with chronic liver diseases [63]. These reports suggest the possibility that GTCs can target certain types of RTKs in a variety of cell types; however, the precise mechanisms underlying the GTCs-mediated inhibition of RTKs activation in cancer cells remain to be elucidated.

8 Mechanisms mediating the inhibition of RTKs activation and intracellular signaling pathways by GTCs

One possible mechanism by which the inhibition of RTKs activation by GTCs could be explained is through the "sealing" and "trapping" effects of GTCs [64]. Namely, EGCG covers the cell surface and directly interrupts the binding of EGF to EGFR [38]. EGCG has also been shown to bind directly to EGF and VEGF, thus preventing these growth factors from interacting with their corresponding receptors and activating downstream signaling cascades [38, 65]. In addition, EGCG may also inhibit the activation of RTKs by affecting the expression levels of their ligands. The expression levels of the EGFR family ligands EGF and heregulin have been shown to be downregulated by EGCG treatment in CRC cells [17]. EGCG also decreases the levels of IGF-1, IGF-2, and VEGF, which might be associated with decreased ERK and Akt activities, in CRC and HCC cells [16, 17, 50]. These findings could partly explain the inhibitory effects of GTCs on the activation of RTKs in various types of cancer cells [16, 17, 38–43, 49, 50].

Several studies have also provided evidence that GTCs can directly target the kinase activity of RTKs and their intracellular signaling pathways and transcription factors. EGCG was shown to competitively bind to the ATP binding site of IGF-1R and block downstream signaling [66]. Sah et al. [67] demonstrated that EGCG directly inhibits ERK and Akt kinases in immortalized human cervical cells. In addition, EGCG was shown to play a role in the direct inhibition of the activation of ERK and mitogen-activated protein kinase kinase-1 (MEK1) and of the association with Raf-1 with MEK1, and in the inhibition of AP-1 activity in H-ras-transformed mouse epidermal cells [24, 25]. EGCG also exerted antiproliferative effects on H-ras-transformed rat intestinal epithelial cells [68]. These reports seem to be significant when considering the prevention of CRC by GTCs because Ras (KRAS) gene mutations occur frequently in this malignancy [69]. Moreover, administration of EGCG through the drinking water significantly decreased small intestinal tumor formation in Apc[Min/+] mice, a recognized mouse model for human intestinal cancer, by reducing the expression of the phosphorylated form of Akt and ERK proteins in small intestinal tumors [70]. Administration of EGCG through the drinking water also suppressed tumor

836   M. Shimizu et al.

Mol. Nutr. Food Res. 2011, *55*, 832–843

formation in APC$^{Min/+}$ mice by decreasing the levels of basic fibroblast growth factor in small intestinal tissue samples [71]. These reports are important because the mutation of the APC gene, a tumor suppressor gene, is critically implicated in human colorectal carcinogenesis [72]. Altogether, these results suggest that GTCs might exert antitumor and chemopreventive effects by binding, probably with relatively low affinity, to multiple cellular targets (Fig. 1). Moreover, these results also demonstrate the potential of GTCs as an effective chemopreventive agent against CRC in patients bearing APC and/or Ras gene mutations.

## 9   Lipid rafts: a promising target of EGCG

Evidence exists that several plasma membrane-associated RTKs, including EGFR, IGF-1R, and VEGFR2, are closely associated with the detergent-insoluble ordered membrane domains called "lipid rafts," which play a critical role as signal processing hubs. The localization of RTKs to lipid rafts appears to modulate both their ligand binding and tyrosine kinase activities [73–76]. Lipid organization is also considered to play a fundamental role in receptor internalization [77]. Recent studies show that lipid rafts provide a platform for a 67-kDa LR that binds EGCG, thus affecting the uptake of EGCG [21, 22, 78, 79]. The expression of the 67-kDa LR is found to be upregulated in various types of human cancers, including CRC [80], and to directly correlate with the malignant potential via activation of multiple signal transduction pathways such as MAPK [81, 82]. Therefore, EGCG may presumably mediate its cancer-preventive activity by targeting the 67-kDa LR [83].

In addition, on the basis of our recent series of studies [84–86], we presume that targeting lipid rafts is one of the most relevant mechanisms of EGCG in exerting its anticancer and chemopreventive properties (Fig. 2). EGFR activation was shown to only occur in the lipid raft fraction, whereas total cellular EGFR is present in the non-raft membrane fraction in HT29 cells. In these cells, EGCG
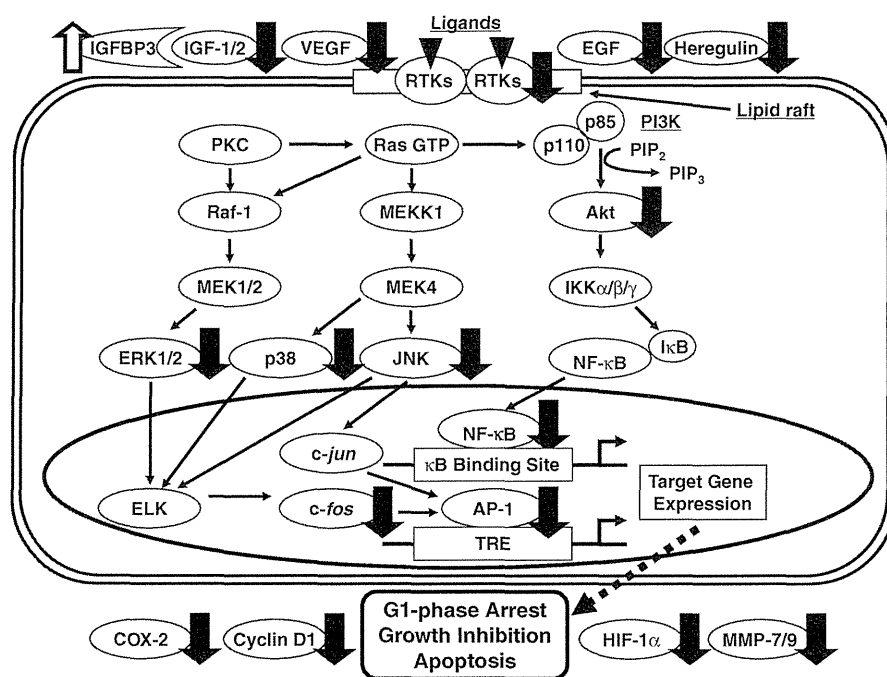
Figure 1. Effects of GTCs on RTKs and their intracellular signaling pathways. Activation of RTKs including EGFR, IGF-1R, and VEGFR2 by specific ligands leads to the induction of their intrinsic tyrosine kinase activities and autophosphorylation of tyrosine residues. The activated RTKs then create docking sites for downstream effector molecules such as Ras, Raf-1, and PI3K, which subsequently stimulate several intracellular processes. Activated Raf-1 stimulates MEK and its signaling cascade, resulting in the phosphorylation of the MAPK protein ERK. In its active state, MAPK activates a variety of transcription factors, including ELK and c-Jun, and subsequently promotes the expression of target genes by stimulating the transcriptional activity of AP-1, a dimeric complex that comprises members of the Jun and Fos families of transcription factors. The activation of PI3K triggers the synthesis of the lipid PIP$_3$, which activates the downstream pathways that involve Akt. The NF-κB family of transcription factors, which is important in cell survival, is one of the functional targets of Akt. EGCG inhibits the activation of certain RTKs, which takes place in lipid rafts (gray box), as well as the activation of the MAPK cascade, such as Ras/Raf/MEK/ERK/JNK pathways and PI3K pathways. Molecules that appear to be cellular targets for EGCG are indicated by a black arrow (downregulation) or by a white arrow (upregulation), respectively. These multiple effects of EGCG result in the induction of apoptosis and cell cycle arrest in the G$_0$–G$_1$ phase, thus inhibiting cell proliferation in cancer cells.

