Send Orders of Reprints at bspsaif@emirates.net.ae

 Current Drug Targets, 2012, *13*, 1842-1857

Cancer Chemoprevention with Green Tea Catechins: From Bench to Bed

Yohei Shirakami[1,2], Masahito Shimizu[1,*] and Hisataka Moriwaki[1]

[1]*Department of Gastroenterology, Gifu University Graduate School of Medicine, Gifu, Japan;* [2]*Department of Informative Clinical Medicine, Gifu University Graduate School of Medicine, Gifu, Japan*

Abstract: Many epidemiological studies and a large number of experimental studies using a variety of animal models have observed that consumption or administration of green tea appears to exert cancer chemopreventive activity. Based on the results of numerous laboratory cell culture investigations, several mechanisms have been hypothesized to underlie the anti-cancer activity of green tea catechins, especially that of (–)-epigallocatechin-3-gallate (EGCG), the most abundant and active constituent in green tea. These mechanisms include promotion of anti-oxidant activity, inhibition of NF-κB and AP-1, regulation of the cell cycle, inhibition of receptor tyrosine kinase pathways, control of epigenetic modifications, and modulation of the immune system. Several recent interventional studies examining the anti-carcinogenic properties of green tea catechins in humans have yielded promising results that suggest the possibility of their application to human clinical trials. This review article analyzes the results of these studies to explicate the effects of consumption or administration of green tea and its constituents on malignancies observed to date and discuss future directions in this research field.

Keywords: Cancer chemoprevention, green tea catechins, receptor tyrosine kinases.

1. INTRODUCTION

Currently the second most commonly consumed beverage worldwide after water, tea has been consumed since ancient times, when its leaves were originally used for medicinal purposes [1, 2]. Produced from the dried leaves of the tea plant *Camellia sinensis*, tea is generally classified into the three main types—green, black, and oolong—depending on the nature of its processing [3]. Approximately 80% of tea produced in the world is black tea, which is generally consumed in Western and several Asian countries, and 20% is green tea, which is mainly consumed in Asia and Middle East [4]. Among the type of tea, green tea has been most extensively studied for its health benefits, including cancer chemoprevention [5].

Tea contains polyphenols, important constituents that include catechins and flavonoids, such as flavonol, theogallin, theanine, and methylxanthine. Of the major catechinsin green tea, which include (–)-epigallocatechin-3-gallate (EGCG), (–)-epicatechin-3-gallate, (–)-epigallocatechin (EGC), and (–)-epicatechin (EC), EGCG is the most abundant, accounting for 50% to 80% of total catechin constituent [5]. Recently, tea extracts and constituents, especially EGCG, have garnered much attention from researchers and even the general public because of their possible preventive effects in the treatment of chronic diseases, such as cardiovascular disease, obesity, and malignancy. EGCG is also reported to provide beneficial effects against other diseases, including diabetes mellitus, stroke, Parkinson disease, and Alzheimer disease [6-9]. Although the evidence is inconclusive, many epidemiological investigations have indicated that drinking green tea provides chemopreventive effects against malignancies at various organ sites [10-13]. A number of *in vitro* culture experiments and *in vivo* animal studies have suggested that diverse mechanisms underlie the cancer chemopreventive effects of green tea and EGCG, including promotion of antioxidant activity [14, 15]; alteration of cell-cycle regulatory proteins [16]; and inhibition of the DNA methyltransferase [1, 17], mitogen-activated protein kinase (MAPK), and receptor tyrosine kinase (RTK) pathways [1, 18, 19]. To provide more insight into these effects and mechanisms, the present review discusses the recent research data collected from investigation of the effect of consumption or administration of green tea catechins on cancer risk and development and the possible mechanisms by which they exert a cancer chemopreventive effect. This review also briefly summarizes the results of human interventional studies to assess the potential of the clinical application of their results to cancer chemoprevention.

2. EPIDEMIOLOGICAL STUDIES

The effects of consumption of green tea on the risk of cancer have been extensively investigated in many countries. While only some of these studies have shown that consumption of green tea provides a protective effect against cancer, and human epidemiological studies on the cancer-preventive effects of green tea consumption have not yet offered conclusive results, experimental *in vitro* studies and *in vivo* animal carcinogenesis studies have consistently demonstrated that green tea catechins appear to have some efficacy in inhibiting a large variety of malignancies. The following sections discuss the findings regarding the effect of green tea

*Address correspondence to this author at the Department of Gastroenterology, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan; Tel: +81-58-230-6308; Fax: +81-58-230-6310;
E-mail: shimim-gif@umin.ac.jp

1873-5592/12 $58.00+.00

consumption on the risk of malignant disease of the lung, breast, esophagus, stomach, colorectum, and prostate and they are summarized with other organ sites in (Table **1**).

Lung Cancer

One of the most common cancers in the world, lung cancer is generally attributed to smoking, which is universally recognized as its major cause. Recently, various molecular targeted agents have been developed and applied to the treatment of lung cancer. Many epidemiological studies have investigated the relationship between green tea intake and risk of lung cancer, including a systematic review that evaluated the association between green tea consumption and lung cancer risk [20]. Among them, several reported a significantly decreased risk of lung cancer with high green tea intake [21], while others reported a significantly increased risk [22] or no association [20, 23, 24]. The study by Nakachi *et al.* [21] was the only prospective cohort studies that found a significantly decreased risk for lung cancer with green tea. On the basis of the follow-up study, they revealed decreased relative risk of lung cancer incidence for those consuming over 10 cups a day compared with those consuming below 3 cups (RR = 0.33, 95% CI = 0.11-0.94). However, smoking or exposure to second-hand smoking could have been a confounding factor in these epidemiological investigations [25]. Several recent studies have observed that green tea consumption provides a protective effect against lung cancer only in specific subpopulations. In a population-based case-control study of the association between green tea consumption and lung cancer risk among women living in Shanghai, China [26]. Zhong *et al.* observed a statistically significant, dose-dependent reduction in risk in women who regularly consumed tea compared to those who did not regularly consume tea among non-smokers, but no association between green tea intake and lung cancer risk in smokers.

Breast Cancer

Cancer of the breast tissue is the most common cancer in females worldwide. Although the majority of human cases occur in women, breast cancer can develop in men in rare cases. A recent meta-analysis of seven epidemiological studies of green tea and breast cancer reported that several studies found an inverse association between green tea intake (more than 3 cups a day) and breast cancer risk of incidence or recurrence while others found no association [27]. An analysis of case-control studies of breast cancer incidence demonstrated an inverse association (pooled RR = 0.81, 95% CI = 0.75-0.88) while no association was found among cohort studies of breast cancer incidence. In contrast, a recent prospective cohort study in Japan that evaluated the association between green tea intake and breast cancer risk found no inverse association, regardless of hormone receptor-defined subtype or menopausal status [28]. Moreover, a similar prospective cohort study in China found a statistically significantly more positive association between risk of breast cancer and green tea consumption in post-menopausal women who had begun drinking green tea at a younger age compared to those who had not [29]. Although the results of many epidemiological studies are inconsistent with the experimental evidence suggesting the protective effect of green tea catechins on breast carcinogenesis, many studies have identified an inverse association between green tea consumption and breast cancer risk in post-menopausal women. The observed positive correlation between higher estrogen levels and increased breast cancer risk, as well as between lower circulating estrogen levels and green tea consumption [30, 31], suggests that ability to lower estrogen levels might be a mechanism by which green tea consumption exerts a protective effect against post-menopausal breast cancer.

Several studies have also suggested that genetic polymorphism can affect the anti-cancer effect provided by green tea intake. Among them, a study conducted by Wu *et al.* [32] found that green tea consumption reduced breast cancer risk in subjects with a low levels of allele of catechol-*O*-methyltransferase activity, which may associated with increased bioavailability of green tea catechins. In another study, green tea consumption was observed to reduce risk of breast cancer in subjects with high levels of angiotensin-converting enzyme activity [33]. Green tea consumption also appears to have an inhibitory effect on breast cancer recurrence, with two prospective cohort studies in Japan observing a significant decrease in risk of breast cancer recurrence with green tea consumption [12, 34].

