

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,

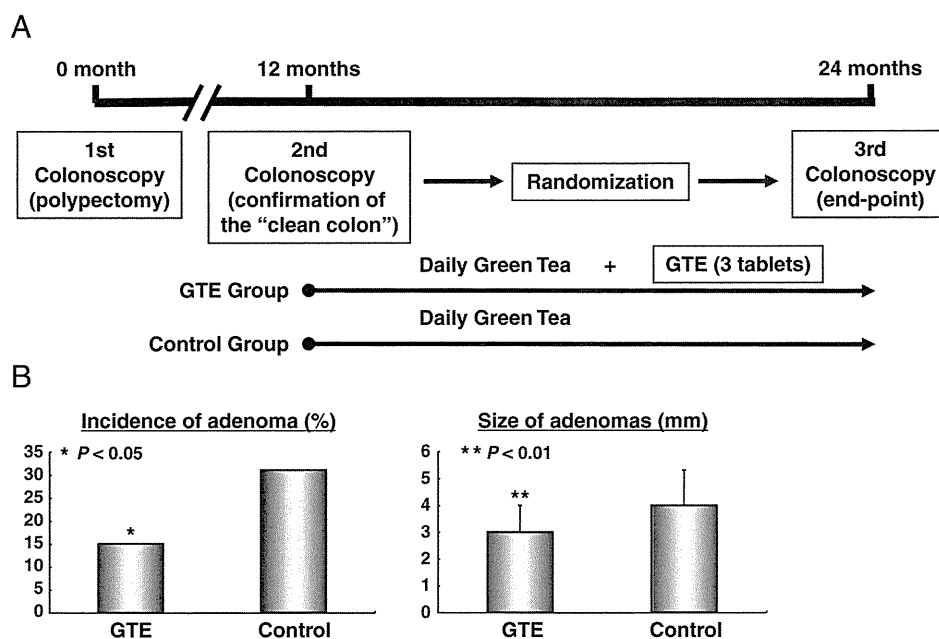


Figure 3. Pilot study revealing the preventive effect of GTE on metachronous adenomas after polypectomy. (A) Study design. The study included 136 participants who underwent endoscopic resection of one or more colorectal adenomas. Twelve months later, the participants received another total colonoscopy 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 chemopreventive 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.

This manuscript is dedicated to our mentor Prof. I. B. Weinstein with loving memories. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (No. 22790638 to M. S. and No. 21590838 to H. M.) and by Grant-in-Aid for the 3rd Term Comprehensive 10-year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan.

The authors have declared no conflict of interest.

12 References

- [1] Yang, C. S., Maliakal, P., Meng, X., Inhibition of carcinogenesis by tea. *Annu. Rev. Pharmacol. Toxicol.* 2002, **42**, 25–54.
- [2] Yang, C. S., Wang, X., Lu, G., Picinich, S. C., Cancer prevention by tea: animal studies, molecular mechanisms and human relevance. *Nat. Rev. Cancer* 2009, **9**, 429–439.
- [3] Shimizu, M., Weinstein, I. B., Modulation of signal transduction by tea catechins and related phytochemicals. *Mutat. Res.* 2005, **591**, 147–160.
- [4] Shimizu, M., Shirakami, Y., Moriwaki, H., Targeting receptor tyrosine kinases for chemoprevention by green tea catechin, EGCG. *Int. J. Mol. Sci.* 2008, **9**, 1034–1049.
- [5] Khan, N., Afaq, F., Saleem, M., Ahmad, N. et al., Targeting multiple signaling pathways by green tea polyphenol (–)-epigallocatechin-3-gallate. *Cancer Res.* 2006, **66**, 2500–2505.
- [6] Okabe, S., Ochiai, Y., Aida, M., Park, K. et al., Mechanistic aspects of green tea as a cancer preventive: effect of components on human stomach cancer cell lines. *Jpn. J. Cancer Res.* 1999, **90**, 733–739.
- [7] Schlessinger, J., Cell signaling by receptor tyrosine kinases. *Cell* 2000, **103**, 211–225.
- [8] Lemmon, M. A., Schlessinger, J., Cell signaling by receptor tyrosine kinases. *Cell* 2010, **141**, 1117–1134.
- [9] Hynes, N. E., Lane, H. A., ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat. Rev. Cancer* 2005, **5**, 341–354.
- [10] Pollak, M. N., Schernhammer, E. S., Hankinson, S. E., Insulin-like growth factors and neoplasia. *Nat. Rev. Cancer* 2004, **4**, 505–518.
- [11] Ellis, L. M., Hicklin, D. J., VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nat. Rev. Cancer* 2008, **8**, 579–591.
- [12] Shimizu, M., Fukutomi, Y., Ninomiya, M., Nagura, K. et al., Green tea extracts for the prevention of metachronous colorectal adenomas: a pilot study. *Cancer Epidemiol. Biomarkers Prev.* 2008, **17**, 3020–3025.
- [13] Shimizu, M., Shirakami, Y., Sakai, H., Adachi, S. et al., (–)-Epigallocatechin gallate suppresses azoxymethane-induced colonic premalignant lesions in male C57BL/Ks J-db/db mice. *Cancer Prev. Res.* 2008, **1**, 298–304.
- [14] Shimizu, M., Sakai, H., Shirakami, Y., Yasuda, Y. et al., Preventive effects of (–)-epigallocatechin gallate on diethylnitrosamine-induced liver tumorigenesis in obese and diabetic C57BL/KsJ-db/db mice. *Cancer Prev. Res.* 2011, **4**, 396–403.
- [15] Shirakami, Y., Shimizu, M., Tsurumi, H., Hara, Y. et al., EGCG and Polyphenon E attenuate inflammation-related mouse colon carcinogenesis induced by AOM plus DDS. *Mol. Med. Rep.* 2008, **1**, 355–361.
- [16] Shirakami, Y., Shimizu, M., Adachi, S., Sakai, H. et al., (–)-Epigallocatechin gallate suppresses the growth of human hepatocellular carcinoma cells by inhibiting activation of the vascular endothelial growth factor-vascular endothelial growth factor receptor axis. *Cancer Sci.* 2009, **100**, 1957–1962.
- [17] Shimizu, M., Shirakami, Y., Sakai, H., Yasuda, Y. et al., (–)-Epigallocatechin gallate inhibits growth and activation of the VEGF/VEGFR axis in human colorectal cancer cells. *Chem. Biol. Interact.* 2010, **185**, 247–252.

- [18] Hou, Z., Sang, S., You, H., Lee, M. J. et al., Mechanism of action of (–)-epigallocatechin-3-gallate: auto-oxidation-dependent inactivation of epidermal growth factor receptor and direct effects on growth inhibition in human esophageal cancer KYSE 150 cells. *Cancer Res.* 2005, *65*, 8049–8056.
- [19] Han, C., Screening of anticarcinogenic ingredients in tea polyphenols. *Cancer Lett.* 1997, *114*, 153–158.
- [20] Rodriguez, S. K., Guo, W., Liu, L., Band, M. A. et al., Green tea catechin, epigallocatechin-3-gallate, inhibits vascular endothelial growth factor angiogenic signaling by disrupting the formation of a receptor complex. *Int. J. Cancer* 2006, *118*, 1635–1644.
- [21] Tachibana, H., Koga, K., Fujimura, Y., Yamada, K., A receptor for green tea polyphenol EGCG. *Nat. Struct. Mol. Biol.* 2004, *11*, 380–381.
- [22] Umeda, D., Yano, S., Yamada, K., Tachibana, H., Green tea polyphenol epigallocatechin-3-gallate signaling pathway through 67-kDa laminin receptor. *J. Biol. Chem.* 2008, *283*, 3050–3058.
- [23] Leone, M., Zhai, D., Sareth, S., Kitada, S. et al., Cancer prevention by tea polyphenols is linked to their direct inhibition of antiapoptotic Bcl-2-family proteins. *Cancer Res.* 2003, *63*, 8118–8121.
- [24] Chung, J. Y., Park, J. O., Phyu, H., Dong, Z., Yang, C. S., Mechanisms of inhibition of the Ras-MAP kinase signaling pathway in 30.7b Ras 12 cells by tea polyphenols (–)-epigallocatechin-3-gallate and theaflavin-3,3'-digallate. *FASEB J.* 2001, *15*, 2022–2024.
- [25] Chung, J. Y., Huang, C., Meng, X., Dong, Z., Yang, C. S., Inhibition of activator protein 1 activity and cell growth by purified green tea and black tea polyphenols in H-ras-transformed cells: structure–activity relationship and mechanisms involved. *Cancer Res.* 1999, *59*, 4610–4617.
- [26] Coffey, R. J., Jr., Shipley, G. D., Moses, H. L., Production of transforming growth factors by human colon cancer lines. *Cancer Res.* 1986, *46*, 1164–1169.
- [27] Markowitz, S. D., Molkentin, K., Gerbic, C., Jackson, J. et al., Growth stimulation by coexpression of transforming growth factor- α and epidermal growth factor-receptor in normal and adenomatous human colon epithelium. *J. Clin. Invest.* 1990, *86*, 356–362.
- [28] Malecka-Panas, E., Kordek, R., Biernat, W., Tureaud, J. et al., Differential activation of total and EGF receptor (EGF-R) tyrosine kinase (tyr-k) in the rectal mucosa in patients with adenomatous polyps, ulcerative colitis and colon cancer. *Hepato-gastroenterology* 1997, *44*, 435–440.
- [29] Ito, Y., Takeda, T., Sakon, M., Tsujimoto, M. et al., Expression and clinical significance of erb-B receptor family in hepatocellular carcinoma. *Br. J. Cancer* 2001, *84*, 1377–1383.
- [30] Hakam, A., Yeatman, T. J., Lu, L., Mora, L. et al., Expression of insulin-like growth factor-1 receptor in human colorectal cancer. *Hum. Pathol.* 1999, *30*, 1128–1133.
- [31] Scharf, J. G., Braulke, T., The role of the IGF axis in hepatocarcinogenesis. *Horm. Metab. Res.* 2003, *35*, 685–693.
- [32] Huynh, H., Chow, P. K., Ooi, L. L., Soo, K. C., A possible role for insulin-like growth factor-binding protein-3 auto-crine/paracrine loops in controlling hepatocellular carcinoma cell proliferation. *Cell Growth Differ.* 2002, *13*, 115–122.
- [33] Aishima, S., Basaki, Y., Oda, Y., Kuroda, Y. et al., High expression of insulin-like growth factor binding protein-3 is correlated with lower portal invasion and better prognosis in human hepatocellular carcinoma. *Cancer Sci.* 2006, *97*, 1182–1190.
- [34] Cascinu, S., Staccioli, M. P., Gasparini, G., Giordani, P. et al., Expression of vascular endothelial growth factor can predict event-free survival in stage II colon cancer. *Clin. Cancer Res.* 2000, *6*, 2803–2807.
- [35] Giatromanolaki, A., Koukourakis, M. I., Sivridis, E., Chlouverakis, G. et al., Activated VEGFR2/KDR pathway in tumour cells and tumour associated vessels of colorectal cancer. *Eur. J. Clin. Invest.* 2007, *37*, 878–886.
- [36] Ng, I. O., Poon, R. T., Lee, J. M., Fan, S. T. et al., Microvessel density, vascular endothelial growth factor and its receptors Flt-1 and Flk-1/KDR in hepatocellular carcinoma. *Am. J. Clin. Pathol.* 2001, *116*, 838–845.
- [37] Dhar, D. K., Naora, H., Yamanoi, A., Ono, T. et al., Requisite role of VEGF receptors in angiogenesis of hepatocellular carcinoma: a comparison with angiopoietin/Tie pathway. *Anticancer Res.* 2002, *22*, 379–386.
- [38] Liang, Y. C., Lin-shiau, S. Y., Chen, C. F., Lin, J. K., Suppression of extracellular signals and cell proliferation through EGF receptor binding by (–)-epigallocatechin gallate in human A431 epidermoid carcinoma cells. *J. Cell Biochem.* 1997, *67*, 55–65.
- [39] Masuda, M., Suzui, M., Weinstein, I. B., Effects of epigallocatechin-3-gallate on growth, epidermal growth factor receptor signaling pathways, gene expression, and chemosensitivity in human head and neck squamous cell carcinoma cell lines. *Clin. Cancer Res.* 2001, *7*, 4220–4229.
- [40] Masuda, M., Suzui, M., Lim, J. T., Deguchi, A. et al., Epigallocatechin-3-gallate decreases VEGF production in head and neck and breast carcinoma cells by inhibiting EGFR-related pathways of signal transduction. *J. Exp. Ther. Oncol.* 2002, *2*, 350–359.
- [41] Masuda, M., Suzui, M., Lim, J. T., Weinstein, I. B., Epigallocatechin-3-gallate inhibits activation of HER-2/neu and downstream signaling pathways in human head and neck and breast carcinoma cells. *Clin. Cancer Res.* 2003, *9*, 3486–3491.
- [42] Shimizu, M., Deguchi, A., Lim, J. T., Moriwaki, H. et al., (–)-Epigallocatechin gallate and polyphenon E inhibit growth and activation of the epidermal growth factor receptor and human epidermal growth factor receptor-2 signaling pathways in human colon cancer cells. *Clin. Cancer Res.* 2005, *11*, 2735–2746.
- [43] Shimizu, M., Deguchi, A., Joe, A. K., McKoy, J. F. et al., EGCG inhibits activation of HER3 and expression of cyclooxygenase-2 in human colon cancer cells. *J. Exp. Ther. Oncol.* 2005, *5*, 69–78.