Mol. Nutr. Food Res. 2011, *55*, 832–843

837
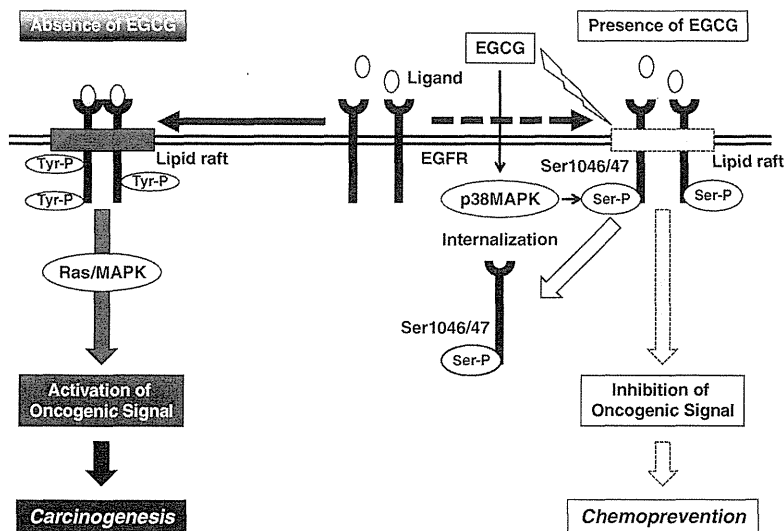
Figure 2. Hypothetical scheme indicating the inhibitory effect of EGCG on the activation of EGFR by inducing the alteration of lipid rafts and internalization of EGFR in CRC cells. EGCG alters the organization of lipid rafts and inhibits the dimerization and activation of EGFR. EGCG also promotes the internalization of nonactivated monomer EGFR into the cytosol through phosphorylation of EGFR at serine 1046/47 by the activation of p38 MAPK. As a result, EGCG decreases the levels of phosphorylated EGFR and inhibits the activation of EGFR signaling, which acts as an oncogenic signal in CRC cells.

inhibits the binding of EGF to EGFR and its subsequent dimerization and activation by reducing the levels of EGFR in lipid rafts [84]. Our group also found that EGCG induces alterations in membrane organization, resulting in the internalization of the inactivated form of EGFR into endosomes and the suppression of CRC cell growth [85]. EGCG also induces the internalization and subsequent degradation of EGFR through the phosphorylation of the receptor, which is associated with the activation of p38 MAPK by EGCG [86]. These findings strongly suggest that EGCG inhibits EGF binding to EGFR and dimerization/activation by causing an alteration in the lipid organization of the plasma membrane [87]. Given the fact that a majority of RTKs function on lipid rafts, this mechanism might explain, at least in part, the ubiquitous inhibitory effects of EGCG on a variety of RTKs. In addition, EGFR internalization triggered by EGCG might also be a possible mechanism mediating the anticancer effect of this agent and other related compounds.

10 Possible clinical applications of GTCs

Several studies have used animal models of carcinogenesis to show the significant chemopreventive effects of GTCs. On the contrary, the results of epidemiological studies investigating the effects of tea consumption on the risk of human cancer have been inconclusive [1, 2]. This might be associated with different factors such as human genetic variability, lifestyle, amount and type of tea consumed, and the diversity in cancer etiologies. Among these factors, the quantity and quality of tea consumed appears to be one of the most important variables affecting the relationship between tea consumption and cancer risk reduction. Prospective cohort studies in Japan showed that daily consumption of 10 cups of tea (equivalent to 2.5 g green tea extract (GTE) is required for the cancer-preventive effect [88,

89]. Green tea consumption in specific quantities is also associated with reduced risk of esophageal and breast cancers [90, 91].

On the other hand, some intervention studies provide a clear evidence for the chemopreventive and probable anticancer progression effects of tea preparations. Early double-blind intervention trials showed that oral administration of mixed tea products significantly decreases the size of leukoplakia, an oral precancerous mucosa lesion, suggesting that tea may have a protective effect in oral cancers [92]. A recent double-blind, placebo-controlled study in Italy revealed that the progression of high-grade prostate intraepithelial neoplasia to prostate cancer can be effectively prevented by oral administration of GTCs (600 mg/day for 1 year) [93]. Furthermore, an interesting clinical trial demonstrated that the serum levels of IGF-1, VEGF, and HGF were significantly decreased by the administration of Polyphenon E in prostate cancer patients [94]. These findings support a potential role for GTCs in the prevention and/or treatment of human malignancies.