Esophageal Cancer

Several epidemiological investigations have examined the potential protective effect of tea consumption on esophageal cancer risk. In most of these studies and a review article, however, the tea temperature and type were not analyzed and discussed. Such an oversight is problematical, as consuming hot beverages could damage the esophageal epithelia, leading to increased risk of esophageal cancer [35]. A case-control study in Shanghai, China indicated that consumption of green tea at a "burning-hot" temperature is associated with a statistically significantly increased risk of esophageal cancer compared to consumption of green tea at a lower temperature [36]. In a similar study examining the effect of green tea temperature on the risk of esophageal cancer in a high-incidence area of esophageal cancer in China, Wu *et al.* [37] found that consumption of high-temperature hot tea is associated with a statistically significantly increased risk of esophageal cancer compared to non-consumption of tea. A prospective cohort study in Japan demonstrated that subjects who typically consume green tea at high temperatures have a statistically significant higher risk of mortality due to esophageal cancer compared to those who consume green tea at moderate temperatures [38]. Most of these studies have observed an increased risk of esophageal cancer with consumption of high-temperature beverages, regardless of the beverage type. In accordance, a systemic review reported that most of the studies show that a significantly increased risk of esophageal cancer is associated with very hot tea intake [39]. These findings support the existence of the adverse thermal effect of tea consumption, specifically its association with esophageal cancer development.

Further confounding factors in the association between tea consumption and esophageal cancer risk was revealed by a prospective study in Shanghai, China, which found that tea consumers tend to smoke cigarettes and drink alcoholic beverages, recognized as two established risk factors for esophageal cancer [40]. Specifically, the study found that 77%

Table 1. Numbers of epidemiological studies on green tea consumption and the risk of human cancers. data were obtained from articles searched by PubMed since 1980

Organ Site	Cohort Studies		Case-Control Studies	
	Risk Reduction	No Risk Reduction	Risk Reduction	No Risk Reduction
Lung	0	4	3	3
Esophagus	0	2	3	6
Stomach	2	6	8	9
Liver	1	1	2	0
Pancreas	0	2	2	1
Kidney and Bladder	0	1	1	4
Large intestine	3	5	4	3
Breast	3	5	4	1
Prostate	2	1	2	0
Ovary	1	0	2	0
Other	2	2	7	1

of men who smoke cigarettes and drink alcoholic beverages consumed green tea on a daily basis [40]. This phenomenon may account for the findings of a case-control study of esophageal cancer in China conducted by Gao *et al.* [13, 36], which indicated a statistically significantly reduced risk of esophageal cancer with green tea consumption in women (OR = 0.50, 95% CI = 0.30-0.83) but not in men (OR = 0.80, 95% CI = 0.58-1.09). This gender difference in the relationship between green tea consumption and esophageal cancer might be due to the residual confounding effect of smoking and alcohol drinking, as Chinese women rarely use tobacco or alcohol. Indeed, when the data collected from subjects who smoked cigarettes and/or drank alcoholic beverages were analyzed separately from those who neither smoked nor drank, a statistically significant decreased risk of esophageal cancer with green tea consumption was observed in both men and women [13, 36].

Stomach Cancer

Among the large number of case-control and cohort studies that have examined the relationship between green tea consumption and risk of stomach cancer, many case-control studies and several cohort studies have indicated reduced risk of stomach cancer with green tea consumption [41, 42]. Of note is a nested case-control study in a prospective cohort of Chinese men that found an inverse relationship between urinary levels of green tea catechins, such as EGC and EC, and stomach cancer [43]. This study provided direct evidence of the cancer preventive effects of green tea catechins on human gastric carcinogenesis. In a recent pooled analysis of six cohort studies, Inoue *et al.* [44] found an inverse association between green tea consumption (more than 5 cups a day) and gastric cancer risk in women (multivariate-adjusted pooled HR = 0.79, 95% CI = 0.65-0.96). A recent meta-analysis examining the quantitative relationship between green tea consumption and risk of gastric cancer reported that the case-control (summary OR = 0.73, 95% CI = 0.64-0.83) but not the cohort studies (summary RR = 1.04, 95% CI = 0.93-1.17) indicated an inverse association between consumption and risk [45].

Colorectal Cancer

Many population-based investigations have observed that green tea treatment appears to provide some protection against colon cancer. Although several studies have examined the relationship between colorectal cancer risk and tea consumption [46-49], none has yielded clear conclusions. A prospective cohort study on green tea intake and colorectal cancer incidence and mortality was conducted recently in Japan [50]. In this study, after up to 6 years of follow-up, in comparison with <1 cup of green tea a day, subjects with consumption of ≥4 cups a day exhibited a HR of 0.35 (95% CI = 0.08–1.55) for death from colorectal cancer [50]. A meta-analysis of studies that investigated the relationship between green tea intake and colorectal cancer risk reported that although several case-control studies found an inverse relationship between green tea consumption and colon cancer risk (summary OR = 0.74, 95% CI = 0.60-0.93), several cohort studies found no association between green tea consumption and rectal cancer risk (summary RR = 0.99, 95% CI = 0.79-1.24) [51]. Likewise, a study that examined the relationship between urinary levels of specific tea catechins and their metabolites and colorectal cancer risk also found no relationship between urinary green tea catechins and rectal cancer risk, but did find a negative correlation between urinary catechin levels and colon cancer risk [52].

Prostate Cancer

Typically developing in men over the age of fifty, prostate cancer is the most common malignancy experienced by men throughout the world. The detection rate of this cancer

is higher in developed countries, where it is generally treated by surgery, hormonal, and/or radiation therapy. Among the many epidemiological studies that have examined the association between green tea intake and prostate cancer risk, two case-control studies found an inverse relationship between green tea consumption and prostate cancer risk [53, 54]. In the first study, compared with non-drinkers, regular green tea drinkers showed an OR of 0.28 (95% CI = 0.17-0.47) and increasing amount of green tea consumption with decreased risk of prostate cancer [53]. However, both studies employed hospitalized inpatients as control subjects, and one study found the association to be inverse but statistically non-significant [54]. Although three recent prospective cohort studies found no association between green tea consumption and prostate cancer risk [55-57], one stratified analysis by disease stage showed a dose-dependent inverse association between green tea consumption and risk of advanced prostate cancer [57]. Although epidemiological studies have not yet clarified the chemopreventive effects of green tea constituents, such findings suggest that administration of these constituents may reduce the growth of prostate tumors, as do the findings an interventional study examining the effect of green tea components on prostate carcinogenesis that is discussed in a later section.

3. ANTI-CANCER ACTIVITY OF GREEN TEA AND ITS CONSTITUENTS

Green tea and its constituents have been reported to show anti-cancer activity in human cell-line laboratory studies as well as chemically or genetically induced animal-carcinogenesis models that examined the lungs, skin, oral cavity, esophagus, stomach, liver, pancreas, bladder, small and large intestine, mammary gland, and prostate [8, 58-60]. The following sections describe the mechanisms hypothesized to underlie the anti-cancer activity of green tea catechins at these sites and the activities in animal models are summarized in (Table **2**).

Incubation of human small-cell lung carcinoma cells with the green tea catechin EGCG for 24 hours has been observed to result in reduced telomerase activity and reduction of caspase-3 and -9 activity [61]. Supporting this observation, Milligan *et al.* [62] reported that EGCG treatment inhibits non-small cell lung cancer (NSCLC) cell proliferation in cell lines that are sensitive to as well as cell lines that are resistant to erlotinib, one of the molecular targeted agents in the treatment of lung cancer. A study of the effects of combined erlotinib and EGCG treatment in SCID mice found that it resulted in greater inhibition of cell proliferation and colony formation compared to treatment with either agent alone, as well as that it significantly delayed the growth of NSCLC xenografts [62]. Several studies have also found that chemically induced lung carcinogenesis in various murine models could be significantly decreased by green tea administration [63-66]. A study on the effect of administration of standardized polyphenol Polyphenon E (PolyE), which contains 65% EGCG, 25% other catechins, and 0.6% caffeine, extracted from green tea found that it significantly reduced incidence and multiplicity of lung adenocarcinoma [63] and progression of lung adenoma into adenocarcinoma in A/J mice [64]. Further examining the gene expression changes caused by administration of green tea catechins in a chemically induced lung-tumorigenesis mouse model, Lu *et al.* [64] identified 88 genes that are differentially expressed in tumors but not in normal tissues, and found that their differential expression could be reversed by treatment with green tea catechins.