- [44] Gupta, R. A., Dubois, R. N., Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. *Nat. Rev. Cancer* 2001, 1, 11–21.
- [45] Zahorowska, B., Crowe, P. J., Yang, J. L., Combined therapies for cancer: a review of EGFR-targeted monotherapy and combination treatment with other drugs. *J. Cancer Res. Clin. Oncol.* 2009, 135, 1137–1148.
- [46] Zhang, X., Zhang, H., Tighiouart, M., Lee, J. E. et al., Synergistic inhibition of head and neck tumor growth by green tea (–)-epigallocatechin-3-gallate and EGFR tyrosine kinase inhibitor. *Int. J. Cancer* 2008, 123, 1005–1014.
- [47] Amin, A. R., Khuri, F. R., Chen, Z. G., Shin, D. M., Synergistic growth inhibition of squamous cell carcinoma of the head and neck by erlotinib and epigallocatechin-3-gallate: the role of p53-dependent inhibition of nuclear factor-kappaB. *Cancer Prev. Res.* 2009, 2, 538–545.
- [48] Milligan, S. A., Burke, P., Coleman, D. T., Bigelow, R. L. et al., The green tea polyphenol EGCG potentiates the antiproliferative activity of c-Met and epidermal growth factor receptor inhibitors in non-small cell lung cancer cells. *Clin. Cancer Res.* 2009, 15, 4885–4894.
- [49] Shimizu, M., Deguchi, A., Hara, Y., Moriwaki, H. et al., EGCG inhibits activation of the insulin-like growth factor-1 receptor in human colon cancer cells. *Biochem. Biophys. Res. Commun.* 2005, 334, 947–953.
- [50] Shimizu, M., Shirakami, Y., Sakai, H., Tatebe, H. et al., EGCG inhibits activation of the insulin-like growth factor (IGF)/IGF-1 receptor axis in human hepatocellular carcinoma cells. *Cancer Lett.* 2008, 262, 10–18.
- [51] Adhami, V. M., Siddiqui, I. A., Ahmad, N., Gupta, S. et al., Oral consumption of green tea polyphenols inhibits insulin-like growth factor-I-induced signaling in an autochthonous mouse model of prostate cancer. *Cancer Res.* 2004, 64, 8715–8722.
- [52] Adhami, V. M., Siddiqui, I. A., Sarfaraz, S., Khwaja, S. I. et al., Effective prostate cancer chemopreventive intervention with green tea polyphenols in the TRAMP model depends on the stage of the disease. *Clin. Cancer Res.* 2009, 15, 1947–1953.
- [53] Yasuda, Y., Shimizu, M., Sakai, H., Iwasa, J. et al., (–)-Epigallocatechin gallate prevents carbon tetrachloride-induced rat hepatic fibrosis by inhibiting the expression of the PDGFRbeta and IGF-1R. *Chem. Biol. Interact.* 2009, 182, 159–164.
- [54] Yoshida, H., Tateishi, R., Arakawa, Y., Sata, M. et al., Benefit of interferon therapy in hepatocellular carcinoma prevention for individual patients with chronic hepatitis C. *Gut* 2004, 53, 425–430.
- [55] Shiratori, Y., Imazeki, F., Moriyama, M., Yano, M. et al., Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. *Ann. Intern. Med.* 2000, 132, 517–524.
- [56] Lee, Y. K., Bone, N. D., Strega, A. K., Shanafelt, T. D. et al., VEGF receptor phosphorylation status and apoptosis is modulated by a green tea component, epigallocatechin-3-gallate (EGCG), in B-cell chronic lymphocytic leukemia. *Blood* 2004, 104, 788–794.
- [57] Zhang, Q., Tang, X., Lu, Q., Zhang, Z. et al., Green tea extract and (–)-epigallocatechin-3-gallate inhibit hypoxia- and serum-induced HIF-1alpha protein accumulation and VEGF expression in human cervical carcinoma and hepatoma cells. *Mol. Cancer Ther.* 2006, 5, 1227–1238.
- [58] Jung, Y. D., Kim, M. S., Shin, B. A., Chay, K. O. et al., EGCG, a major component of green tea, inhibits tumour growth by inhibiting VEGF induction in human colon carcinoma cells. *Br. J. Cancer* 2001, 84, 844–850.
- [59] Kataoka, H., Hamasuna, R., Itoh, H., Kitamura, N., Kono, M., Activation of hepatocyte growth factor/scatter factor in colorectal carcinoma. *Cancer Res.* 2000, 60, 6148–6159.
- [60] Takeuchi, H., Bilchik, A., Saha, S., Turner, R. et al., c-MET expression level in primary colon cancer: a predictor of tumor invasion and lymph node metastases. *Clin. Cancer Res.* 2003, 9, 1480–1488.
- [61] Larsen, C. A., Dashwood, R. H., Suppression of Met activation in human colon cancer cells treated with (–)-epigallocatechin-3-gallate: minor role of hydrogen peroxide. *Biochem. Biophys. Res. Commun.* 2009, 389, 527–530.
- [62] Larsen, C. A., Dashwood, R. H., (–)-Epigallocatechin-3-gallate inhibits Met signaling, proliferation, and invasiveness in human colon cancer cells. *Arch. Biochem. Biophys.* 2010, 501, 52–57.
- [63] Sakata, R., Ueno, T., Nakamura, T., Sakamoto, M. et al., Green tea polyphenol epigallocatechin-3-gallate inhibits platelet-derived growth factor-induced proliferation of human hepatic stellate cell line LI90. *J. Hepatol.* 2004, 40, 52–59.
- [64] Kitano, K., Nam, K. Y., Kimura, S., Fujiki, H., Imanishi, Y., Sealing effects of (–)-epigallocatechin gallate on protein kinase C and protein phosphatase 2A. *Biophys. Chem.* 1997, 65, 157–164.
- [65] Kondo, T., Ohta, T., Igura, K., Hara, Y. et al., Tea catechins inhibit angiogenesis in vitro, measured by human endothelial cell growth, migration and tube formation, through inhibition of VEGF receptor binding. *Cancer Lett.* 2002, 180, 139–144.
- [66] Li, M., He, Z., Ermakova, S., Zheng, D. et al., Direct inhibition of insulin-like growth factor-I receptor kinase activity by (–)-epigallocatechin-3-gallate regulates cell transformation. *Cancer Epidemiol. Biomarkers Prev.* 2007, 16, 598–605.
- [67] Sah, J. F., Balasubramanian, S., Eckert, R. L., Rorke, E. A., Epigallocatechin-3-gallate inhibits epidermal growth factor receptor signaling pathway. Evidence for direct inhibition of ERK1/2 and AKT kinases. *J. Biol. Chem.* 2004, 279, 12755–12762.
- [68] Peng, G., Wargovich, M. J., Dixon, D. A., Anti-proliferative effects of green tea polyphenol EGCG on Ha-Ras-induced transformation of intestinal epithelial cells. *Cancer Lett.* 2006, 238, 260–270.
- [69] Lievre, A., Bachet, J. B., Le Corre, D., Boige, V. et al., KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res.* 2006, 66, 3992–3995.