The successful prevention of the development of colorectal adenomas, the precancerous lesions for CRC, after polypectomy was shown in a pilot study in which the administration of GTE (1.5 g/day for 1 year) in patients who had undergone polypectomy for colorectal adenomas reduced the development of metachronous colorectal adenomas in comparison with patients who did not take this supplement. The size of relapsed adenomas was also significantly smaller in the GTE supplemented group in comparison with the control untreated group (Fig. 3) [12]. The absence of any serious adverse events as a consequence of GTCs administration in these trials [12, 93] is a significant finding for the consideration of the use of GTCs as "chemopreventive" in clinical practice. In addition, the results of these clinical trials [12, 92] also suggest the possibility that cancers that develop in the digestive tract,
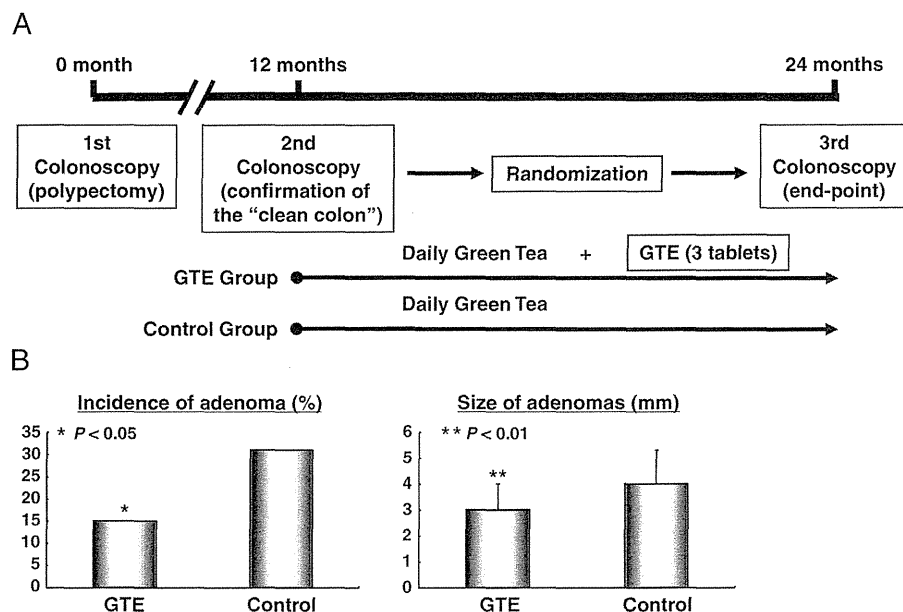
Figure 3. Pilot study revealing the preventive effect of GTE on metachronous adenomas after polypectomy. (A) Study design. The study included 136 participants who underwent endoscopic resection of one or more colorectal adenomas. Twelve months later, the participants received another total colonoscopy to confirm the absence of remaining endoscopically detectable adenoma (confirmation of the "clean colon"). The participants were then randomized into two groups while maintaining a daily green tea drinking; the GTE group (71 patients) was given three GTE tablets per day for 12 months and the control group (65 patients) received no supplement. After 12 months of GTE supplements, a follow-up (end-point) colonoscopy was conducted in 125 patients (60 in the GTE group and 65 in the control group) to test for the presence of new adenomas. One tablet of GTE (500 mg), which contains 52.5 mg EGCG, 12.3 mg (−)-epicatechin, 34.6 mg (−)-epigallocatechin, 11.1 mg (−)-epicatechin gallate, and 15.7 mg caffeine, is equivalent to approximately two Japanese-size cups of green tea. (B) Effects of the GTE supplement on the incidence and the size of metachronous adenomas at the end-point colonoscopy. Left panel: the incidence of metachronous adenomas was 31% (20 of 65) in the control group and 15% (9 of 60) in the GTE group (relative risk, 0.49; 95% confidence interval, 0.24–0.99: $*p<0.05$). Right panel: the size of relapsed adenomas was 4.0 ± 1.3 mm in the control group and 3.0 ± 1.0 mm in the GTE group ($**p<0.01$).

such as in the oral cavity, esophagus, stomach, and colorectum, might be more effective targets for chemoprevention using GTCs, because direct contact ("exposure") with the digestive tract seems to be a key factor for the cancer-preventive activity of polyphenolic compounds [95].

An interventional study using GTCs in a high-risk group of individuals for HCC has been conducted in a high aflatoxin exposure area in China. In this double-blinded and placebo-controlled phase IIa chemoprevention trial, administration of GTCs in these individuals, who were seropositive for both HBs-Ag and aflatoxin-albumin adducts, effectively reduced the levels of urinary 8-hydroxydeoxyguanosine, a surrogate marker of oxidative DNA damage [96]. Daily GTCs administration also modulated aflatoxin biomarkers in this trial [97]; however, whether GTCs ultimately prevent the development of HCC has yet to be clarified. In addition, HCC development is frequently associated with chronic inflammation and subsequent cirrhosis of the liver induced by a persistent infection with hepatitis viruses. Increasing evidence also indicates that obesity and related metabolic abnormalities, especially insulin resistance, raise the risk of HCC [98, 99],

whereas GTCs seem to have antiobesity and antidiabetic effects [100]. Therefore, well-designed interventional trials should be conducted to examine whether GTCs prevent the development of HCC in high-risk patients with viral liver cirrhosis and obesity. Recent rodent experiments showing the antifibrotic [53] and chemopepreventive effects of EGCG in obesity-related liver tumorigenesis [14] might encourage the use of GTCs for such patients.

11   Concluding remarks

In concluding this review, it should be mentioned that the concentrations of EGCG used in some of the cell culture experiments (20–100 µM) aiming to elucidate the mechanisms of action of this agent are higher than the plasma and tissue concentrations observed in human trials or in mice in cancer chemoprevention experiments [101]. Therefore, it remains unclear and thus requires careful consideration whether the information obtained from in vitro studies with high EGCG concentrations can be directly extrapolated to cancer chemoprevention in animals and humans. On the

other hand, a number of high-affinity EGCG-binding proteins, including IGF-1R, have been revealed by recent affinity chromatography studies [66, 102, 103]. These studies indicate that EGCG does in fact bind to target proteins at low concentrations, although relatively high concentrations are required to exert its physiological functions. Moreover, for consideration of GTCs in the clinical practice, it should be emphasized that EGCG can inhibit the activation of EGFR at low micromolar concentrations [42, 43, 85] that are considered within the physiologically relevant range for human exposure [104]. Furthermore, EGCG preferentially inhibits the growth of cancer cells without affecting the growth of the corresponding normal cells [16, 42, 50, 105].

A possible explanation for these phenomena is the concept of "oncogene addiction" according to which cancers associated with multiple genetic, epigenetic, and chromosomal abnormalities are usually dependent on or "addicted" to one or a few genes for both maintenance of the malignant phenotype and cell survival and, therefore, targeting only one or a few of these aberrant molecules might be effective to inhibit carcinogenesis and growth of cancer cell [106, 107]. It is likely that EGCG preferentially inhibits growth and induces apoptosis in cancer cells by blocking the activity of one or a few of "addicted" oncogenic factors, including abnormalities in RTKs.

Tea is currently considered one of the most promising dietary agents for the prevention and treatment of many diseases, especially cancer. The present review provides evidence that the effects of GTCs on the inhibition of carcinogenesis are mediated, at least in part, by the regulation of the activity of certain RTKs and their related intracellular signaling pathways; this observation does not exclude other mechanisms that may also play critical roles in mediating the anticancer and cancer chemopreventive effects of these agents [1, 2]. The safety and efficacy of GTCs demonstrated in recent intervention studies [12, 93] could be crucial for the clinical application of GTCs as chemopreventive agents.

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842   M. Shimizu et al.

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Chemoprevention of hepatocellular carcinoma by acyclic retinoid

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TABLE OF CONTENTS

1. ABSTRACT

The prognosis for patients with hepatocellular carcinoma (HCC) is poor and effective prevention strategies are urgently required. Here, we review abnormalities in the expression and function of retinoids and their receptors, and how they play a critical role in the development of HCC. In particular, a malfunction of RXRα due to phosphorylation by Ras-MAPK signaling pathway is profoundly associated with liver carcinogenesis and thus may be a promising target for HCC chemoprevention. Acyclic retinoid (ACR), a synthetic retinoid, inhibits Ras-MAPK activation and RXRα phosphorylation, thereby suppressing growth in HCC-derived cells. In clinical trials, ACR has been shown to improve patient survival by preventing viral HCC development, a possible manifestation of the concept of "clonal deletion" therapy. "Combination chemoprevention" with ACR as the key drug has great potential to become an effective strategy for the prevention of liver carcinogenesis. In summary, both basic and clinical research strongly suggest that ACR plays a critical role in preventing the development of HCC and that "clonal deletion" therapy is one of the most practical approaches for this purpose.

2. INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, accounting for 500,000 to 600,000 deaths per year. The development of HCC is frequently associated with chronic inflammation and subsequent cirrhosis of the liver induced by persistent infection with hepatitis B virus (HBV) or hepatitis C virus (HCV). This fact indicates that HCC is a major health problem in Eastern as well as Western countries where hepatitis viral infection is endemic, and the incidence is increasing (1-3). However, in spite of strenuous efforts to develop effective methods of diagnosis and treatment, there has been limited improvement in the prognosis for this malignancy. A major obstacle for HCC therapy is the high frequency of tumor recurrence after curative treatment; the recurrence rate at 5 years after definitive therapy may exceed 70% (4, 5). At present, there are no effective chemotherapeutic agents for this malignancy. Therefore, there is a critical need to develop more effective strategies for the chemoprevention and chemotherapy of HCC to improve the prognosis for patients with this malignancy; for this purpose, we must elucidate the molecular mechanisms underlying hepatocarcinogenesis. Among the several causal factors for the development of HCC,
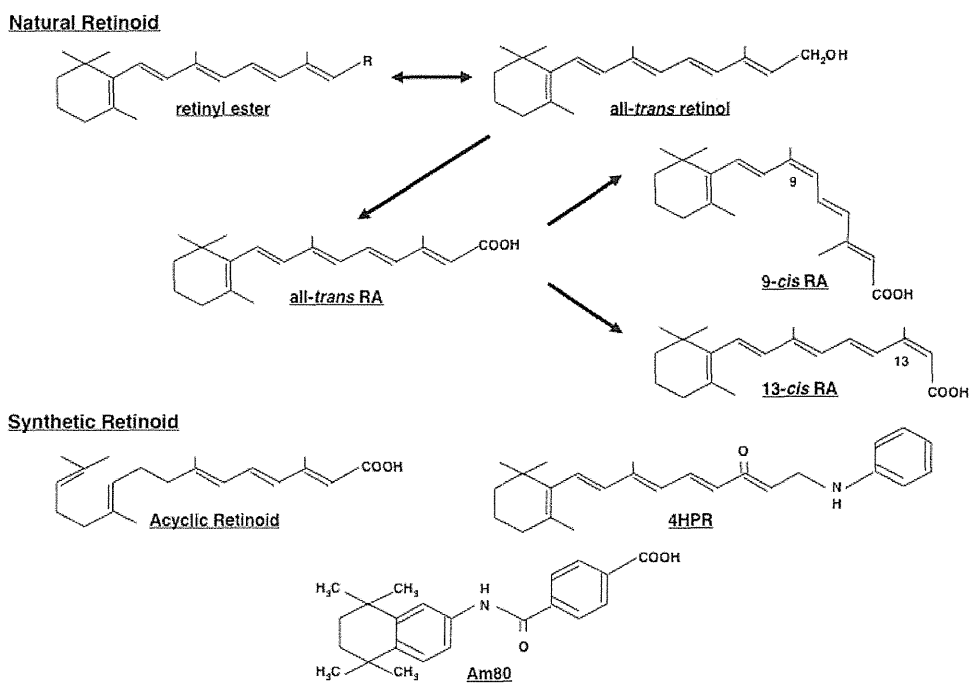
Figure 1. Chemical structures of natural and representative synthetic retinoids. Retinyl esters (mainly retinyl palmitate, R: fatty acid), stored in the liver stellate cells, are hydrolyzed to retinol. Retinoic acid (RA) is biosynthesized from retinol via the intermediate metabolite retinal by oxidization in the cells of peripheral tissues. Three well-known isomers of RA, all-*trans* RA, 9-*cis* RA, and 13-*cis* RA activate retinoid receptor, RARs, whereas only 9-*cis* RA activates the other receptor, RXRs. All-*trans* RA inhibits proliferation and induces granulocytic differentiation in leukemic cells of acute promyelocytic leukemia and thus is a first-line drug for this disease. A number of synthetic retinoids have been developed for pharmacological applications including cancer chemoprevention. ACR and N-(4-hydroxyphenyl) retinamide (4HPR) successfully prevented the development of HCC and breast cancer, respectively, in clinical trials. Am80 (Tamibarotene) is approved for relapsed or refractory acute promyelocytic leukemia in Japan.

phosphorylation of retinoid X receptor-α (RXRα) by the Ras-MAPK signaling pathway is considered to play a key role (6-9).

Because of the high incidence of recurrence and the development of secondary tumors (4, 5), the curative treatment for HCC is difficult once this malignancy has developed. The high risk group, including patients infected with hepatitis, are easily identified, however. Therefore, cancer chemoprevention, an approach wherein a natural or synthetic chemical compound works to arrest or reverse premalignancies via physiological pathways (10), is one of the most promising strategies for the treatment of HCC, particularly hepatitis virus-positive patients. We previously reported that, in clinical trials, the administration of acyclic retinoid (ACR), a novel synthetic retinoid which targets phosphorylated RXRα (11-13), reduced the incidence of post-therapeutic HCC recurrence and improved patient survival (14-17). In this article, we review evidence that a malfunction of RXRα due to phosphorylation is closely involved in liver carcinogenesis. We also show the pleiotropic effects of ACR in the inhibition of HCC and suppression of cancer growth, especially focusing on the

inhibition of RXRα phosphorylation and induction of RARβ and p21^{CIP1} expression. In addition, the possibility of "combination chemoprevention", which uses ACR as a key drug, and the concept of "clonal deletion" therapy, a practical approach to preventing HCC development, are also discussed.

3. RETINOIDS AND THEIR RECEPTORS

Vitamin A and its functional analogues, collectively termed retinoids, exert fundamental effects on the regulation of epithelial cell growth, differentiation, and development (18, 19). Retinoids consist of several molecular species, including retinoic acid (RA, an active metabolite that binds to its nuclear receptor), retinol (a transport form in the plasma), and retinylesters (a storage form in the tissues). In addition, large numbers of synthetic retinoids, including ACR, have been developed (Figure 1). Retinoids exert their biological functions primarily by regulating gene expression through 2 distinct nuclear receptors, the retinoic acid receptors (RARs) and RXRs, which are both composed of 3 subtypes (α, β, and γ) that are characterized by a modular domain structure. Nuclear retinoid receptors are ligand-dependent transcription

factors; after ligand binding, RXRs form a 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 (18, 19). In addition to RARs, RXRs also form heterodimers with other nuclear receptors including peroxisome proliferator-activated receptors (PPARs), which control energy homeostasis by modulating glucose and lipid metabolism and transport (20). Therefore, RXRs play a fundamental role in controlling normal cell proliferation and metabolism, and act as master regulators of nuclear receptors (19). These facts suggest that retinoid receptors, especially RXRs, are exciting pharmacological targets for the therapies of various human diseases, including cancer and metabolic disease (21, 22).