Two studies found that EGCG treatment decreases vascular endothelial growth factor (VEGF) production in MDA-MB-231 human breast cancer cells by inhibiting signal transduction of cell-surface receptor pathways [67, 68]. A similar study observed that EGCG treatment inhibits cell viability, induces apoptosis, and suppresses angiogenesis in MDA-MB-231 cells by reduction of VEGF expression [69]. Likewise, Thangapazham *et al.* [70] observed that treatment with green tea polyphenols and EGCG can suppress proliferation of MDA-MB-231 cells by inhibiting cell growth and inducing apoptosis. Using a Sprague-Dawley rat model in which mammary tumorigenesis had been induced using the 7, 12-dimethylbenz(a)anthracene, one study found that green-tea catechin treatment reduced the volume of mammary tumors [71], while a similar study found that EGCG treatment slightly decreased the incidence and multiplicity of the development of *N*-nitrosomethylurea-induced mammary tumors [72].

Among the researchers who have investigated the effect of treatment with green tea or its constituents, Chen *et al.* [73] found that HT-29 human colon cancer cell growth is inhibited by EGCG treatment. In a study comparing the FHC cell line with a normal human fetal colon cell line, our research group reported that both EGCG and PolyE preferentially inhibit the growth of various human colon cancer cells, including Caco2, HCT116, HT29, SW480, and SW837 [74], with the IC50s of approximately 30 to 80 μM after 48 hours of treatment. We also observed that the growth of xenografts inoculated with the SW837 cell can be significantly inhibited by EGCG treatment [75]. In a study employing a Wister rat model in which colon cancer had been chemically induced using 1,2-dimethylhydrazine, green tea consumption was found to inhibit the formation of aberrant crypt foci (ACF), premalignant lesions of the intestine [76]. Our recent studies demonstrated that EGCG and PolyE administration suppresses inflammation-related colon carcinogenesis induced using azoxymethane (AOM) and dextran sodium sulfate (DSS) in ICR mice [77]. This experimental animal model is considered a model of colonic carcinogenesis development resulting from chronic intestinal inflammation due to inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease. Employing genetically modified animal models to investigate the effect of EGCG consumption on colon carcinogenesis, our research group also found that consumption of water supplemented with EGCG causes a significant decrease in the total number of ACF and β-catenin accumulated crypts (BCACs), as also a type of premalignant lesion of the colon, in male C57BL/KsJ-*db/db* mice, an animal model genetically modified to develop obesity and diabetes mellitus [78]. More recently, we observed that EGCG administration exerts significant anti-cancer activity in genetically modified rats in which colon carcinogenesis had been induced using AOM and DSS (unpublished data).

In a recent study using a transgenic adenocarcinoma of the mouse prostate (TRAMP) model, researchers at Case

Table 2. Numbers of studies on anti-cancer activities of green tea and its constituents in animal models. data were obtained from articles of animal carcinogenesis models searched by PubMed since 1980. the number of xenograft studies is not included

Organ Site	Inhibitory Effects	No Inhibitory Effects
Lung	10	2
Skin	8	-
Oral cavity	3	-
Esophagus	2	-
Stomach	3	-
Liver	5	-
Pancreas	2	-
Bladder	2	-
Small intestine	4	1
Large intestine	8	4
Breast	3	2
Prostate	4	1

Western Reserve University found that oral infusion of the polyphenol fraction extracted from green tea significantly suppresses tumor incidence and burden in the prostate [79]. Similar inhibitory effects of oral administration of green tea catechins on prostate tumor formation have been observed in a related mouse model [80]. Two studies using xenograft models inoculated with human prostate cancer cells exhibited decreased xenograft tumor growth with administration of green tea extracts or polyphenols [81, 82].

4. POSSIBLE ANTI-CANCER MECHANISMS OF GREEN TEA CATECHINS

Green tea catechins, especially EGCG, have been extensively investigated in order to clarify their anti-cancer mechanisms. Several of the variety of mechanisms by which EGCG has been hypothesized to exert biological molecular activity in human cancer cells are described in the following sections.

Anti-Oxidant Activity

The ability of green tea catechins to function as anti-oxidants has been well demonstrated. In one study, green tea catechins were shown to exert strong anti-oxidant action by quenching free radical species and chelate transition metals [14]. The anti-oxidant activity of EGCG is due to the presence of phenolic groups, which are sensitive to oxidation and able to generate quinine, and is further increased by the presence of the trihydroxyl structure [14, 83]. Due to these characteristics, EGCG is believed to exert the same level of anti-oxidant activity exerted by powerful radical scavengers.

Nevertheless, investigation of EGCG anti-oxidant activity in animal models and human subjects has been inconclusive. The direct anti-oxidant effects of tea catechins *in vivo* have been primarily observed only under conditions of high oxidative stress, such as ulcerative colitis and hepatitis [14]. Treatment with EGCG has also been observed to decrease haptic levels of lipid peroxidation and protein carbonylation in aged but not in young rats [84, 85]. In a recent review of the anti-oxidant effects of green tea consumption in controlled interventional studies, Ellinger *et al.* [86] reported that although these studies concluded that regular intake of at least 0.6 to 1.5 l/day green tea increases plasma anti-oxidant capacity and provides protection against DNA damage in healthy subjects, they provided limited *ex vivo* and *in vivo* evidence that it provides anti-oxidant effects in cancer prevention. Therefore, green tea administration only clearly appears to play an anti-cancer role in subjects exposed to conditions of increased oxidative stress.

Induction of Apoptosis and Cell-Cycle Arrest

Programmed cell death, known as apoptosis, is hypothesized to play an essential role in the elimination of cancerous cells and act as an important protective mechanism against malignancy [9]. Many recent studies have indicated that EGCG treatment induces cell-cycle arrest during the G_1 phase through regulating expression of cyclin D1, cdk4, $p21^{CIP1}$, and $p27^{KIP1}$, and induces apoptosis through generation of reactive oxygen species and caspase-3 and -9 activation [87]. On the other hand, Fujiki *et al.* [88] demonstrated that treatment with EGCG and other tea polyphenols inhibits cellular growth of PC-9 human lung cancer cells through cell-cycle arrest during the G_2/M phase. In a study of the effect of EGCG treatment on human head and neck squamous carcinoma (HNSCC) cell lines, EGCG administration at a concentration of 10 μg/ml was found to increase the proportion of HNSCC at the G_1 phase, decrease cyclin D1 protein levels, and increase $p21^{CIP1}$ and $p27^{KIP1}$ proteins

levels [89]. Another study found that EGCG treatment decreases the levels of Bcl-2 and Bcl-$_X$L proteins and enhances the levels of Bax proteins before promoting caspase-3 activation [90]. In a study of the HT29 human colon cancer cell line, treatment with either EGCG or PolyEat a concentration of 20 μg/ml was found to increase the number of cells during the G_1 phase, induce apoptosis, decrease cyclin D1 and Bcl-$_X$L protein levels, and increase caspase-3 and -9 activity [74]. In an *in vivo* examination using an obese and diabetic mice model in which colon carcinogenesis had been induced using the carcinogen AOM, we found that administration of EGCG suppresses colon neoplastic lesions and causes a marked decrease in the level of cyclin D1 protein in colonic mucosa [78]. These results suggest that green tea and its constituents exert anti-cancer activity by inducing cellular growth arrest and apoptosis through various mechanisms.