- [70] Ju, J., Hong, J., Zhou, J. N., Pan, Z. et al., Inhibition of intestinal tumorigenesis in Apcmin/+ mice by (–)-epigallocatechin-3-gallate, the major catechin in green tea. *Cancer Res.* 2005, *65*, 10623–10631.
- [71] Sukhthankar, M., Yamaguchi, K., Lee, S. H., McEntee, M. F. et al., A green tea component suppresses posttranslational expression of basic fibroblast growth factor in colorectal cancer. *Gastroenterology* 2008, *134*, 1972–1980.
- [72] Powell, S. M., Zilz, N., Beazer-Barclay, Y., Bryan, T. M. et al., APC mutations occur early during colorectal tumorigenesis. *Nature* 1992, *359*, 235–237.
- [73] Patra, S. K., Dissecting lipid raft facilitated cell signaling pathways in cancer. *Biochim. Biophys. Acta* 2008, *1785*, 182–206.
- [74] Pike, L. J., Han, X., Gross, R. W., Epidermal growth factor receptors are localized to lipid rafts that contain a balance of inner and outer leaflet lipids: a shotgun lipidomics study. *J. Biol. Chem.* 2005, *280*, 26796–26804.
- [75] Remacle-Bonnet, M., Garrouste, F., Baillat, G., Andre, F. et al., Membrane rafts segregate pro- from anti-apoptotic insulin-like growth factor-I receptor signaling in colon carcinoma cells stimulated by members of the tumor necrosis factor superfamily. *Am. J. Pathol.* 2005, *167*, 761–773.
- [76] Oshikawa, J., Urao, N., Kim, H. W., Kaplan, N. et al., Extracellular SOD-derived H₂O₂ promotes VEGF signaling in caveolae/lipid rafts and post-ischemic angiogenesis in mice. *PLoS One* 2010, *5*, e10189.
- [77] Puri, C., Tosoni, D., Comai, R., Rabellino, A. et al., Relationships between EGFR signaling-competent and endocytosis-competent membrane microdomains. *Mol. Biol. Cell* 2005, *16*, 2704–2718.
- [78] Fujimura, Y., Yamada, K., Tachibana, H., A lipid raft-associated 67 kDa laminin receptor mediates suppressive effect of epigallocatechin-3-O-gallate on FcεpsilonRI expression. *Biochem. Biophys. Res. Commun.* 2005, *336*, 674–681.
- [79] Tachibana, H., Fujimura, Y., Yamada, K., Tea polyphenol epigallocatechin-3-gallate associates with plasma membrane lipid rafts: lipid rafts mediate anti-allergic action of the catechin. *Biofactors* 2004, *21*, 383–385.
- [80] Sanjuan, X., Fernandez, P. L., Miquel, R., Munoz, J. et al., Overexpression of the 67-kD laminin receptor correlates with tumour progression in human colorectal carcinoma. *J. Pathol.* 1996, *179*, 376–380.
- [81] Givant-Horwitz, V., Davidson, B., Reich, R., Laminin-induced signaling in tumor cells: the role of the M(r) 67,000 laminin receptor. *Cancer Res.* 2004, *64*, 3572–3579.
- [82] Givant-Horwitz, V., Davidson, B., Reich, R., Laminin-induced signaling in tumor cells. *Cancer Lett.* 2005, *223*, 1–10.
- [83] Tachibana, H., Molecular basis for cancer chemoprevention by green tea polyphenol EGCG. *Forum Nutr.* 2009, *61*, 156–169.
- [84] Adachi, S., Nagao, T., Ingolfsson, H. I., Maxfield, F. R. et al., The inhibitory effect of (–)-epigallocatechin gallate on activation of the epidermal growth factor receptor is associated with altered lipid order in HT29 colon cancer cells. *Cancer Res.* 2007, *67*, 6493–6501.
- [85] Adachi, S., Nagao, T., To, S., Joe, A. K. et al., (–)-Epigallocatechin gallate causes internalization of the epidermal growth factor receptor in human colon cancer cells. *Carcinogenesis* 2008, *29*, 1986–1993.
- [86] Adachi, S., Shimizu, M., Shirakami, Y., Yamauchi, J. et al., (–)-Epigallocatechin gallate downregulates EGF receptor via phosphorylation at Ser1046/1047 by p38 MAPK in colon cancer cells. *Carcinogenesis* 2009, *30*, 1544–1552.
- [87] Masuda, M., Wakasaki, T., Toh, S., Shimizu, M., Adachi, S., Chemoprevention of head and neck cancer by green tea extract: EGCG-the role of EGFR signaling and “lipid raft”. *J. Oncol.* 2011, *2011*, 540148.
- [88] Fujiki, H., Suganuma, M., Imai, K., Nakachi, K., Green tea: cancer preventive beverage and/or drug. *Cancer Lett.* 2002, *188*, 9–13.
- [89] Nakachi, K., Matsuyama, S., Miyake, S., Suganuma, M. et al., Preventive effects of drinking green tea on cancer and cardiovascular disease: epidemiological evidence for multiple targeting prevention. *Biofactors* 2000, *13*, 49–54.
- [90] Gao, Y. T., McLaughlin, J. K., Blot, W. J., Ji, B. T. et al., Reduced risk of esophageal cancer associated with green tea consumption. *J. Natl. Cancer Inst.* 1994, *86*, 855–858.
- [91] Zhang, M., Holman, C. D., Huang, J. P., Xie, X., Green tea and the prevention of breast cancer: a case-control study in Southeast China. *Carcinogenesis* 2007, *28*, 1074–1078.
- [92] Li, N., Sun, Z., Han, C., Chen, J., The chemopreventive effects of tea on human oral precancerous mucosa lesions. *Proc. Soc. Exp. Biol. Med.* 1999, *220*, 218–224.
- [93] Bettuzzi, S., Brausi, M., Rizzi, F., Castagnetti, G. et al., Chemoprevention of human prostate cancer by oral administration of green tea catechins in volunteers with high-grade prostate intraepithelial neoplasia: a preliminary report from a one-year proof-of-principle study. *Cancer Res.* 2006, *66*, 1234–1240.
- [94] McLarty, J., Bigelow, R.L., Smith, M., Elmajian, D. et al., Tea polyphenols decrease serum levels of prostate-specific antigen, hepatocyte growth factor, and vascular endothelial growth factor in prostate cancer patients and inhibit production of hepatocyte growth factor and vascular endothelial growth factor in vitro. *Cancer Prev. Res.* 2009, *2*, 673–682.
- [95] Stoner, G. D., Foodstuffs for preventing cancer: the preclinical and clinical development of berries. *Cancer Prev. Res.* 2009, *2*, 187–194.
- [96] Luo, H., Tang, L., Tang, M., Billam, M. et al., Phase IIa chemoprevention trial of green tea polyphenols in high-risk individuals of liver cancer: modulation of urinary excretion of green tea polyphenols and 8-hydroxydeoxyguanosine. *Carcinogenesis* 2006, *27*, 262–268.
- [97] Tang, L., Tang, M., Xu, L., Luo, H. et al., Modulation of aflatoxin biomarkers in human blood and urine by green tea polyphenols intervention. *Carcinogenesis* 2008, *29*, 411–417.

- [98] El-Serag, H. B., Rudolph, K. L., Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007, 132, 2557–2576.
- [99] Muto, Y., Sato, S., Watanabe, A., Moriwaki, H. et al., Overweight and obesity increase the risk for liver cancer in patients with liver cirrhosis and long-term oral supplementation with branched-chain amino acid granules inhibits liver carcinogenesis in heavier patients with liver cirrhosis. *Hepatol. Res.* 2006, 35, 204–214.
- [100] Kao, Y. H., Chang, H. H., Lee, M. J., Chen, C. L., Tea, obesity, and diabetes. *Mol. Nutr. Food Res.* 2006, 50, 188–210.
- [101] Yang, C. S., Sang, S., Lambert, J. D., Lee, M. J., Bioavailability issues in studying the health effects of plant polyphenolic compounds. *Mol. Nutr. Food Res.* 2008, 52, S139–151.
- [102] Ermakova, S., Choi, B. Y., Choi, H. S., Kang, B. S. et al., The intermediate filament protein vimentin is a new target for epigallocatechin gallate. *J. Biol. Chem.* 2005, 280, 16882–16890.
- [103] He, Z., Tang, F., Ermakova, S., Li, M. et al., Fyn is a novel target of (–)-epigallocatechin gallate in the inhibition of JB6 Cl41 cell transformation. *Mol. Carcinog.* 2008, 47, 172–183.
- [104] Chow, H. H., Cai, Y., Hakim, I. A., Crowell, J. A. et al., Pharmacokinetics and safety of green tea polyphenols after multiple-dose administration of epigallocatechin gallate and polyphenon E in healthy individuals. *Clin. Cancer Res.* 2003, 9, 3312–3319.
- [105] Ahmad, N., Gupta, S., Mukhtar, H., Green tea polyphenol epigallocatechin-3-gallate differentially modulates nuclear factor kappaB in cancer cells versus normal cells. *Arch. Biochem. Biophys.* 2000, 376, 338–346.
- [106] Weinstein, I. B., Cancer. Addiction to oncogenes – the Achilles heal of cancer. *Science* 2002, 297, 63–64.
- [107] Weinstein, I. B., Joe, A., Oncogene addiction. *Cancer Res.* 2008, 68, 3077–3080.