4. ABNORMALITIES IN THE RETINOID/RETINOID RECEPTOR AXIS AND HCC

Because retinoids and their receptors play an essential role in normal cell proliferation, differentiation, and death (regulation of apoptosis), abnormalities in the expression and function of these molecules, especially RXRα and RARβ, are strongly associated with the development of various human malignancies including HCC. For instance, the *RARβ* gene is an HBV integration site and its expression is markedly decreased in human HCC (23, 24). In the chemical-induced rat liver carcinogenesis model, both RARβ protein and mRNA levels are also decreased in HCC (25). These findings are interesting because among the retinoid receptors, RARβ is thought to be one of the most important receptors in the regulation of cell growth and apoptosis (26).

The expression of RXRα is also decreased not only in HCC and liver cell adenoma, but also in glutathione *S*-transferase placental form-positive foci, a precancerous HCC lesion in the chemical hepatocarcinogenesis model in rats (25). These findings suggest that the repression of RXRα occurs even in the early stage of liver carcinogenesis. Moreover, recent studies have revealed that liver carcinogenesis is accompanied by an accumulation of the phosphorylated (*i.e.* inactivated) form of RXRα (p-RXRα) (27). Specifically, RXRα protein is anomalously phosphorylated at serine and threonine residues, and accumulates in both human HCC tissue and HCC cell lines (9). Phosphorylation at serine 260 of RXRα, a MAPK consensus site, is closely associated with its retarded degradation, low transcriptional activity, and the promotion of cancer cell growth; the abrogation of phosphorylation by a MAPK inhibitor restores the degradation of RXRα in a ligand-dependent manner (9, 11). In addition, although RXRα is unphosphorylated and highly ubiquitinated in a normal liver, rendering it sensitive to proteasome-mediated degradation, p-RXRα is resistant to ubiquitination and proteasome-mediated degradation in both human HCC tissues and a human HCC cell line (28). Furthermore, the phosphorylation of RXRα abolishes its ability to form heterodimers with RARβ, and this is associated with uncontrolled cell growth and resistance to

retinoids (29). These findings suggest that the accumulation of p-RXRα, (*i.e.*, non-functional RXRα) may interfere with the function of normal RXRα in a dominant-negative manner, thereby playing a critical role in the development of HCC (Figure 2). There are also some reports that show the analogous effects of phosphorylated RXRα in the negative modulation of its heterodimeric binding partners (30-32). Therefore, the inhibition of RXRα phosphorylation and the restoration of its heterodimeric activity with other nuclear receptors may be an effective and important strategy for the prevention and treatment of certain types of human diseases, especially malignant disorders including HCC (6-8, 33-35).

5. ACR IN HCC CHEMOPREVENTION: EXPERIMENTAL STUDIES

ACR, which was initially developed as an agonist for both RXR and RAR (36, 37), has been demonstrated to produce several beneficial effects on the prevention of HCC development and inhibition of growth in HCC cells (ACR is the same substance as NIK-333 and Peretinoin; Kowa Pharmaceutical Co., Tokyo, Japan; See Figure 3). In rodent studies, ACR inhibits both chemical-induced hepatocarcinogenesis in rats and spontaneously occurring HCC in mice (38). ACR also inhibits growth of HCC-derived cells by inducing cell proliferation and apoptosis, which effects seem to be associated with upregulation of RARβ expression (13, 36, 39-44). In human HCC and squamous carcinoma cells, ACR causes cell cycle arrest in G_0-G_1, increased cellular levels of $p21^{CIP1}$, and decreased levels of cyclin D1 and the phosphorylated form of retinoblastoma proteins (44-46). These findings suggest that RARβ and $p21^{CIP1}$ are one of the critical targets of ACR with respect to growth inhibition and apoptotic induction in cancer cells.

Recent *in vivo* and *in vitro* studies have indicated that ACR not only binds to RXR and RAR, but also reduces the development of HCC and inhibits cancer growth by targeting growth factors and their corresponding receptor tyrosine kinases (RTKs), which play a critical role in activation of the Ras-MAPK signaling pathway (41, 46-50). These reports are significant because the activated Ras-MAPK pathway phosphorylates RXRα, thus contributing to the development of HCC (9, 27). In addition, ACR also restores RXRα function by inactivating the Ras-MAPK signaling system, leading to the dephosphorylation of RXRα, although 9-*cis* RA failed to suppress ERK and RXRα phosphorylation (11). Therefore, ACR, which targets the RTK-Ras-MAPK signaling pathway and RXRα phosphorylation, is a promising agent for the chemoprevention of HCC. The role of RXRα phosphorylation in liver carcinogenesis and its inhibition by ACR are schematically represented in Figure 2.