Inhibition of NK-κBand AP-1

Recent studies have indicated that nuclear factor-κB (NF-κB), a transcriptional factor known to be closely associated with cancer development, has a significant role in suppressing apoptosis in cancer cells [91]. In one study, Masuda *et al.* [67] demonstrated that EGCG treatment inhibits the activation of NF-κBin H891 human HNSCCcells and MDA-MB-231 human breast cancer cells. EGCG treatment also down-regulates NF-κB through inducing kinase expression in PC-9 human lung cancer cells [88]. Moreover, EGCG treatment has been shown to inhibit not only NF-κB activity in human colon cancer cells [91] but also inhibit NF-κB nuclear translocation in A431 epidermoid carcinoma cells in a dose- and time-dependent manner [16]. Nuclear translocation of NF-κB occurs when NF-κB is activated, which leads to numerous forms of gene expression related to the suppression of apoptosis and the induction of cellular transformation, proliferation, invasion, metastasis, chemo-resistance, radio-resistance, innate immunity, and inflammation.

Activator protein-1 (AP-1), another transcription factor that regulates forms of gene expression associated with apoptosis and proliferation, also appears to promote cellular proliferation through up-regulating cyclin D1 gene expression and down-regulating tumor-suppressor genes, such as p53, $p21^{CIP1}$, and $p16^{INK4a}$ [91]. EGCG treatment has been observed to inhibit AP-1 activation and cell transformation in the JB6 mouse epidermal cell line [92] and Ras-activated AP-1 activity in the H-ras-transformed JB6 cell line [93]. We have also observed that EGCG treatment inhibits the transcriptional activity of AP-1 and NF-κB promoters, as examined by reporter assay [94], and that EGCG or PolyE treatment causes a dose-dependent inhibition of AP-1 and NF-κB luciferase reporter activity in the HT29 human colon cancer cell line [74]. These findings indicate that inhibition of the NF-κB and/or AP-1 pathways is a possible mechanism underlying the anti-cancer activity of green tea catechins.

Inhibition of Receptor Tyrosine Kinases

Recent studies have indicated that receptor tyrosine kinases (RTKs), which play an essential role in cellular proliferation and apoptosis, are possible targets of green tea catechins in cancer prevention [95, 96]. RTKs and their multiple downstream signaling pathways, including the Ras/extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways, regulate the expression of multiple target genes associated with cellular proliferation and apoptosis [97, 98]. Binding of specific ligands, such as growth factors and cytokines, to the extracellular domain of RTKs stimulates their intrinsic tyrosine kinase activity and triggers phosphorylation of specific tyrosine residues, which leads to the creation of docking sites for downstream targets [97, 98]. As such, the activation of cell-surface RTKs and their downstream signaling play pivotal roles in the regulation of various fundamental processes in normal cells.

Cancerous cells often exhibit inappropriate activation of RTKs due to several mechanisms, including gene mutation and overexpression [99, 100]. The epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptors, a subfamily of four closely related RTKs—EGFR (ErbB-1), human epidermal growth factor receptor (HER) 2/neu (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4)—while insulin-like growth factor-1 receptor (IGF-1R) and vascular endothelial growth factor receptor (VEGFR) belong to a separate family of RTKs. Abnormalities in several RTKs, especially EGFR, IGF-1R, and VEGFR2, are strongly related to malignant properties in various organ sites [99, 100]. This association, combined with supporting preclinical and clinical evidence, indicates that EGFR and HER2 are valid therapeutic targets in the treatment of malignancies, including lung and breast cancer [101]. Green tea catechins have been demonstrated to impact several RTKs in a beneficial manner, a detailed discussion of which can be found in previous reviews [18, 95] and in the following sections.

As previously described, delivery of green tea polyphenols in drinking water at a 0.1% concentration has been found to inhibit the development of prostate cancer and distant metastatic lesions in TRAMP mice [79]. This treatment is hypothesized to decrease IGF-1 levels and restore IGF-binding protein-3 (IGFBP-3) levels in a manner associated with reduced levels of PI3K and phosphorylated forms of Akt and ERK [79, 102]. Similar experimental results were observed in a study of obese and diabetic *db/db* mice in which colon carcinogenesis had been induced [78]. Observation that *in vitro* treatment with 20 μg/ml of EGCG decreases levels of the activated form of IGF-1R protein and IGF-1 while increasing levels of IGFBP-3 in SW837 human colon cancer cells and HepG2 human hepatoma cells further suggests that EGCG exerts inhibitory action on the IGF/IGF-1R axis [103, 104].

Several studies have also reported observation of the anti-cancer effects of EGCG on the VEGF/VEGFR axis. In one study of human HNSCC and breast cancer cells, EGCG treatment was observed to inhibit the production of VEGF through inhibiting activation of signal transducer and activator of transcription(STAT)-3 and NF-κB [67]. In another study, EGCG treatment was observed to inhibit phosphorylation of both VEGFR1 and VEGFR2 and induce apoptosis in B-cell chronic lymphocytic leukemia cells [105]. Our research group demonstrated that EGCG suppresses growth of xenografts generated from HuH7 human hepatoma cells and SW837 human colon cancer cells by suppressing VEGFR2, ERK, and Akt activation and VEGF expression [75, 106].

Several studies conducted by researchers at Columbia University demonstrate that EGCG treatment not only inhibits its activation of EGFR and HER2 and decreases the frequency of their downstream signaling in human HNSCC, breast cancer, and colon cancer cell lines [67, 68, 74, 89] but also inhibits activation of HER3 in SW837 cells [94]. They also found that EGCG and PolyE treatment decreases levels of phosphorylated forms of EGFR and HER2 proteins, subsequently causing a decrease in the phosphorylation of ERK and Akt proteins [74], effects that are summarized schematically in Fig. (**1**). Based on their results, the research group indicated a promising target of EGCG for its anti-cancer mechanisms associated with RTKs, particularly detergent-insoluble ordered plasma membrane domains known as "lipid rafts", which play a critical role as signal processing hubs of RTKs.

Adachi *et al.* [107, 108] found that EGCG alters lipid organization on the plasma membrane and induces the internalization of EGFR into endosomes, preventing ligands from binding to EGFR. Such degradation of EGFR following internalization has been found to be induced by phosphorylation of the receptor at serine 1046/1047, which is related with the activation of p38 MAPK caused by EGCG [109]. This newly suggested mechanism of EGCG activity may be able to account for ubiquitous effects of EGCG on modulating various types of RTKs, as most of RTKs function on lipid rafts, and explain why EGCG might alter lipid organization on the entire cellular membrane, including lipid rafts.

Inhibition of Cyclooxygenase-2 Overexpression

Cyclooxygenase (COX), the key regulatory enzyme for prostaglandin synthesis, is transcribed from two distinct genes, COX-1 and COX-2. While COX-1 is constitutively expressed in many tissues, COX-2 expression is regulated by various factors, such as mitogens, tumor promoters, cytokines, and growth factors. Overexpression of COX-2 has been observed in a variety of pre-malignant and malignant conditions, including colon, liver, pancreatic, breast, lung, bladder, skin, stomach, head and neck, and esophageal cancer [110]. EGCG has been reported to inhibit mitogen-stimulated COX-2 expression in both androgen-sensitive LNCaP and androgen-insensitive PC-3 human prostate carcinoma cells [111], down-regulate COX-2 expression in TPA-stimulated human mammary cells [112], and decrease COX-2 expression in the SW837 human colon cancer cell line [94]. In a recent study using a mouse model in which inflammation-