Chemoprevention of hepatocellular carcinoma by acyclic retinoid

Masahito Shimizu, Hiroyasu Sakai, Hisataka Moriwaki

Department of Medicine, Gifu University Graduate School of Medicine, Gifu, Japan 501-1194

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Retinoids and their receptors
4. Abnormalities in the retinoid/retinoid receptor axis and HCC
5. ACR in HCC chemoprevention: Experimental studies
6. ACR in HCC chemoprevention: Clinical studies
7. "Clonal deletion" therapy for HCC
8. "Combination chemoprevention" of HCC using ACR as the key drug
9. Perspective
10. Acknowledgements
11. References

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 RXR α 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 RXR α 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,

Chemoprevention of HCC by ACR

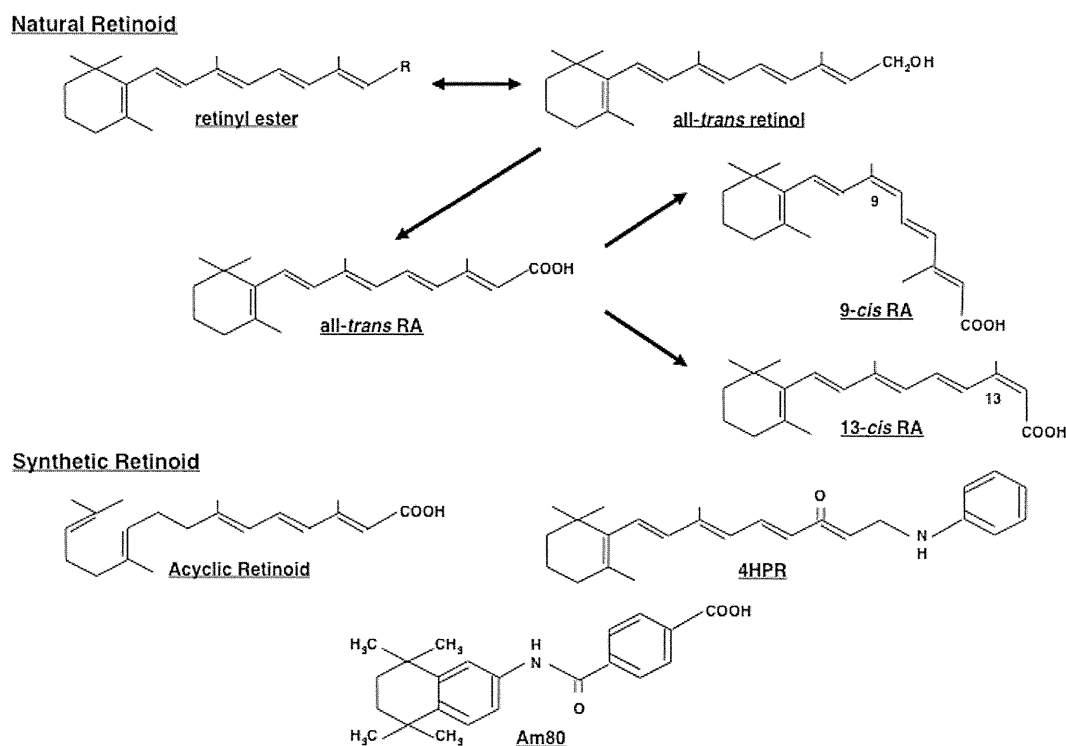


Figure 1. Chemical structures of natural and representative synthetic retinoids. Retinyl esters (mainly retinyl palmitate, R: fatty acid), stored in the liver stellate cells, are hydrolyzed to retinol. Retinoic acid (RA) is biosynthesized from retinol via the intermediate metabolite retinal by oxidation 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 (RXR α) 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 RXR α (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 RXR α 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 RXR α phosphorylation and induction of RAR β 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

Chemoprevention of HCC by ACR

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 RXR α and RAR β , are strongly associated with the development of various human malignancies including HCC. For instance, the *RAR β* 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 RAR β protein and mRNA levels are also decreased in HCC (25). These findings are interesting because among the retinoid receptors, RAR β is thought to be one of the most important receptors in the regulation of cell growth and apoptosis (26).

The expression of RXR α 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 RXR α 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 RXR α (p-RXR α) (27). Specifically, RXR α 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 RXR α , 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 RXR α in a ligand-dependent manner (9, 11). In addition, although RXR α is unphosphorylated and highly ubiquitinated in a normal liver, rendering it sensitive to proteasome-mediated degradation, p-RXR α is resistant to ubiquitination and proteasome-mediated degradation in both human HCC tissues and a human HCC cell line (28). Furthermore, the phosphorylation of RXR α abolishes its ability to form heterodimers with RAR β , and this is associated with uncontrolled cell growth and resistance to

retinoids (29). These findings suggest that the accumulation of p-RXR α , (*i.e.*, non-functional RXR α) may interfere with the function of normal RXR α 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 RXR α in the negative modulation of its heterodimeric binding partners (30-32). Therefore, the inhibition of RXR α 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 RAR β expression (13, 36, 39-44). In human HCC and squamous carcinoma cells, ACR causes cell cycle arrest in G₀-G₁, 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 RAR β 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 RXR α , thus contributing to the development of HCC (9, 27). In addition, ACR also restores RXR α function by inactivating the Ras-MAPK signaling system, leading to the dephosphorylation of RXR α , although 9-*cis* RA failed to suppress ERK and RXR α phosphorylation (11). Therefore, ACR, which targets the RTK-Ras-MAPK signaling pathway and RXR α phosphorylation, is a promising agent for the chemoprevention of HCC. The role of RXR α 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

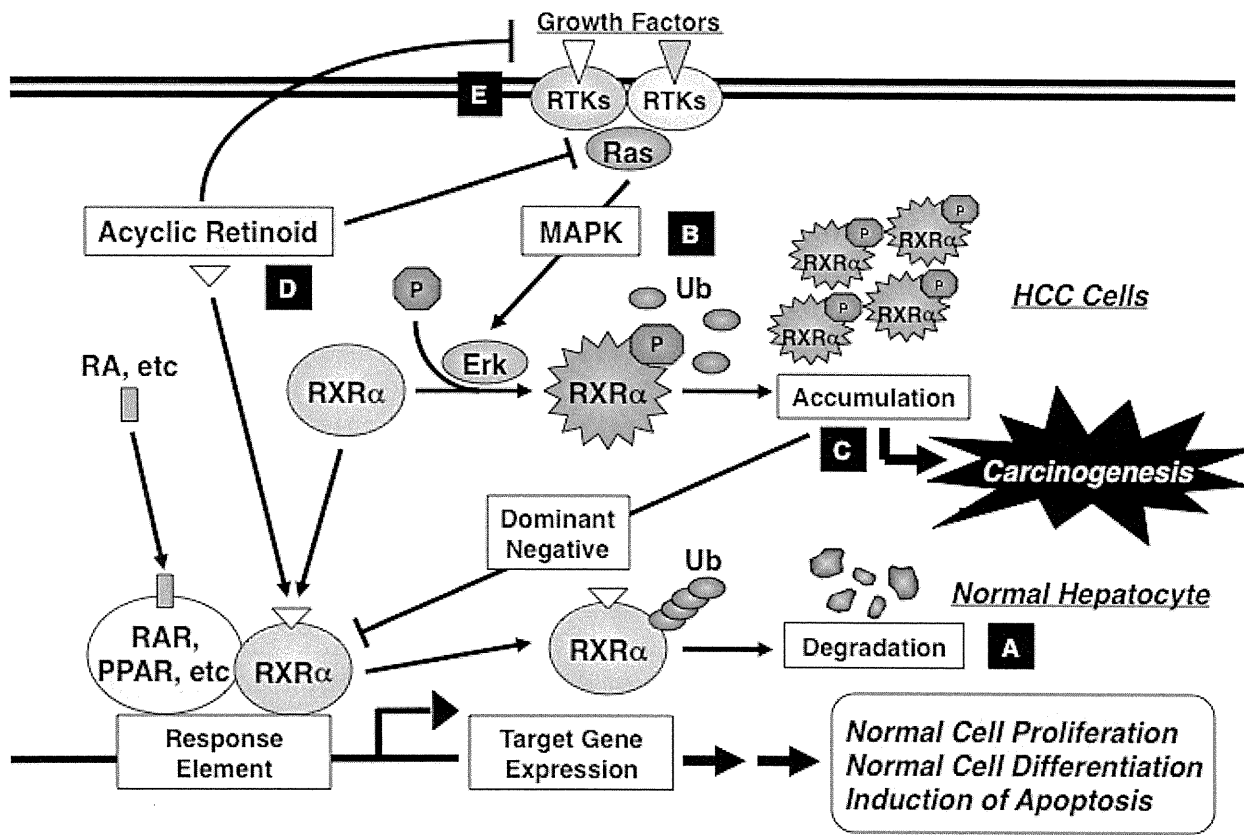


Figure 2. Retinoid-refractoriness due to phosphorylation of RXR α and its restoration by ACR in liver carcinogenesis. In normal hepatocytes, when ACR binds to and activates RXR α , it forms homo- and/or heterodimers with other nuclear receptors including RARs and PPARs, and then activates the expression of target genes that regulate normal cell proliferation and differentiation by binding to the specific response element. Thereafter, RXR α is rapidly ubiquitinated (Ub) and degraded via the proteasome pathway (A). In HCC cells, the Ras-MAPK pathway is highly activated and phosphorylates RXR α at serine residues, thus impairing dimer formation and the subsequent transactivation functions of the receptor (B). Furthermore, non-functional phosphorylated RXR α (p-RXR α) is sequestered from ubiquitin/proteasome-mediated degradation, and accumulates in liver cells, interfering with the physiological function of the remaining unphosphorylated RXR α in a dominant negative manner, thereby playing a critical role in liver carcinogenesis (C). ACR is not only a ligand for RXR α but also suppresses the Ras-MAPK signaling pathway, inhibiting RXR α 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 RXR α 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