6. ACR IN HCC CHEMOPREVENTION: CLINICAL STUDIES

An early phase randomized, controlled clinical trial tested the chemopreventive effect of ACR on
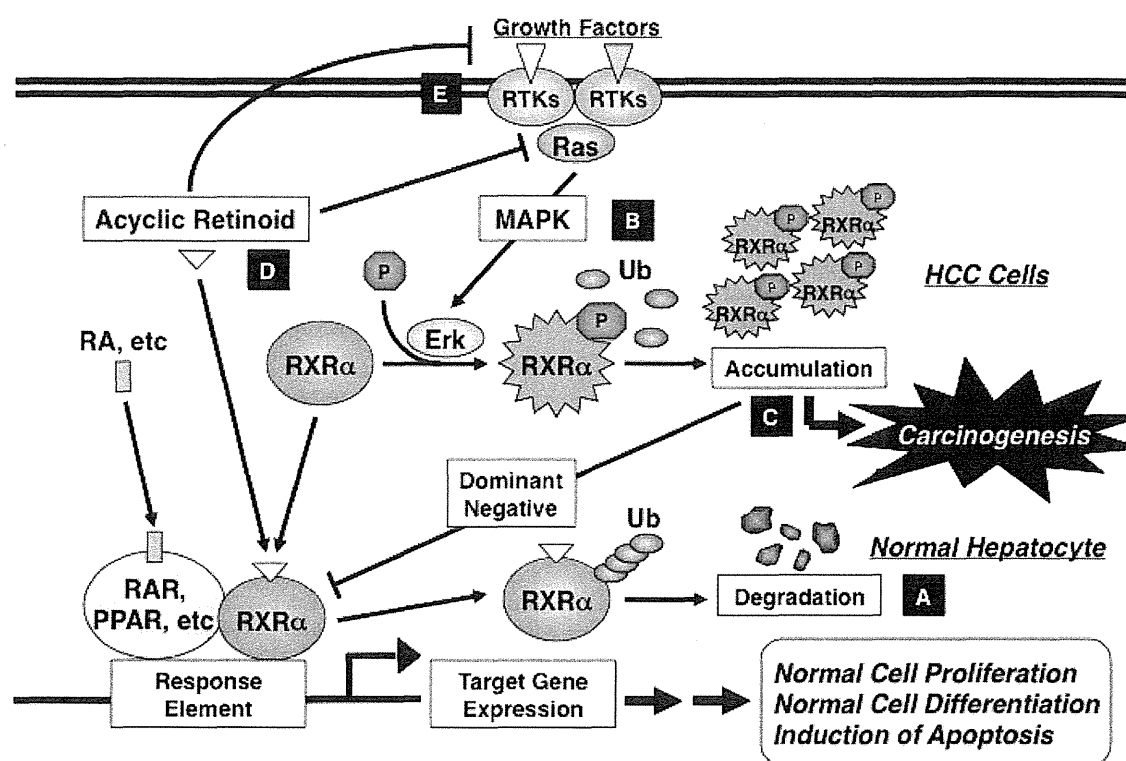
Figure 2. Retinoid-refractoriness due to phosphorylation of RXRα and its restoration by ACR in liver carcinogenesis. In normal hepatocytes, when ACR binds to and activates RXRα, it forms homo- and/or heterodimers with other nuclear receptors including RARs and PPARs, and then activates the expression of target genes that regulate normal cell proliferation and differentiation by binding to the specific response element. Thereafter, RXRα is rapidly ubiquitinated (Ub) and degraded via the proteasome pathway (A). In HCC cells, the Ras-MAPK pathway is highly activated and phosphorylates RXRα at serine residues, thus impairing dimer formation and the subsequent transactivation functions of the receptor (B). Furthermore, non-functional phosphorylated RXRα (p-RXRα) is sequestered from ubiquitin/proteasome-mediated degradation, and accumulates in liver cells, interfering with the physiological function of the remaining unphosphorylated RXRα in a dominant negative manner, thereby playing a critical role in liver carcinogenesis (C). ACR is not only a ligand for RXRα but also suppresses the Ras-MAPK signaling pathway, inhibiting RXRα phosphorylation, restoring the function of the receptor, and thus activating the transcriptional activity of the responsive element (D). ACR also directly or indirectly inhibits the ligand (growth factors)-dependent RTK activities (E), which also contributes to the inhibition of Erk and RXRα phosphorylation and suppression of growth in HCC cells.
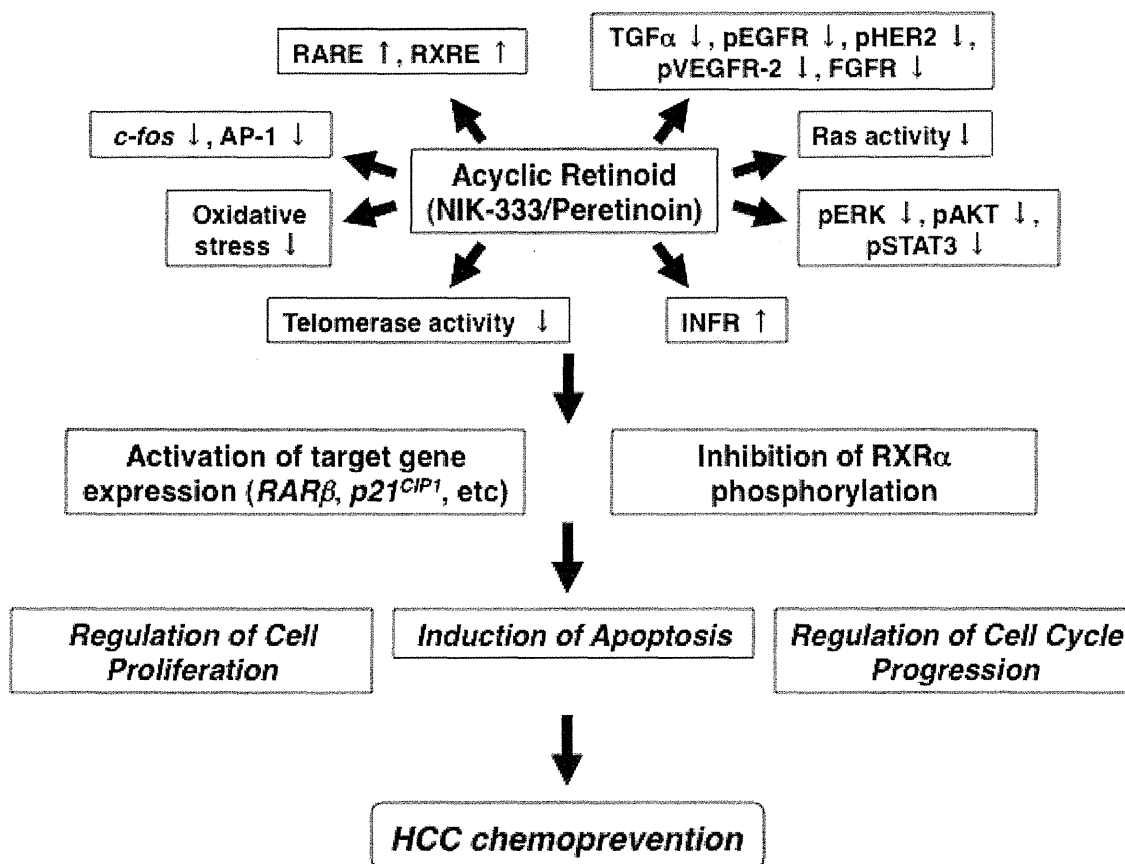
secondary HCC in patients who received anti-cancer treatment for an initial HCC (14-16). In this trial, oral administration of ACR (600 mg per day) for 12 months significantly reduced the incidence of secondary HCC after a median follow-up period of 38 months (P = 0.04) (14), and improved both incidence (P = 0.002) and survival (P = 0.04) after a median follow-up period of 62 months (15). Relative risk of the development of secondary HCC and death were 0.31 (95% confidence interval, 0.12 to 0.78) and 0.33 (0.11 to 0.79), respectively (14, 15). Moreover, the preventive effects of ACR lasted up to 199 weeks after randomization or 151 weeks after completion of ACR administration (16).

A phase II/III trial of ACR confirmed its effectiveness in preventing secondary HCC in hepatitis C

virus-positive patients in a multicenter, large-scale (n = 401) randomized placebo-controlled trial; oral administration of 600 mg of ACR per day was tolerated and had a strong effect on the prevention of secondary HCC with a hazard ratio of 0.27 (0.07 to 0.96) after 2 years (17). The results of these clinical trials suggest that ACR is a novel first-line therapy to reduce the development of secondary HCC.