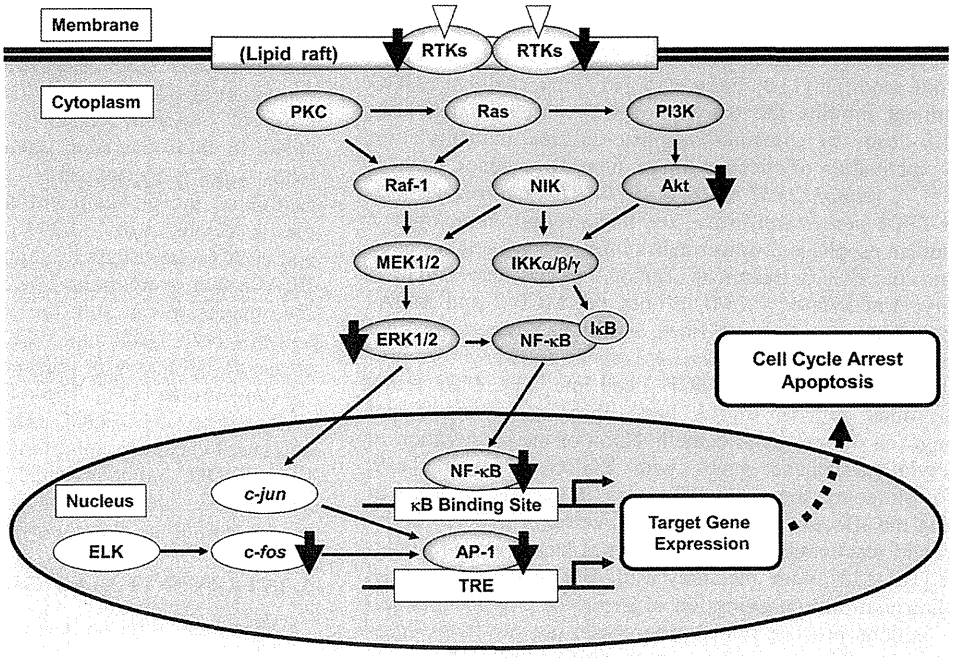


Fig. (1). Effects of green tea catechins on RTKs and their intracellular signaling pathways. Activation of RTKs including EGFR, IGF-1R, and VEGFR2 by specific ligands leads to the induction of their intrinsic tyrosine kinase activities and auto-phosphorylation of tyrosine residues. The activated RTKs 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 activation of 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, as well as the activation of the MAPK cascade, such as Ras/Raf/MEK/ERK pathways and PI3K pathways. Molecules that appear to be cellular targets for EGCG are indicated by a black arrow (downregulation). 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.

related colon carcinogenesis had been induced using AOM and DSS, our research group observed that oral administration of EGCG and PolyE has an inhibitory effect on COX-2 expression in the colon epithelium [77]. These recent findings suggest that development of a compound that can inhibit COX-2 expression, preferably without affecting COX-1 expression, is a promising strategy for cancer chemoprevention [113].

Epigenetic Modification

Epigenetics is the study of the reversible heritable gene expression changes that occur without altering DNA sequences. These changes play a role in the regulation of general gene expression and contribute to carcinogenesis by affecting histone modification, alteration of chromatin structure, and control of the expression of non-coding microRNA [114]. MicroRNA has recently been found to function as a key regulator of the expression of a variety of genes. Epigenetic silencing of DNA-repairing and tumor-suppressor genes, which is typically caused by hypermethylation and occurs during early stages of carcinogenesis, is frequently associated with various diseases [115].

The effects of EGCG, which has been established as an epigenetic modulator in cancer cells, on epigenetic alteration have been primarily attributed to DNA methylation. However, a recent study revealed that EGCG also functions as a histone modifier [116]. Fang *et al.* [117] reported that EGCG treatment inhibits the activity of DNA methyltransferase, which leads to cytosine-phosphate-guanine demethylation and reactivation of silenced tumor-suppressor genes, such as $p16^{INK4a}$, *retinoic acid receptor-β (RARβ), O6-methyl guanine-DNA methyltransferase*, and human *mutL homologue 1* in human esophageal cancer cells. Other *in vitro* studies have found that EGCG treatment promotes partial demethylation of hypermethylated *RARβ* in both the MCF-7 and MDA-MB-231 breast cancer cell lines, as well as a time-dependent decrease in *human telomerase reverse transcriptase* promoter methylation in the MCF-7 cell line [114, 118]. However, other *in vivo* studies have observed no significant changes in the extent of demethylation or the reactivation of methylation-silenced genes with EGCG treatment [119, 120]. Moreover, the results of several *in vivo* studies investigating the effects of EGCG on the reversal of hypermethylation and reactivation of silenced genes have been inconclusive [121-123], with one study using a TRAMP mice model finding that oral consumption of green tea polyphenols neither inhibits prostate tumor progression nor promotes dose-dependent alterations in DNA methylation status [122].

Modulation of the Immune System

Immunity is defined as the ability to fight against various abnormal agents or conditions within the body to prevent the development of infections and diseases, including cancer [124]. Immunity in humans is provided by the immune system, which consists of organs, including the spleen and thymus, as well as the lymph nodes and bone marrow, which regulate the activity of immune system by specific immunocytes [125]. Green tea consumption has been reported to enhance humoral and cell-mediated immunity, resulting in decreased risk of certain cancers [126].

Although inflammation is largely an immune-system response, inappropriate inflammation frequently becomes a cause of disease. EGCG has been reported to be a potent anti-inflammatory compound with therapeutic potential, with many animal studies having observed that administration of green tea polyphenols decreases inflammation. Among them, a study conducted by Yang *et al.* [127] demonstrated that production of tumor necrosis factor (TNF) caused by lipopolysaccharide injection can be decreased by administration of green tea polyphenols.Likewise, our research group found that EGCG and PolyE administration decreases levels of various inflammatory cytokines, including TNF-α, in the colon epithelium and suppresses inflammation-related colon carcinogenesis induced by AOM and DSS injection in a mouse colon cancer model [77]. These findings suggest that administration of green tea and its constituents might have a beneficial effect on inflammatory disorders, possibly, at least in the case of EGCG, by exerting anti-inflammatory activity *via* inhibition of NF-□B activation [58].

Indoleamine 2, 3-dioxygenase (IDO) is a tryptophan catabolic enzyme believed to play an important role in induction of immune tolerance [128]. In an investigation of the effects of EGCG on the expression IDO induced by interferon (IFN)-□, we found that EGCG treatment causes a significant decrease in IFN-□-induced expression of IDO in colon cancer cells in a dose-dependent manner and significantly inhibits IDO enzymatic activity by suppressing the activation of STAT1 [129]. These results are consistent with those of previous studies using human oral cancer cells [130]. In an *in vivo* study examining the effects of treatment with 1-methyltryptophan (1-MT), an IDO inhibitor, and EGCG on the development of AOM-induced colonic neoplastic lesions in male F344 rats, we found that both 1-MT and EGCG significantly suppress production of ACF and BCACs that overexpress IDO protein; that EGCG decreases IDO expression in both the colonic epithelium and stroma; and that both 1-MT and EGCG significantly inhibit the increase of IDO activity induced by AOM injection in the serum and stroma [131]. These findings and those of our recent study suggest that EGCG might exert anti-cancer effects on colorectal cancer by inhibiting both the expression and function of IDO, indicating that IDO inhibitors, such as 1-MT and EGCG, are potential agents for anti-tumor immunomodulation in the chemoprevention of colorectal cancer.

5. INTERVENTIONAL STUDIES

Treatment with tea catechins, including EGCG, appears to offer many potential clinical advantages relative to treatment with other traditional anti-cancer agents. Moreover, tea catechins offer the advantages of being globally available from common beverages; being able to be isolated inexpensively; and, based on their long history of safety as beverages, having a high level of safety. Although several studies have reported observation of tea-catechin-induced toxicity, a number of animal experiments and human interventional studies have observed no adverse events, even with administration of relatively high doses [132, 133].