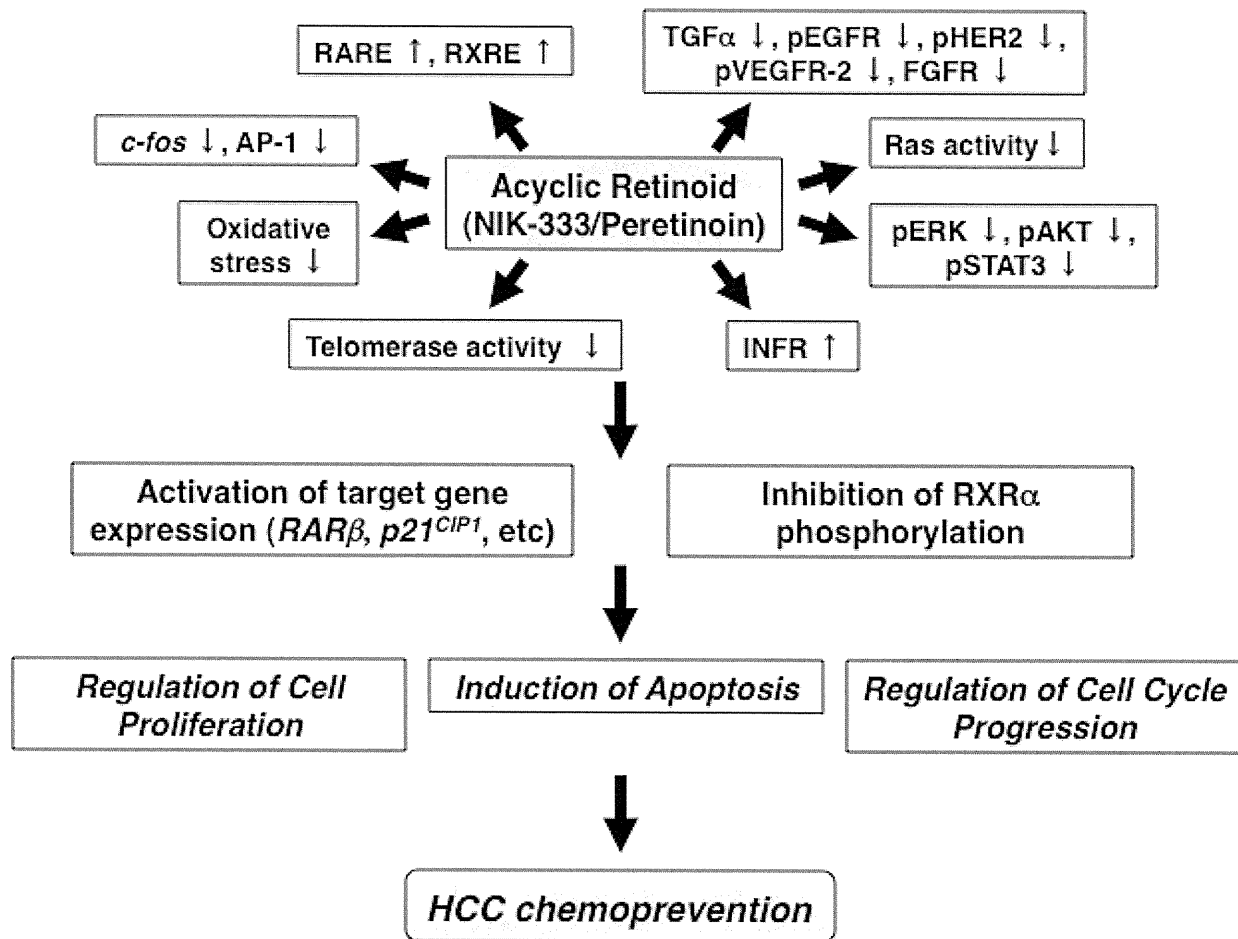


Figure 3. Pleiotropic effects of ACR to prevent HCC development. One of the main effects of ACR is to activate the expression of its target genes, such as *RARβ* and *p21^{CIP1}*, by upregulating the promoter activity of RARE and RXRE. In addition, ACR suppresses cancer cell growth by inhibiting activation 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-

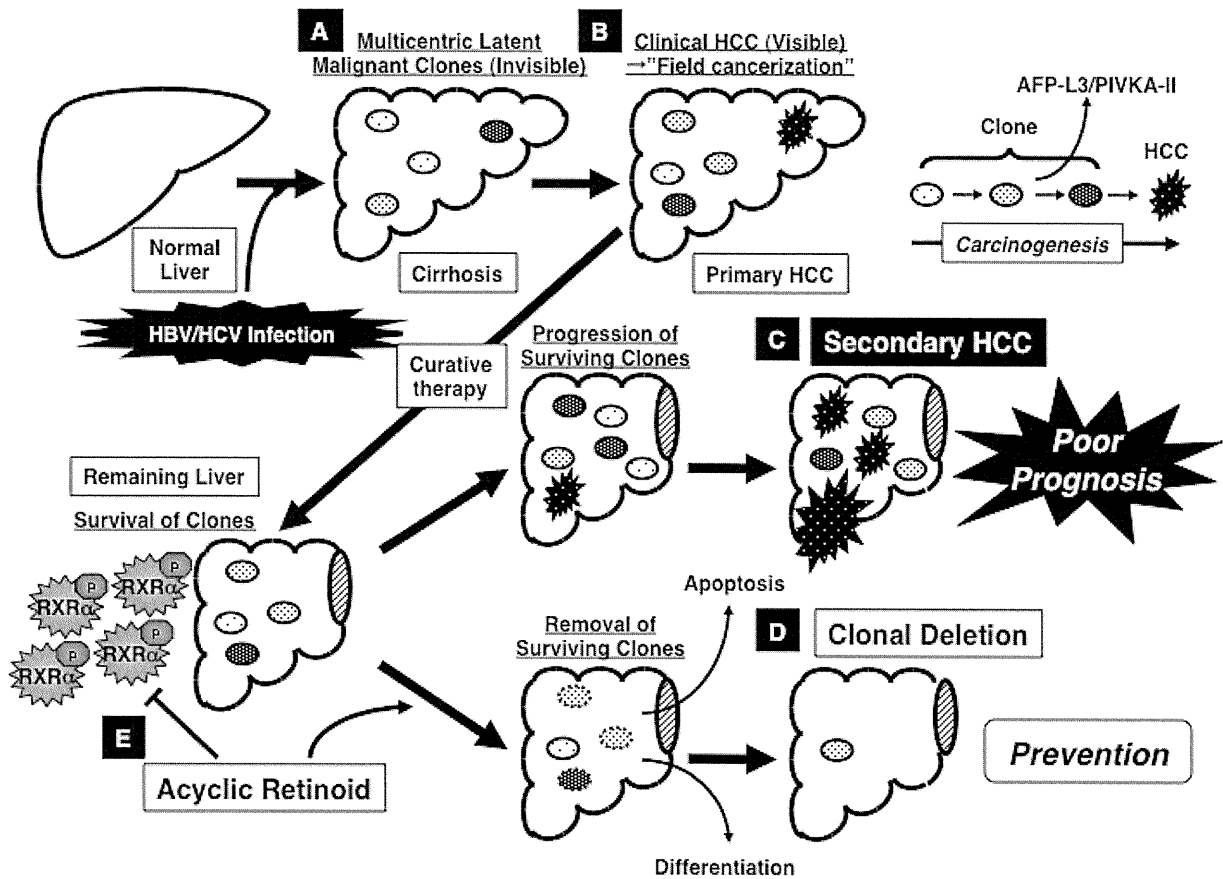


Figure 4. The concept of “clonal deletion” therapy for HCC chemoprevention. Persistent inflammation caused by hepatitis viral infection transforms the liver into a “precancerous field”, which consists of multiple latent malignant clones that arise through multicentric carcinogenesis and are clinically 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 RXR α (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 K₂ (VK₂) synergistically inhibits cell growth and induces apoptosis in HCC cells

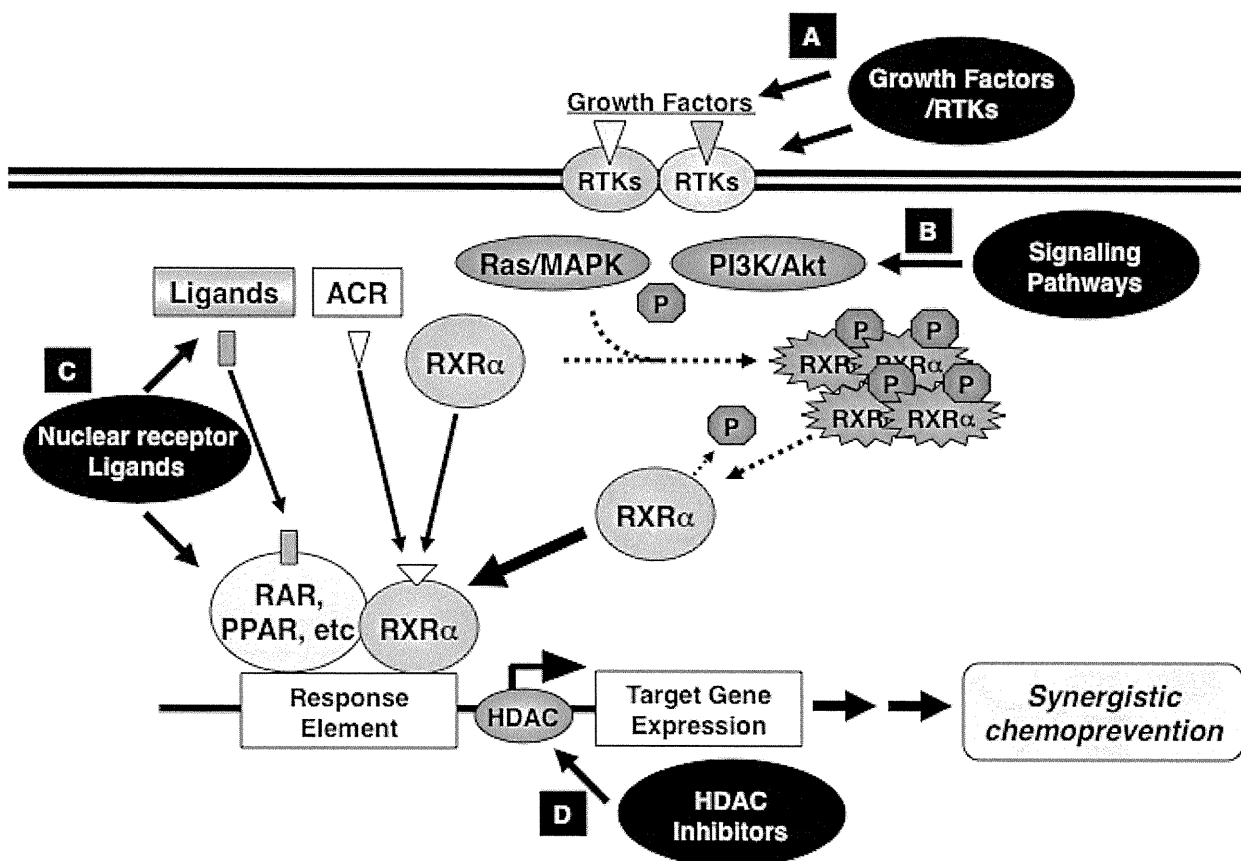


Figure 5. The possibility of “combination chemoprevention” for HCC using ACR as the key agent. Dephosphorylation of RXR α and subsequent restoration of the function of this nuclear receptor are critical to prevent the development of HCC. Therefore, the agents which target growth factor and their corresponding RTKs (A), as well as their related signaling pathways (B), including the Ras-MAPK and PI3K-Akt signaling pathways that phosphorylate RXR α , 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 VK₂ 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 RXR α . Indeed, ACR and VK₂ cooperatively inhibit activation of the Ras-MAPK signaling pathway, thus suppressing the phosphorylation of RXR α 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 RXR α . Combined treatment with ACR plus valproic acid, a histone deacetylase (HDAC) inhibitor, acts synergistically to induce apoptosis and G₀-G₁ cell cycle arrest in HCC cells by inhibiting phosphorylation of RXR α , ERK, Akt, and GSK-3 β proteins (13).

In addition to dephosphorylation of RXR α , 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

Chemoprevention of HCC by ACR

RAR β 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 RXR α phosphorylation is profoundly involved in liver carcinogenesis and thus may be a critical target for HCC chemoprevention. Clinical trials reveal that ACR, which inhibits RXR α phosphorylation but induces RAR β 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.