7. "CLONAL DELETION" THERAPY FOR HCC

Liver carcinogenesis is characteristically multicentric in nature, a phenomenon which is expressed by the term "field cancerization" (51). The poor prognosis for HCC, which is associated with a high incidence of recurrence and development of secondary tumors, is

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RARE ↑, RXRE ↑          TGFα ↓, pEGFR ↓, pHER2 ↓,
                             pVEGFR-2 ↓, FGFR ↓

c-fos ↓, AP-1 ↓                                    Ras activity ↓
                    Acyclic Retinoid
Oxidative           (NIK-333/Peretinoin)      pERK ↓, pAKT ↓,
stress ↓                                          pSTAT3 ↓

        Telomerase activity ↓          INFR ↑


Activation of target gene              Inhibition of RXRα
expression (RARβ, p21^CIP1, etc)       phosphorylation


Regulation of Cell      Induction of Apoptosis      Regulation of Cell Cycle
Proliferation                                              Progression


                    HCC chemoprevention
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Figure 3. Pleiotropic effects of ACR to prevent HCC development. One of the main effects of ACR is to activate the expression of its target genes, such as *RARβ* and *p21^{CIP1}*, by upregulating the promoter activity of RARE and RXRE. In addition, ACR suppresses cancer cell growth by inhibiting activation and expression of some types of RTKs, including EGFR, HER2, VEGFR-2, and FGFR, which contribute to the subsequent inhibition of Ras-MAPK activation and RXRα phosphorylation. Phosphorylation of Akt and Stat3 proteins are also inhibited by ACR. Induction of RARβ and restoration of the function of RXRα due to dephosphorylation by ACR leads to cooperative regulation of cell proliferation, cell cycle progression, and induction of apoptosis, thus preventing the development of HCC. ACR also induces the expression of IFN receptor (INFR), inhibits transcriptional activity of c-*fos* and AP-1 promoters, and down-regulates telomerase activity in HCC and squamous cell carcinoma cells. ACR also suppresses liver tumorigenesis by repressing oxidative stress. Detailed discussion of these findings may be found in previous articles (6-8, 11-13, 36-50, 53, 58, 60-62).

particularly relevant to field cancerization. Once a liver is exposed to continuous carcinogenic insults, such as hepatitis viral infection and alcohol toxicity, the whole exposed liver is regarded as a precancerous lesion which possesses multiple as well as independent premalignant or latent malignant clones. Hence, even if the first cancer is diagnosed and removed early, the next clone essentially arises to form a secondary HCC. Therefore, the most effective strategy for HCC chemoprevention is the deletion of latent malignant clones (clonal deletion) and inhibition of the evolution of such clones (clonal inhibition) before they expand into clinically detectable tumors. We have proposed that implementation of this novel concept, "clonal deletion" therapy, which is defined as the removal of latent malignant (or premalignant) clones that are invisible by

diagnostic imaging from the liver when it is in a hypercarcinogenic state, is fundamental to the chemoprevention of HCC (Figure 4) (6-8).

ACR has been used to effectively demonstrate this concept in the clinical setting. In the clinical trial, serum levels of lectin-reactive α-fetoprotein factor 3 (AFP-L3), which indicates the presence of latent (*i.e.*, invisible) malignant clones in the remnant liver, were significantly reduced by 12-month administration of ACR (52). This observation indicates that ACR eliminates or removes the AFP-L3 producing premalignant clones from the remnant liver before they expanded into clinically detectable (*i.e.*, visible) tumors, thereby inhibiting secondary HCC. Moreover, ACR suppressed the appearance of serum AFP-
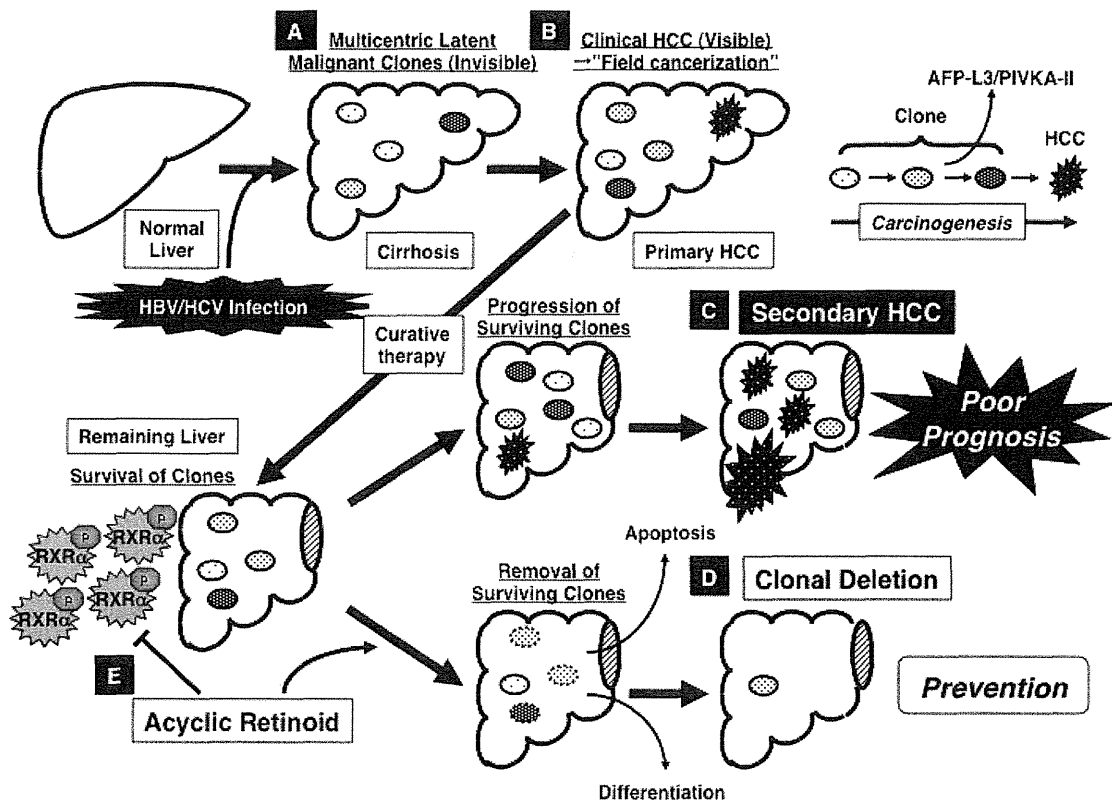
Figure 4. The concept of "clonal deletion" therapy for HCC chemoprevention. Persistent inflammation caused by hepatitis viral infection transforms the liver into a "precancerous field", which consists of multiple latent malignant clones that arise through multicentric carcinogenesis and are clinically undetectable by image analysis (invisible) (A). These multiple clones demonstrate different grades of malignancy in the cirrhotic liver and, at some point, turn into clinical (visible) HCC ("field cancerization") (B). Even when primary HCC is found and removed early, the other clones survive in the remaining liver and grow into secondary HCC, 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 deletion of such transformed clones by inducing cell differentiation or apoptosis before they expand into clinically detectable tumors (the concept of "clonal deletion" therapy) (D). ACR, which targets phosphorylated RXRα (E), prevents the recurrence and development of secondary HCC via the mechanism described by this concept; ACR decreased the serum levels of AFP-L3 and PIVKA-II, which are produced by latent malignant clones, thus demonstrating the eradication and inhibition of these clones. Once such clones are deleted, the preventive effect on HCC lasts several years without continuous administration of ACR. Therefore, ACR can significantly improve the survival rate of such patients.

L3 in patients whose AFP-L3 levels were negative at trial enrollment, whereas the number of patients whose serum AFP-L3 appeared *de novo* was significantly increased in the placebo group; these patients had a significantly higher risk of secondary HCC (52). This finding suggests that, in addition to elimination, ACR actively inhibits the development of AFP-L3-producing clones, which have the potential to become HCC. This is one of the reasons why only a short-term administration (12 months) of ACR exerted a long-term preventive effect on HCC development for several years after termination of treatment (16). It takes several years for the next cancer clones to arise clinically once they are eliminated or inhibited. Therefore, the promise of clonal deletion seems to be therapeutic

rather than preventive, and ACR prevents the development of HCC by this mechanism.