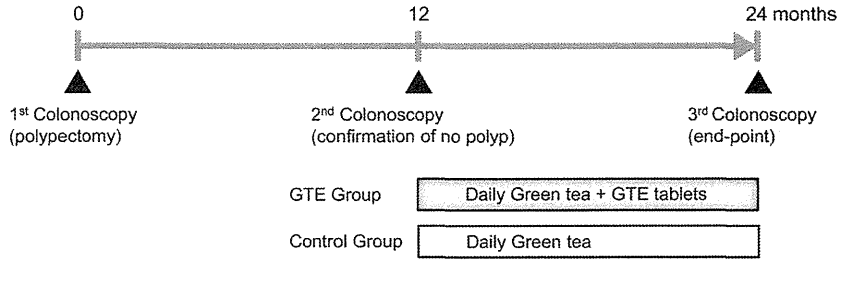
Nevertheless, only a few clinical trials have investigated cancer chemoprevention by administration of tea catechins and/or EGCG. Among them, one interventional study found

that oral administration of mixed tea products causes a significant decrease in the size of leukoplakia, a precancerous lesion of the oral mucosa [134]. This study was a double-blind trial and the treated group was administered with 3 g mixed tea products for 6 months. Another found that delivery of green tea extracts either topically in ointment form or orally in capsule form at a dose of 200 mg for 12 weeks can effectively treat human papilloma virus-infected cervical lesions [135]. In a randomized, double-blind, placebo-controlled study, Bettuzzi *et al.* [136] found that oral administration of 600 mg/day of green tea catechins for 12 months can inhibit the progression of high-grade prostate intraepithelial neoplasia (PIN) to prostate cancer. Moreover, in a long-term follow-up study of a subset of the participants, they also observed continued inhibition of development of prostate cancer from PIN even two years after initial treatment with green tea catechins [137]. These findings provide support of the beneficial effects of green tea catechins on the prevention of human malignancies. Although three other interventional studies investigating the efficacy of green tea against prostate cancer found no evidence of beneficial effects [138-140], these studies included patients with established prostate malignancies, whereas Bettuzzi *et al.* examined only patients with premalignant lesions. This discrepancy in findings regarding patients at different stages of malignancy suggests that treatment with green tea catechins might be effective at only very early stages of cancer development, and should thus be primarily considered as agents in chemoprevention.

In a recent pilot study of the effects of treatment with green tea extracts on the development of colorectal adenoma, a pre-cancerous lesion of the colon, in patients who had undergone polypectomy for removal of colorectal adenomas [141], our research group found that administration of 1.5 g/day of green tea extracts for 1 year suppressed the development of metachronous colorectal adenoma to a significant extent compared to an untreated control group Fig. (**2**). We also found that the relapsed adenomas in the experimental group were significantly smaller in size compared to those of the untreated control group, and observed no adverse reactions in the experimental group. The very encouraging findings of our study and those of Bettuzzi *et al.* [136] suggest the possibility of their application to human clinical trials. A similar trial for investigating the effect of green tea extract on recurrence of colorectal adenomas is being conducted, which is planned to have much more participants and longer observation period [142].

One potential mechanism underlying the cancer chemopreventive effect of tea is the anti-oxidant activity of green tea catechins. In a phase II randomized controlled interventional trial evaluating the efficacy of regular green tea consumption in reducing DNA damage in heavy smokers, consumption of 4 cups/day of decaffeinated green tea for 4

A



B

Characteristic	GTE Group	Control Group	*P*
Total no. of patients	60	65	–
No. of patients with metachronous adenomas (%)	9 (15)	20 (31)	<0.05
Size of relapsed adenomas (mm), mean ± SD	3.0 ± 1.0	4.0 ± 1.3	<0.001

Fig. (2). Pilot study revealing the preventive effect of green tea extract (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 3 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 52.5mg EGCG, 12.3mg EC, 34.6mg EGC, 11.1mg (–)-epicatechingallate, and 15.7 mg caffeine, is equivalent to approximately 2 Japanese-size cups of green tea. (**B**) Effects of the GTE supplementation on the incidence and the size of metachronous adenomas at the end-point colonoscopy. The incidence of metachronous adenomas was 15% (9 of 60) in the GTE group and 31% (20 of 65) in the control group. The size of relapsed adenomas was significantly smaller in the GTE group compared to the control group.

months led to a statistically significant decrease in levels of urinary 8-hydroxydeoxyguanosine (8-OHdG), a surrogate biomarker of oxidative DNA damage, compared with baseline levels [143]. However, another study found evidence of an inverse association between green tea consumption and lung cancer risk in smokers [26], suggesting that the antioxidant properties of green tea play a limited role in decreasing lung cancer risk and, thus, the existence of an alternative mechanism by which green tea decreases risk. Nevertheless, a double-blind, placebo-controlled phase IIa trial of the effect of administration of green tea catechins to residents of a region with high levels of aflatoxin, a risk factor for developing hepatocellular carcinoma (HCC), found that green tea catechin administration not only significantly decreased levels of urinary 8-OHdG in subjects who had tested seropositive for both HBs-Ag and aflatoxin-albumin adducts [144] but also modulated aflatoxin biomarkers [145]. Interventional studies, including fairly recent publications [146], with green tea on malignancies and cancer-related clinical conditions are summarized in (Table **3**).

Although many studies have used animal models of carcinogenesis to demonstrate the significant cancer chemopreventive effects of green-tea catechin administration, the results of epidemiological studies examining the effects of green tea consumption on the risk of various human cancers have been inconclusive, as described in previous sections. This discrepancy in results between laboratory experiments and human epidemiological studies has been attributed to a variety of confounding factors, including variations in human genetics, cancer etiology, and lifestyle, as well as in the quantity, quality, and type of tea intake [13]. Among these factors, the amount and type of tea consumption appear to affect the correlation between tea consumption and cancer risk reduction to a significant extent. Prospective cohort studies conducted in Japan indicated that daily consumption of at least 10 cups of tea, equivalent to consumption of 2.5 g of green tea extract, is required to derive a cancer preventive effect [21, 147]. Green tea appears to be more consistently related with reduced risk of cancer, at the very least gastrointestinal and lung cancer in nonsmokers, than black tea, possibly because of the relatively higher concentrations of tea catechins in green tea [13].

The existence of other confounding factors, such as smoking, alcohol intake, and obesity, may be responsible for the inconsistent results among epidemiological studies that analyzed the anti-cancer effects of green tea catechins. For instance, smoking and alcohol intake have been found to significantly affect the development of esophageal and liver cancer [40, 148], while the development of liver cancer, especially HCC, is often related to chronic haptic inflammation induced by a persistent infection with hepatitis viruses. In addition, increasing evidence indicates that obesity and associated metabolic abnormalities, especially insulin resistance, increase the risk of HCC [149, 150]. Since green tea catechins appear to provide anti-obesity and anti-diabetic action, additional interventional studies should analyze whether administration of green tea catechins prevents the development of HCC in obese, high-risk patients with chronic hepatic inflammation. To collect explicit and definitive data regarding the chemopreventive effects of green tea catechin administration in the prevention of many types of malignancies in various organ sites, well-designed cohort studies and carefully conducted interventional trials should be performed with large numbers of subjects and designed to eliminate any potential confounding factors.

6. CONCLUDING REMARKS

As described in the present review, the anti-cancer effects of green tea catechins, especially EGCG, and their molecular mechanisms have been observed in a variety of animal models and cell culture experiments using various cancer cell lines. The results of many epidemiological and interventional studies indicate that administration of green tea catechins has clinical relevance and efficacy in human cancer chemoprevention. As the bioavailability and plasma concentration of green tea catechins following tea consumption are critically important factors, they must be considered when reviewing the results of *in vivo* experimental and human clinical studies of the absorption, distribution, and metabolism of green tea catechins [151]. In several cell culture experiments, the concentrations of EGCG required to observe biological effects were much higher than the plasma and tissue concentrations detected in human trials or experimental animal studies [127]. In fact, the concentrations examined in previous *in vitro* studies have ranged from 20 to 100 μM, exceeding the concentrations achievable in plasma and tissues by 10- to 100-fold [25, 152]. In consequence of their extensive metabolism in humans, tea catechins usually reach plasma concentrations only in the low micro-molar range following typical tea consumption [153]. Hence, it remains unclear whether the findings obtained from *in vitro* studies using much higher concentrations of EGCG can be directly extrapolated to animal and human cancer chemoprevention. In this regard, recent affinity chromatography studies have revealed that although EGCG appears to bind to a number of high-affinity EGCG-binding proteins, including IGF-1R, at low concentrations, relatively high concentrations of EGCG are required for exertion of its physiological functions [154-156]. Another important clinical consideration is that the direct contact provided by oral administration likely leads to much higher levels of green tea catechins in the epithelia of the oral-digestive tract compared to other means of administration, indicating that tea catechins might be even more effective against malignancies in oral and gastrointestinal tissues.