10. ACKNOWLEDGEMENTS

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (No. 18790457 to M. S. and No. 17015016 to H. M.).

11. REFERENCES

1. El-Serag, H. B.; Rudolph, K. L. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 132:2557-2576 (2007)
2. Parikh, S.; Hyman, D. Hepatocellular cancer: a guide for the internist. *Am J Med* 120:194-202 (2007)
3. Ince, N.; Wands, J. R. The increasing incidence of hepatocellular carcinoma. *N Engl J Med* 340:798-799 (1999)
4. Kumada, T.; Nakano, S.; Takeda, I.; Sugiyama, K.; Osada, T.; Kiriya, S.; Sone, Y.; Toyoda, H.; Shimada, S.; Takahashi, M.; Sassa, T. Patterns of recurrence after initial treatment in patients with small hepatocellular carcinoma. *Hepatology* 25:87-92 (1997)
5. Koda, M.; Murawaki, Y.; Mitsuda, A.; Ohyama, K.; Horie, Y.; Suou, T.; Kawasaki, H.; Ikawa, S. Predictive factors for intrahepatic recurrence after percutaneous ethanol injection therapy for small hepatocellular carcinoma. *Cancer* 88:529-537 (2000)
6. Shimizu, M.; Takai, K.; Moriwaki, H. Strategy and mechanism for the prevention of hepatocellular carcinoma: phosphorylated retinoid X receptor alpha is a critical target for hepatocellular carcinoma chemoprevention. *Cancer Sci* 100:369-374 (2009)
7. Moriwaki, H.; Shimizu, M.; Okuno, M.; Nishiwaki-Matsushima, R. Chemoprevention of liver carcinogenesis with retinoids: Basic and clinical aspects. *Hepatol Res* 37 Suppl 2:S299-302 (2007)
8. Okuno, M.; Kojima, S.; Akita, K.; Matsushima-Nishiwaki, R.; Adachi, S.; Sano, T.; Takano, Y.; Takai, K.; Obara, A.; Yasuda, I.; Shiratori, Y.; Okano, Y.; Shimada, J.; Suzuki, Y.; Muto, Y.; Moriwaki, H. Retinoids in liver fibrosis and cancer. *Front Biosci* 7:d204-218 (2002)
9. Matsushima-Nishiwaki, R.; Okuno, M.; Adachi, S.; Sano, T.; Akita, K.; Moriwaki, H.; Friedman, S. L.; Kojima, S. Phosphorylation of retinoid X receptor alpha at serine 260 impairs its metabolism and function in human hepatocellular carcinoma. *Cancer Res* 61:7675-7682 (2001)
10. Sporn, M. B.; Newton, D. L. Chemoprevention of cancer with retinoids. *Fed Proc* 38:2528-2534 (1979)
11. Matsushima-Nishiwaki, R.; Okuno, M.; Takano, Y.; Kojima, S.; Friedman, S. L.; Moriwaki, H. Molecular mechanism for growth suppression of human hepatocellular carcinoma cells by acyclic retinoid. *Carcinogenesis* 24:1353-1359 (2003)
12. Kanamori, T.; Shimizu, M.; Okuno, M.; Matsushima-Nishiwaki, R.; Tsurumi, H.; Kojima, S.; Moriwaki, H. Synergistic growth inhibition by acyclic retinoid and vitamin K2 in human hepatocellular carcinoma cells. *Cancer Sci* 98:431-437 (2007)
13. Tatebe, H.; Shimizu, M.; Shirakami, Y.; Sakai, K.; Yasuda, Y.; Tsurumi, H.; Moriwaki, H. Acyclic retinoid synergises with valproic acid to inhibit growth in human hepatocellular carcinoma cells. *Cancer Lett* 285:210-217 (2009)
14. Muto, Y.; Moriwaki, H.; Ninomiya, M.; Adachi, S.; Saito, A.; Takasaki, K. T.; Tanaka, T.; Tsurumi, K.; Okuno, M.; Tomita, E.; Nakamura, T.; Kojima, T. Prevention of second primary tumors by an acyclic retinoid, polyprenoic acid, in patients with hepatocellular carcinoma. Hepatoma Prevention Study Group. *N Engl J Med* 334:1561-1567 (1996)
15. Muto, Y.; Moriwaki, H.; Saito, A. Prevention of second primary tumors by an acyclic retinoid in patients with hepatocellular carcinoma. *N Engl J Med* 340:1046-1047 (1999)
16. Takai, K.; Okuno, M.; Yasuda, I.; Matsushima-Nishiwaki, R.; Uematsu, T.; Tsurumi, H.; Shiratori, Y.; Muto, Y.; Moriwaki, H. Prevention of second primary tumors by an acyclic retinoid in patients with hepatocellular carcinoma. Updated analysis of the long-term follow-up data. *Intervirol* 48:39-45 (2005)
17. Okita, K.; Matsui, O.; Kumada, H.; Tanaka, K.; Kaneko, S.; Moriwaki, H.; Izumi, N.; Okusaka, T.; Ohashi, Y.; Makuuchi, M. Effect of peretinoin on recurrence of

Chemoprevention of HCC by ACR

hepatocellular carcinoma (HCC): Results of a phase II/III randomized placebo-controlled trial. Peretinoin Study Group. *J Clin Oncol* 28 Suppl 7s:4024 (2010)

18. Germain, P.: Chambon, P.: Eichele, G.: Evans, R. M.: Lazar, M. A.: Leid, M.: De Lera, A. R.: Lotan, R.: Mangelsdorf, D. J.: Gronemeyer, H. International Union of Pharmacology. LX. Retinoic acid receptors. *Pharmacol Rev* 58:712-725 (2006)

19. Germain, P.: Chambon, P.: Eichele, G.: Evans, R. M.: Lazar, M. A.: Leid, M.: De Lera, A. R.: Lotan, R.: Mangelsdorf, D. J.: Gronemeyer, H. International Union of Pharmacology. LXIII. Retinoid X receptors. *Pharmacol Rev* 58:760-772 (2006)

20. Michalik, L.: Auwerx, J.: Berger, J. P.: Chatterjee, V. K.: Glass, C. K.: Gonzalez, F. J.: Grimaldi, P. A.: Kadowaki, T.: Lazar, M. A.: O'Rahilly, S.: Palmer, C. N.: Plutzky, J.: Reddy, J. K.: Spiegelman, B. M.: Staels, B.: Wahli, W. International Union of Pharmacology. LXI. Peroxisome proliferator-activated receptors. *Pharmacol Rev* 58:726-741 (2006)

21. Altucci, L.: Gronemeyer, H. The promise of retinoids to fight against cancer. *Nat Rev Cancer* 1:181-193 (2001)

22. Altucci, L.: Leibowitz, M. D.: Ogilvie, K. M.: De Lera, A. R.: Gronemeyer, H. RAR and RXR modulation in cancer and metabolic disease. *Nat Rev Drug Discov* 6:793-810 (2007)

23. de The, H.: Marchio, A.: Tiollais, P.: Dejean, A. A novel steroid thyroid hormone receptor-related gene inappropriately expressed in human hepatocellular carcinoma. *Nature* 330:667-670 (1987)

24. Sever, C. E.: Locker, J. Expression of retinoic acid alpha and beta receptor genes in liver and hepatocellular carcinoma. *Mol Carcinog* 4:138-144 (1991)

25. Ando, N.: Shimizu, M.: Okuno, M.: Matsushima-Nishiwaki, R.: Tsurumi, H.: Tanaka, T.: Moriwaki, H. Expression of retinoid X receptor alpha is decreased in 3'-methyl-4-dimethylaminoazobenzene-induced hepatocellular carcinoma in rats. *Oncol Rep* 18:879-884 (2007)

26. Alvarez, S.: Germain, P.: Alvarez, R.: Rodriguez-Barrios, F.: Gronemeyer, H.: de Lera, A. R. Structure, function and modulation of retinoic acid receptor beta, a tumor suppressor. *Int J Biochem Cell Biol* 39:1406-1415 (2007)

27. Matsushima-Nishiwaki, R.: Shidoji, Y.: Nishiwaki, S.: Yamada, T.: Moriwaki, H.: Muto, Y. Aberrant metabolism of retinoid X receptor proteins in human hepatocellular carcinoma. *Mol Cell Endocrinol* 121:179-190 (1996)

28. Adachi, S.: Okuno, M.: Matsushima-Nishiwaki, R.: Takano, Y.: Kojima, S.: Friedman, S. L.: Moriwaki, H.: Okano, Y. Phosphorylation of retinoid X receptor

suppresses its ubiquitination in human hepatocellular carcinoma. *Hepatology* 35:332-340 (2002)

29. Yoshimura, K.: Muto, Y.: Shimizu, M.: Matsushima-Nishiwaki, R.: Okuno, M.: Takano, Y.: Tsurumi, H.: Kojima, S.: Okano, Y.: Moriwaki, H. Phosphorylated retinoid X receptor alpha loses its heterodimeric activity with retinoic acid receptor beta. *Cancer Sci* 98:1868-1874 (2007)

30. Lee, H. Y.: Suh, Y. A.: Robinson, M. J.: Clifford, J. L.: Hong, W. K.: Woodgett, J. R.: Cobb, M. H.: Mangelsdorf, D. J.: Kurie, J. M. Stress pathway activation induces phosphorylation of retinoid X receptor. *J Biol Chem* 275:32193-32199 (2000)

31. Solomon, C.: White, J. H.: Kremer, R. Mitogen-activated protein kinase inhibits 1,25-dihydroxyvitamin D3-dependent signal transduction by phosphorylating human retinoid X receptor alpha. *J Clin Invest* 103:1729-1735 (1999)

32. Macoritto, M.: Nguyen-Yamamoto, L.: Huang, D. C.: Samuel, S.: Yang, X. F.: Wang, T. T.: White, J. H.: Kremer, R. Phosphorylation of the human retinoid X receptor alpha at serine 260 impairs coactivator(s) recruitment and induces hormone resistance to multiple ligands. *J Biol Chem* 283:4943-4956 (2008)

33. Yamazaki, K.: Shimizu, M.: Okuno, M.: Matsushima-Nishiwaki, R.: Kanemura, N.: Araki, H.: Tsurumi, H.: Kojima, S.: Weinstein, I. B.: Moriwaki, H. Synergistic effects of RXR alpha and PPAR gamma ligands to inhibit growth in human colon cancer cells -phosphorylated RXR alpha is a critical target for colon cancer management. *Gut* 56:1557-1563 (2007)