8. "COMBINATION CHEMOPREVENTION" OF HCC USING ACR AS THE KEY DRUG

Combination therapy is often advantageous because it provides the potential for synergistic effects between specific drugs; ACR is no exception in this regard. For instance, ACR acts synergistically with interferon (IFN)-β in suppressing growth and inducing apoptosis in human HCC cell lines via upregulation of type 1 IFN receptor and Stat1 expression by ACR (53). The combination of ACR plus vitamin K_2 (VK_2) synergistically inhibits cell growth and induces apoptosis in HCC cells
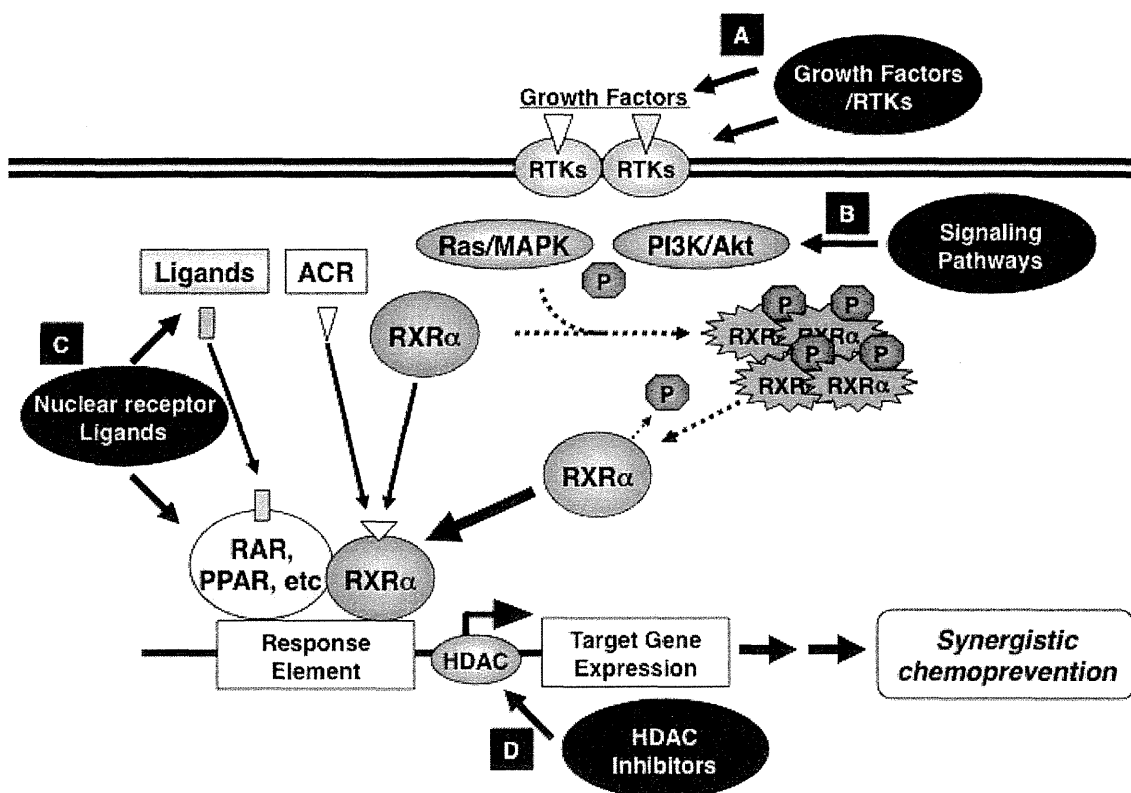
Figure 5. The possibility of "combination chemoprevention" for HCC using ACR as the key agent. Dephosphorylation of RXRα and subsequent restoration of the function of this nuclear receptor are critical to prevent the development of HCC. Therefore, the agents which target growth factor and their corresponding RTKs (A), as well as their related signaling pathways (B), including the Ras-MAPK and PI3K-Akt signaling pathways that phosphorylate RXRα, might be good partners for ACR to exert synergistic effects on the chemoprevention of HCC. The ligands for the nuclear receptors, which form heterodimers with RXR such as RAR and PPAR (C), are also able to enhance 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, which suggests that the combination of ACR plus HDAC inhibitors may also be a promising regimen for HCC chemoprevention (D).

without affecting the growth of normal human hepatocytes (12). 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 (54-57). Therefore, we assume that "combination chemoprevention" using ACR as the key agent may be a useful strategy to prevent the development of HCC.

The expected mechanisms of ACR-based combination chemoprevention are schematically summarized in Figure 5. Initially, specific agents that target the Ras-MAPK signaling pathway and its upstream RTKs are among the most promising partners for ACR because these agents dephosphorylate RXRα. Indeed, ACR and VK_2 cooperatively inhibit activation of the Ras-MAPK signaling pathway, thus suppressing the phosphorylation of RXRα and the growth of HCC cells (12). The combination of 9-cis RA (58) or ACR

(unpublished data) plus trastuzumab, a humanized anti-human epidermal growth factor receptor-2 (HER2) monoclonal antibody, synergistically inhibits growth and induces apoptosis in HCC cells via cooperative inhibition of the activation of HER2 and its downstream signaling molecules, including ERK and Akt, and subsequent dephosphorylation of RXRα. Combined treatment with ACR plus valproic acid, a histone deacetylase (HDAC) inhibitor, acts synergistically to induce apoptosis and G_0-G_1 cell cycle arrest in HCC cells by inhibiting phosphorylation of RXRα, ERK, Akt, and GSK-3β proteins (13).

In addition to dephosphorylation of RXRα, induction of nuclear receptors that dimerize RXR, such as RAR and PPAR (33, 59), and recruitment of their ligands may also exert synergistic growth inhibition in cancer cells when combined with ACR. Both valproic acid (13) and OSI-461 (43), a potent derivative of sulindac sulfone, enhance the ability of ACR to raise the cellular levels of

RARβ and p21^{CIP1}, thereby markedly increasing the RARE and RXRE promoter activities and inducing apoptosis in HCC cells. Therefore, these combinations may also be an effective regimen for the chemoprevention and chemotherapy of HCC.

9. PERSPECTIVE

The prevention of HCC is an urgent task on a global scale, and one of the most practical approaches to the accomplishment of this purpose is "clonal deletion" therapy. Experimental studies strongly suggest that RXRα phosphorylation is profoundly involved in liver carcinogenesis and thus may be a critical target for HCC chemoprevention. Clinical trials reveal that ACR, which inhibits RXRα phosphorylation but induces RARβ expression, is a promising candidate for HCC chemoprevention by putting the concept of "clonal deletion" in practice. ACR-based combination chemoprevention, which is expected to exert synergism, also holds great promise as a master therapeutic for HCC chemoprevention. In conclusion, ACR may play a critical role in preventing HCC development when it is used alone or combined with other drugs and, therefore, early clinical application of this agent is greatly anticipated.

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