In order to obtain high concentrations of tea catechins in the blood and tissues, relatively high doses of tea catechins are often used in human interventional studies. As previously discussed, many studies have reported observation of no adverse reactions with administration of tea polyphenols at doses ranging from 600 to 1800 mg/day [132, 136, 157]. There have been, however, a growing number of case reports of side effects in humans related to intake of green tea dietary supplements, with excess gas, upset stomach, nausea, heartburn, stomachache, abdominal pain, dizziness, headache, muscle pain, and hepatotoxicity (elevated serum transaminase and bilirubin levels) having been reported with doses ranging from 700 to 2100 mg/day[133, 157]. In several case reports, liver biopsy samples indicated portal and periportal inflammation and necrosis [158, 159]. While notable, these side effects were observed in studies that administered high doses of tea catechins in non-traditional forms, such as pills and capsules, rather than *via* consumption of green tea [160].

Table 3. Interventional studies with green tea on malignancies or cancer-related clinical condition. data were obtained from articles searched by PubMed since 1980

Reference	Country	Cancers or Cancer-Related Clinical Condition	Type of Study Design	Green Tea Status and Quantity	Observation Period	No. of Participants	Inhibitory Effect
Ahn *et al.* 2003 [135]	Korea	Human papilloma virus-infected cervical lesions	Placebo-controlled	PolyE ointment and/or PolyE or EGCG capsule 200 mg/day	12 weeks	90	Yes
Bettuzzi *et al.* 2006 [136]	Italy	Prostate cancer from high-grade prostate intraepithelial neoplasia (HG-PIN)	Randomized, double-blind, placebo-controlled	Green tea catechins capsule 600 mg /day	1 year	60	Yes
Brausi *et al.* 2008 [137]	Italy	Prostate cancer from HG-PIN	Placebo-controlled	Green tea catechins capsule 600 mg /day (in the first year)	2 years	< 60	Yes
Jatoi *et al.* 2003 [139]	USA	Prostate cancer	Multi-institutional phase II	Green tea powder 6 g/day	4 months	42	No
Choan *et al.* 2005 [138]	Canada	Prostate cancer	Single-institutional prospective single arm	Green tea extract capsule 500 mg/day	2 months	19	No
McLarty *et al.* 2009 [140]	USA	Prostate cancer	Open-label, single-arm two-stage phase II	PolyE capsule 1.3 g (800 mg EGCG) /day	1 month	26	Yes
Shimizu *et al.* 2008 [141]	Japan	Metachronous colorectal adenoma	Randomized, placebo-controlled	Green tea extracts 1.5 g/day	1 year	125	Yes
Hakim *et al.* 2003 [143]	USA	DNA damage in heavy smokers	Three-arm randomized placebo-controlled phase II	Decaffeinated green tea 4 cups/day	4 months	133	Yes
Luo *et al.* 2006 [144]	USA	DNA damage in subjects with sero-positive for both HBs-Ag and aflatoxin-albumin adducts	Double-blind, placebo-controlled phase IIa	Green tea polyphenol capsule 500 or 1000 mg/day	3 months	124	Yes
Nguyen *et al.* 2012 [146]	USA	Prostate cancer before prostatectomy	Randomized, double-blind, placebo-controlled	PolyE capsule containing 800 mg of EGCG/day	3 weeks	50	No

Significant evidence indicates that consumption of green tea in its whole form provides greater cancer chemoprevention efficacy compared to administration of EGCG alone. Indeed, EGCG has been found to be significantly more efficacious when administered in combination with other tea catechins [147, 161, 162], with our findingsshowing that combined EGCG and EC administration resulted in synergistic inhibition of growth and induction of apoptosis in the HT29 colon cancer cell line [74]. Performing combination assay, we found that the optimal ratio of EGCG to EC for

efficacious anti-cancer action is 60:7, which reflects the relative concentration of EGCG (60%) to EC (7%) of PolyE [74]. This appears to be one reason why PolyE, which contains less EGCG compared to pure EGCG by weight, was observed to exert an anti-cancer effect almost equivalent to that of the same concentration of EGCG alone in our study [74] and other studies that observed the efficacy of total green tea extracts and PolyE in inhibiting tumor development in rodents [163, 164].

As previous research has indicated that EC enhances cellular incorporation of ^{3}H-EGCG in lung cancer cells [162], the mechanism by which combined administration of EC and EGCG exerts these synergistic effects might be increased cellular uptake of EGCG. Among the enhanced anti-tumor effects provided by combinations of green tea extract and other drugs reported by Suganuma *et al.*, combined treatment with green tea extract and sulindac, a COX inhibitor, has been found to enhance inhibition of tumor development in multiple intestinal neoplasia mice [165], and combined treatment with EGCG and celecoxib, another COX inhibitor, to suppress the development of chemically induced lung tumors and enhance the inhibitory effect of EGCG in A/J mice [166]. Cell culture experiments have also revealed that combined treatment with EGCG and celecoxib induces new gene expressions, such as *GADD153* and $p21^{CIP1}$, which are not induced by EGCG or COX inhibitors alone, and also induces stronger apoptosis than that of EGCG or COX inhibitors alone [147, 167-169].

Such observation of the synergistic anti-cancer effects provided by combined administration of green tea catechins and existing anti-cancer drugs has revealed a particularly promising strategy in the prevention and treatment of human malignancies that appears to have no or few side effects. As such, it has recently attracted much interest in the cancer field, in which tea is currently considered one of the most beneficial dietary agents in the prevention and treatment of many lifestyle-related diseases, especially cancer. The present review provides evidence that the effects of treatment with green tea catechins on the inhibition of carcinogenesis are mediated by various possible mechanisms, including anti-oxidant activity, anti-inflammation activity, RTK inhibition, epigenetic modification, and/or immune system modulation. To explicate these molecular mechanisms and their recognized and newly discovered anti-cancer effects, as well as account for the inconsistency and discrepancy between laboratory experiments and human clinical investigations, future research must continue to investigate administration of tea catechins in the prevention and treatment of cancers in various organ sites. The results of laboratory studies should be compared with those of *in vivo* studies to evaluate whether these mechanisms are applicable to cancer chemoprevention in humans and identify the optimal conditions for future interventional studies. For the clinical application of green tea catechins as chemopreventive agents, a greater number of well-designed epidemiological and interventional studies should be conducted to investigate the anti-cancer activity of green tea and its constituents in humans.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

This review was based on studies 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 a Grant-in-Aid for the 3rd-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare of Japan.

ABBREVIATIONS

ACF	=	Aberrant Crypt Foci
AOM	=	Azoxymethane
AP-1	=	Activator Protein-1
BCAC	=	Beta-Catenin Accumulated Crypt
COX	=	Cyclooxygenase
DSS	=	Dextran Sodium Sulfate
EC	=	(–)-epicatechin
EGC	=	(–)-epigallocatechin
EGCG	=	(–)-epigallocatechin-3-gallate
EGFR	=	Epidermal Growth Factor Receptor
ERK	=	Extracellular Signal-Regulated Kinase
HER	=	Human Epidermal Receptor
MAPK	=	Mitogen-Activated Protein Kinase
NF-κB	=	Nuclear Factor kappaB
PI3K	=	Phosphatidylinositol-3-Kinase
PIN	=	Prostate Intraepithelial Neoplasia
PolyE	=	Polyphenon E
RAR	=	Retinoic Acid Receptor
RTK	=	Receptor Tyrosine Kinase
TRAMP	=	Transgenic Adenocarcinoma of the Mouse Prostate
VEGF	=	Vascular Endothelial Growth Factor
VEGFR	=	Vascular Endothelial Growth Factor Receptor
TNF	=	Tumor Necrosis Factor

REFERENCES

[1] Larsen CA, Dashwood RH, Bisson WH. Tea catechins as inhibitors of receptor tyrosine kinases: mechanistic insights and human relevance. Pharmacol Res 2010; 62(6): 457-64.

[2] Siddiqui IA, Asim M, Hafeez BB, Adhami VM, Tarapore RS, Mukhtar H. Green tea polyphenol EGCG blunts androgen receptor function in prostate cancer. FASEB J 2011; 25(4): 1198-207.

[3] Graham HN. Green tea composition, consumption, and polyphenol chemistry. Prev Med 1992; 21(3): 334-50.