34. Kanemura, N.: Tsurumi, H.: Okuno, M.: Matsushima-Nishiwaki, R.: Shimizu, M.: Moriwaki, H. Retinoid X receptor alpha is highly phosphorylated in retinoic acid-resistant HL-60R cells and the combination of 9-cis retinoic acid plus MEK inhibitor induces apoptosis in the cells. *Leuk Res* 32:884-892 (2008)

35. Lattuada, D.: Vigano, P.: Mangioni, S.: Sassone, J.: Di Francesco, S.: Vignali, M.: Di Blasio, A. M. Accumulation of retinoid X receptor-alpha in uterine leiomyomas is associated with a delayed ligand-dependent proteasome-mediated degradation and an alteration of its transcriptional activity. *Mol Endocrinol* 21:602-612 (2007)

36. Yamada, Y.: Shidoji, Y.: Fukutomi, Y.: Ishikawa, T.: Kaneko, T.: Nakagama, H.: Imawari, M.: Moriwaki, H.: Muto, Y. Positive and negative regulations of albumin gene expression by retinoids in human hepatoma cell lines. *Mol Carcinog* 10:151-158 (1994)

37. Araki, H.: Shidoji, Y.: Yamada, Y.: Moriwaki, H.: Muto, Y. Retinoid agonist activities of synthetic geranyl geranoic acid derivatives. *Biochem Biophys Res Commun* 209:66-72 (1995)

Chemoprevention of HCC by ACR

38. Muto, Y.: Moriwaki, H. Antitumor activity of vitamin A and its derivatives. *J Natl Cancer Inst* 73:1389-1393 (1984)
39. Fukutomi, Y.: Omori, M.: Muto, Y.: Ninomiya, M.: Okuno, M.: Moriwaki, H. Inhibitory effects of acyclic retinoid (polyprenonic acid) and its hydroxy derivative on cell growth and on secretion of alpha-fetoprotein in human hepatoma-derived cell line (PLC/PRF/5). *Jpn J Cancer Res* 81:1281-1285 (1990)
40. Nakamura, N.: Shidoji, Y.: Yamada, Y.: Hatakeyama, H.: Moriwaki, H.: Muto, Y. Induction of apoptosis by acyclic retinoid in the human hepatoma-derived cell line, HuH-7. *Biochem Biophys Res Commun* 207:382-388 (1995)
41. Nakamura, N.: Shidoji, Y.: Moriwaki, H.: Muto, Y. Apoptosis in human hepatoma cell line induced by 4,5-didehydro geranylgeranoic acid (acyclic retinoid) via down-regulation of transforming growth factor-alpha. *Biochem Biophys Res Commun* 219:100-104 (1996)
42. Yasuda, I.: Shiratori, Y.: Adachi, S.: Obora, A.: Takemura, M.: Okuno, M.: Shidoji, Y.: Seishima, M.: Muto, Y.: Moriwaki, H. Acyclic retinoid induces partial differentiation, down-regulates telomerase reverse transcriptase mRNA expression and telomerase activity, and induces apoptosis in human hepatoma-derived cell lines. *J Hepatol* 36:660-671 (2002)
43. Shimizu, M.: Suzui, M.: Deguchi, A.: Lim, J. T.: Xiao, D.: Hayes, J. H.: Papadopoulos, K. P.: Weinstein, I. B. Synergistic effects of acyclic retinoid and OSI-461 on growth inhibition and gene expression in human hepatoma cells. *Clin Cancer Res* 10:6710-6721 (2004)
44. Suzui, M.: Shimizu, M.: Masuda, M.: Lim, J. T.: Yoshimi, N.: Weinstein, I. B. Acyclic retinoid activates retinoic acid receptor beta and induces transcriptional activation of p21(CIP1) in HepG2 human hepatoma cells. *Mol Cancer Ther* 3:309-316 (2004)
45. Suzui, M.: Masuda, M.: Lim, J. T.: Albanese, C.: Pestell, R. G.: Weinstein, I. B. Growth inhibition of human hepatoma cells by acyclic retinoid is associated with induction of p21(CIP1) and inhibition of expression of cyclin D1. *Cancer Res* 62:3997-4006 (2002)
46. Shimizu, M.: Suzui, M.: Deguchi, A.: Lim, J. T.: Weinstein, I. B. Effects of acyclic retinoid on growth, cell cycle control, epidermal growth factor receptor signaling, and gene expression in human squamous cell carcinoma cells. *Clin Cancer Res* 10:1130-1140 (2004)
47. Kagawa, M.: Sano, T.: Ishibashi, N.: Hashimoto, M.: Okuno, M.: Moriwaki, K.: Suzuki, R.: Kohno, H.: Tanaka, T. An acyclic retinoid, NIK-333, inhibits N-diethylnitrosamine-induced rat hepatocarcinogenesis through suppression of TGF-alpha expression and cell proliferation. *Carcinogenesis* 25:979-985 (2004)
48. Sano, T.: Kagawa, M.: Okuno, M.: Ishibashi, N.: Hashimoto, M.: Yamamoto, M.: Suzuki, R.: Kohno, H.: Matsushima-Nishiwaki, R.: Takano, Y.: Tsurumi, H.: Kojima, S.: Friedman, S. L.: Moriwaki, H.: Tanaka, T. Prevention of rat hepatocarcinogenesis by acyclic retinoid is accompanied by reduction in emergence of both TGF-alpha-expressing oval-like cells and activated hepatic stellate cells. *Nutr Cancer* 51:197-206 (2005)
49. Shao, R. X.: Otsuka, M.: Kato, N.: Taniguchi, H.: Hoshida, Y.: Moriyama, M.: Kawabe, T.: Omata, M. Acyclic retinoid inhibits human hepatoma cell growth by suppressing fibroblast growth factor-mediated signaling pathways. *Gastroenterology* 128:86-95 (2005)
50. Komi, Y.: Sogabe, Y.: Ishibashi, N.: Sato, Y.: Moriwaki, H.: Shimokado, K.: Kojima, S. Acyclic retinoid inhibits angiogenesis by suppressing the MAPK pathway. *Lab Invest* 90:52-60 (2010)
51. Slaughter, D. P.: Southwick, H. W.: Smejkal, W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer* 6:963-968 (1953)
52. Moriwaki, H.: Yasuda, I.: Shiratori, Y.: Uematsu, T.: Okuno, M.: Muto, Y. Deletion of serum lectin-reactive alpha-fetoprotein by acyclic retinoid: a potent biomarker in the chemoprevention of second primary hepatoma. *Clin Cancer Res* 3:727-731 (1997)
53. Obora, A.: Shiratori, Y.: Okuno, M.: Adachi, S.: Takano, Y.: Matsushima-Nishiwaki, R.: Yasuda, I.: Yamada, Y.: Akita, K.: Sano, T.: Shimada, J.: Kojima, S.: Okano, Y.: Friedman, S. L.: Moriwaki, H. Synergistic induction of apoptosis by acyclic retinoid and interferon-beta in human hepatocellular carcinoma cells. *Hepatology* 36:1115-1124 (2002)
54. Habu, D.: Shiomi, S.: Tamori, A.: Takeda, T.: Tanaka, T.: Kubo, S.: Nishiguchi, S. Role of vitamin K2 in the development of hepatocellular carcinoma in women with viral cirrhosis of the liver. *JAMA* 292:358-361 (2004)
55. Mizuta, T.: Ozaki, I.: Eguchi, Y.: Yasutake, T.: Kawazoe, S.: Fujimoto, K.: Yamamoto, K. The effect of menatetrenone, a vitamin K2 analog, on disease recurrence and survival in patients with hepatocellular carcinoma after curative treatment: a pilot study. *Cancer* 106:867-872 (2006)
56. Ikeda, K.: Arase, Y.: Saitoh, S.: Kobayashi, M.: Suzuki, Y.: Suzuki, F.: Tsubota, A.: Chayama, K.: Murashima, N.: Kumada, H. Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of the primary tumor-A prospective randomized study of hepatitis C virus-related liver cancer. *Hepatology* 32:228-232 (2000)
57. Kubo, S.: Nishiguchi, S.: Hirohashi, K.: Tanaka, H.: Shuto, T.: Yamazaki, O.: Shiomi, S.: Tamori, A.: Oka, H.: Igawa, S.: Kuroki, T.: Kinoshita, H. Effects of long-term

Chemoprevention of HCC by ACR

postoperative interferon-alpha therapy on intrahepatic recurrence after resection of hepatitis C virus-related hepatocellular carcinoma. A randomized, controlled trial. *Ann Intern Med* 134:963-967 (2001)

58. Tatebe, H.: Shimizu, M.: Shirakami, Y.: Tsurumi, H.: Moriwaki, H. Synergistic growth inhibition by 9-cis-retinoic acid plus trastuzumab in human hepatocellular carcinoma cells. *Clin Cancer Res* 14:2806-2812 (2008)

59. Shimizu, M.: Moriwaki, H. Synergistic Effects of PPARgamma Ligands and Retinoids in Cancer Treatment. *PPAR Res* 2008:181047 (2008)

60. Suzui, M.: Sunagawa, N.: Chiba, I.: Moriwaki, H.: Yoshimi, N. Acyclic retinoid, a novel synthetic retinoid, induces growth inhibition, apoptosis, and changes in mRNA expression of cell cycle- and differentiation-related molecules in human colon carcinoma cells. *Int J Oncol* 28:1193-1199 (2006)

61. Sakabe, T.: Tsuchiya, H.: Endo, M.: Tomita, A.: Ishii, K.: Gonda, K.: Murai, R.: Takubo, K.: Hoshikawa, Y.: Kurimasa, A.: Ishibashi, N.: Yanagida, S.: Shiota, G. An antioxidant effect by acyclic retinoid suppresses liver tumor in mice. *Biochem Pharmacol* 73:1405-1411 (2007)

62. Nakagawa, T.: Shimizu, M.: Shirakami, Y.: Tatebe, H.: Yasuda, I.: Tsurumi, H.: Moriwaki, H. Synergistic effects of acyclic retinoid and gemcitabine on growth inhibition in pancreatic cancer cells. *Cancer Lett* 273:250-256 (2009)

Abbreviations: ACR, acyclic retinoid; AFP-L3, lectin-reactive α -fetoprotein factor 3; 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 receptors; 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; VK₂, vitamin K₂

Key Words: Retinoid, HCC, chemoprevention, phosphorylated RXR alpha, Review

Send correspondence to: Masahito Shimizu, Department of Medicine, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu, Japan 501-1194, Tel: 81-58-230-6308, Fax: 81-58-230-6310, E-mail: shimim-gif@umin.ac.jp



Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and Cellular Pharmacology

Rho-kinase inhibitor upregulates migration by altering focal adhesion formation via the Akt pathway in colon cancer cells

Seiji Adachi ^{a,b,*}, Ichiro Yasuda ^a, Masanori Nakashima ^a, Takahiro Yamauchi ^a, Takashi Yoshioka ^c, Yukio Okano ^c, Hisataka Moriwaki ^a, Osamu Kozawa ^b

^a Department of Gastroenterology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan

^b Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan

^c Department of Molecular Pathobiochemistry, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan

ARTICLE INFO

Article history:

Received 16 April 2010

Received in revised form 27 September 2010

Accepted 6 October 2010

Available online 16 October 2010

Keywords:

Rho-kinase

Cell migration

Colon cancer

Focal adhesion

Akt

ABSTRACT

Although Rho-kinase is reportedly implicated in carcinogenesis and the progression of human cancers, its precise mechanism has not been fully elucidated. We recently reported that Rho-kinase negatively regulates epidermal growth factor (EGF)-stimulated cancer progression in SW480 colon cancer cells. In the present study, we investigated the effect of Rho-kinase on the migration of SW480 colon cancer cells and the mechanism underlying the involvement of Rho-kinase. Interestingly, (R)-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide, 2HCl (Y27632), a specific inhibitor of Rho-kinase, dose-dependently enhanced cell migration. SW480 cells spontaneously release vascular endothelial growth factor (VEGF), however, Y27632 had little effect on its release. While Rho-kinase, which is generally phosphorylated in unstimulated cells, was clearly suppressed by Y27632, exogenous VEGF did not affect its phosphorylation. Immunofluorescence microscopy revealed that Y27632 caused a dramatic change in the localization of focal adhesion components, vinculin, phosphorylated caveolin-1 and tyrosine-phosphorylated proteins in SW480 cells. Furthermore, Akt inhibitor restored the loss of vinculin-stained focal adhesion formation induced by Y27632. We also observed similar effects for Y27632 on the migration and localization of focal adhesion components such as vinculin in another colon cancer cell line, HT29. Taken together, these results strongly suggest that Rho-kinase negatively regulates the migration of colon cancer cells by altering focal adhesion formation via the Akt pathway.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Cell invasion into adjacent tissues is a major prognostic factor for advanced colon cancer patients. Abnormal cell migration leads to pathological states such as invasion and metastasis of cancer. The multistep process of cell migration includes the extension of lamellipodia, cell surface protrusions comprised of actin filaments, which are anchored to the underlying substratum by small integrin-dependent focal adhesions (Brew et al., 2009). The cell cytoskeleton is composed of actin filaments and a network of microtubules, and controls cell motility (Ridley et al., 2003). The polymerization of actin pushes against the plasma membrane and provides the force for forward movement. Actin stress fibers generate contractile forces by pulling against focal adhesions to induce retraction of the rear cell membrane, suggesting that stress fibers are important for cell migration (BurrIDGE, 1981). Cytoskeletal proteins such as vinculin, actinin, and several non-receptor protein tyrosine kinases, including

members of the Src family and focal adhesion kinase (FAK), are involved in the organization of focal adhesion complexes (BurrIDGE and Chrzanoska-Wodnicka, 1996; Humphries et al., 2007).

GTPases of the Ras and Rho superfamily act as molecular switches to control a wide range of essential biological pathways in all eukaryotic cells (Bar-Sagi and Hall, 2000). Once they are active, Ras and Rho GTPases interact with target proteins to promote a cellular response (Bar-Sagi and Hall, 2000). Among the molecules in this superfamily, Rho regulates the formation of contractile actin–myosin filaments, which form stress fibers, and maintains focal adhesions at the rear of the cell (Salhia et al., 2005). RhoA reportedly induces the formation of stress fibers and mature focal adhesions through activation of its downstream effectors such as Rho-kinase (Matsui et al., 1996; Pellegrin and Mellor, 2007). Rho GTPases also contribute to the regulation of cell cycle progression, cell motility, and invasive phenotypes (Sahai and Marshall, 2002). Although the overexpression of Rho has been linked to progression of human cancers (Fritz et al., 1999), an opposing effect of Rho in the process of cell migration has also been reported. Dihydromotuporamine C (dhMotC), a macrocyclic alkaloid that inhibits tumor cell invasion, induces the formation of new stress fibers and large focal adhesion complexes that are dispersed around the entire cell periphery. The compound also has

* Corresponding author. Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan. Tel.: +81 58 230 6217; fax: +81 58 230 6218.

E-mail address: seijiadachi0123@gmail.com (S. Adachi).

the ability to activate Rho, and this ability appears to be an important determinant of the anti-invasive activity of dhMotC (McHardy et al., 2004). Therefore, the roles of the Rho GTPases and their downstream effectors in cancer progression and invasion remain controversial.

Rho-kinase is one of numerous targets of Rho (Riento and Ridley, 2003). Accumulating evidence indicates that the Rho/Rho-kinase pathway plays an important role in various cellular functions, such as vascular smooth muscle cell contraction, cell migration and cell proliferation (Sahai and Marshall, 2002). However, the exact role that Rho-kinase plays in cancer cells remains to be elucidated. We recently reported that Rho-kinase negatively regulates epidermal growth factor (EGF)-stimulated cancer progression in SW480 colon cancer cells (Nakashima et al., 2010). In the present study, we investigated the role of Rho-kinase in colon cancer cell migration.

2. Materials and methods

2.1. Materials

(R)-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide, 2HCl (Y27632), and Akt inhibitor were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Recombinant human VEGF was purchased from R&D Systems, Inc. (Minneapolis, MN). Anti-phospho-specific myosin phosphatase targeting subunit-1 (MYPT-1) (Thr850) antibodies were purchased from Upstate (Lake Placid, NY). Anti-MYPT-1 antibodies and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against phospho-specific Akt and Akt were purchased from Cell Signaling, Inc. (Beverly, MA). Antibodies against phospho-caveolin-1 and phosphotyrosine were purchased from BD Transduction Laboratories (San Jose, CA). The ECL Western blot detection system was purchased from GE Healthcare (Buckinghamshire, UK). Other materials and chemicals were obtained from commercial sources. Y27632 was dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect the assay for the Western blot analysis.

2.2. Cell culture

Unless indicated otherwise, SW480 and HT29 human colon cancer cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, San Diego, CA), containing 10% fetal calf serum. Before the experiments, they were incubated in serum free medium for an additional 24 h as described previously (Adachi et al., 2008).

2.3. Measurement of VEGF

The SW480 culture medium was changed to fresh media without serum, and cells were incubated for 0, 12, 24 and 48 h. The respective media were then collected and the VEGF concentration was measured using a human VEGF enzyme-linked immune-sorbent assay (ELISA) kit purchased from R&D Systems, Inc.

2.4. Cell migration assay

Cell migration was assessed using a Boyden chamber (8 mmol/L pores, Transwell®; Corning Costar Corp., Cambridge, MA). The cells (5×10^4 per well) were seeded in the upper chamber, and DMEM containing 10% fetal calf serum and the indicated compounds were added to the bottom chamber. After 48 h incubation at 37 °C, the cells on the upper surface of the membrane were mechanically removed, and the cells that had migrated to the lower surface of the membrane were fixed and stained with hematoxylin. The average number of migrated cells from 5 randomly chosen fields on the lower surface of the membrane was counted. Each experiment was performed in triplicate.

2.5. Western blot analysis

Western blot analyses were performed as described previously (Adachi et al., 2007). In brief, the cells were treated with various concentrations of Y27632 (0–3 μ M) for 60 min and protein extracts were examined by a Western blot analysis. The protein (10 μ g) was fractionated and transferred onto an Immune-Blot PVDF Membrane (Bio-Rad, Hercules, CA). Membranes were blocked with 5% fat-free dry milk in phosphate buffered saline containing 0.1% Tween-20 for 30 min before incubation with the indicated primary antibodies. Peroxidase-labeled antibodies were used as secondary antibodies. The peroxidase activity on the membrane was visualized on X-ray film by means of the ECL Western blot detection system.

2.6. Immunofluorescence microscopy studies

Immunofluorescence microscopy studies were performed as described previously (Adachi et al., 2009a,b). The cells grown on coverslip-bottom dishes were incubated with or without Y27632 (3 μ M) for 60 min at 37 °C. The cells were then fixed with 4% paraformaldehyde for 10 min on ice and exposed to 0.1% Triton X-100 for 10 min to permeabilize the cell membrane. They were then exposed to the indicated primary antibodies, followed by exposure to Alexa Fluor conjugated secondary antibodies and 4',6-diamidino-2-phenylindole (DAPI) (Wako, Tokyo, Japan) for 60 min. Finally, the cells were examined by fluorescence microscopy using a BIOREVO system (BZ-9000) (Keyence, Tokyo, Japan) according to the manufacturer's protocol.

2.7. Densitometric analysis

The densitometric analysis was performed using a scanner and an image analysis software package (Image J ver. 1.32). The background-subtracted signal intensity of each protein signal was normalized to the respective control signal. All data were obtained from at least three independent experiments.

2.8. Statistical analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between the indicated pairs, and $P < 0.05$ was considered to be significant.

3. Results

3.1. Effect of Rho-kinase inhibitor on the migration of SW480 and HT29 colon cancer cells

We first investigated the effect of Y27632, a specific inhibitor of Rho-kinase (Shimokawa and Rashid, 2007), on cell migration in SW480 and HT29 cells. As shown in Fig. 1, we examined cell motility using a Boyden chamber and found that 3 μ M of Y27632 significantly stimulated the migration of SW480 cells (Fig. 1A). Y27632 also dose-dependently enhanced the migration of HT29 cells (Fig. 1B), suggesting a negative role for Rho-kinase in colon cancer cell migration. Of interest, we recently reported the inhibition of Rho-kinase to stimulate colon cancer cell proliferation (Nakashima et al., 2010). These results led us to further investigate the mechanism underlying the involvement of Rho-kinase in colon cancer cell migration.

3.2. Effect of Rho-kinase inhibitor on VEGF release in SW480 colon cancer cells

VEGF has been well documented to be the most potent inducer of angiogenesis, while also promoting several events required for the formation of new blood vessels, such as endothelial cell proliferation,