[4] Katiyar SK, Agarwal R, Mukhtar H. Green tea in chemoprevention of cancer. Compr Ther 1992; 18(10): 3-8.

[5] Siddiqui IA, Adhami VM, Saleem M, Mukhtar H. Beneficial effects of tea and its polyphenols against prostate cancer. Mol Nutr Food Res 2006; 50(2): 130-43.

[6] Grove KA, Lambert JD. Laboratory, epidemiological, and human intervention studies show that tea (Camellia sinensis) may be useful in the prevention of obesity. J Nutr 2010; 140(3): 446-53.

[7] Higdon JV, Frei B. Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. Crit Rev Food Sci Nutr 2003; 43(1): 89-143.

[8] Khan N, Afaq F, Saleem M, Ahmad N, Mukhtar H. Targeting multiple signaling pathways by green tea polyphenol (-)-epigallocatechin-3-gallate. Cancer Res 2006; 66(5): 2500-5.

[9] Shankar S, Ganapathy S, Hingorani SR, Srivastava RK. EGCG inhibits growth, invasion, angiogenesis and metastasis of pancreatic cancer. Front Biosci 2008; 13: 440-52.

[10] Yang CS, Wang H, Li GX, Yang Z, Guan F, Jin H. Cancer prevention by tea: Evidence from laboratory studies. Pharmacol Res 2011; 64(2): 113-22.

[11] Imai K, Suga K, Nakachi K. Cancer-preventive effects of drinking green tea among a Japanese population. Prev Med 1997; 26(6): 769-75.

[12] Nakachi K, Suemasu K, Suga K, Takeo T, Imai K, Higashi Y. Influence of drinking green tea on breast cancer malignancy among Japanese patients. Jpn J Cancer Res 1998; 89(3): 254-61.

[13] Yuan JM, Sun C, Butler LM. Tea and cancer prevention: epidemiological studies. Pharmacol Res 2011; 64(2): 123-35.

[14] Lambert JD, Elias RJ. The antioxidant and pro-oxidant activities of green tea polyphenols: a role in cancer prevention. Arch Biochem Biophys 2010; 501(1): 65-72.

[15] Thawonsuwan J, Kiron V, Satoh S, Panigrahi A, Verlhac V. Epigallocatechin-3-gallate (EGCG) affects the antioxidant and immune defense of the rainbow trout, Oncorhynchus mykiss. Fish Physiol Biochem 2010; 36(3): 687-97.

[16] Gupta S, Hastak K, Afaq F, Ahmad N, Mukhtar H. Essential role of caspases in epigallocatechin-3-gallate-mediated inhibition of nuclear factor kappa B and induction of apoptosis. Oncogene 2004; 23(14): 2507-22.

[17] Lee AH, Fraser ML, Binns CW. Possible role for green tea in ovarian cancer prevention. Future Oncol 2005; 1(6): 771-7.

[18] Shimizu M, Adachi S, Masuda M, Kozawa O, Moriwaki H. Cancer chemoprevention with green tea catechins by targeting receptor tyrosine kinases. Mol Nutr Food Res 2011; 55(6): 832-43.

[19] Singh BN, Shankar S, Srivastava RK. Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. Biochem Pharmacol 2011; 82(12): 1807-21.

[20] Arts IC. A review of the epidemiological evidence on tea, flavonoids, and lung cancer. J Nutr 2008; 138(8): 1561S-6S.

[21] Nakachi K, Matsuyama S, Miyake S, Suganuma M, Imai K. Preventive effects of drinking green tea on cancer and cardiovascular disease: epidemiological evidence for multiple targeting prevention. Biofactors 2000; 13(1-4): 49-54.

[22] Tewes FJ, Koo LC, Meisgen TJ, Rylander R. Lung cancer risk and mutagenicity of tea. Environ Res 1990 Jun; 52(1): 23-33.

[23] Kuriyama S, Shimazu T, Ohmori K, *et al.* Green tea consumption and mortality due to cardiovascular disease, cancer, and all causes in Japan: the Ohsaki study. JAMA 2006; 296(10): 1255-65.

[24] Nagano J, Kono S, Preston DL, Mabuchi K. A prospective study of green tea consumption and cancer incidence, Hiroshima and Nagasaki (Japan). Cancer Causes Control 2001; 12(6): 501-8.

[25] Ju J, Lu G, Lambert JD, Yang CS. Inhibition of carcinogenesis by tea constituents. Semin Cancer Biol 2007; 17(5): 395-402.

[26] Zhong L, Goldberg MS, Gao YT, Hanley JA, Parent ME, Jin F. A population-based case-control study of lung cancer and green tea consumption among women living in Shanghai, China. Epidemiology 2001; 12(6): 695-700.

[27] Ogunleye AA, Xue F, Michels KB. Green tea consumption and breast cancer risk or recurrence: a meta-analysis. Breast Cancer Res Treat 2010; 119(2): 477-84.

[28] Dai Q, Shu XO, Li H, *et al.* Is green tea drinking associated with a later onset of breast cancer? Ann Epidemiol 2010; 20(1): 74-81.

[29] Iwasaki M, Inoue M, Sasazuki S, *et al.* Green tea drinking and subsequent risk of breast cancer in a population to based cohort of Japanese women. Breast Cancer Res 2010; 12(5): R88.

[30] Nagata C, Kabuto M, Shimizu H. Association of coffee, green tea, and caffeine intakes with serum concentrations of estradiol and sex hormone-binding globulin in premenopausal Japanese women. Nutr Cancer 1998; 30(1): 21-4.

[31] Wu AH, Arakawa K, Stanczyk FZ, Van Den Berg D, Koh WP, Yu MC. Tea and circulating estrogen levels in postmenopausal Chinese women in Singapore. Carcinogenesis 2005; 26(5): 976-80.

[32] Wu AH, Yu MC, Tseng CC, Hankin J, Pike MC. Green tea and risk of breast cancer in Asian Americans. Int J Cancer 2003; 106(4): 574-9.

[33] Yuan JM, Koh WP, Sun CL, Lee HP, Yu MC. Green tea intake, ACE gene polymorphism and breast cancer risk among Chinese women in Singapore. Carcinogenesis 2005; 26(8): 1389-94.

[34] Inoue M, Tajima K, Mizutani M, *et al.* Regular consumption of green tea and the risk of breast cancer recurrence: follow-up study from the Hospital-based Epidemiologic Research Program at Aichi Cancer Center (HERPACC), Japan. Cancer Lett 2001; 167(2): 175-82.

[35] Brunning DA. Letter: Oesophageal cancer and hot tea. Lancet 1974; 1(7851): 272.

[36] Gao YT, McLaughlin JK, Blot WJ, Ji BT, Dai Q, Fraumeni JF, Jr. Reduced risk of esophageal cancer associated with green tea consumption. J Natl Cancer Inst 1994; 86(11): 855-8.

[37] Wu M, Liu AM, Kampman E, *et al.* Green tea drinking, high tea temperature and esophageal cancer in high- and low-risk areas of Jiangsu Province, China: a population-based case-control study. Int J Cancer 2009; 124(8): 1907-13.

[38] Kinjo Y, Cui Y, Akiba S, *et al.* Mortality risks of oesophageal cancer associated with hot tea, alcohol, tobacco and diet in Japan. J Epidemiol 1998; 8(4): 235-43.

[39] Islami F, Boffetta P, Ren JS, Pedoeim L, Khatib D, Kamangar F. High-temperature beverages and foods and esophageal cancer risk--a systematic review. Int J Cancer 2009; 125(3): 491-524.

[40] Yuan JM, Ross RK, Wang XL, Gao YT, Henderson BE, Yu MC. Morbidity and mortality in relation to cigarette smoking in Shanghai, China. A prospective male cohort study. JAMA 1996 Jun 5; 275(21): 1646-50.

[41] Sasazuki S, Inoue M, Hanaoka T, Yamamoto S, Sobue T, Tsugane S. Green tea consumption and subsequent risk of gastric cancer by subsite: the JPHC Study. Cancer Causes Control 2004; 15(5): 483-91.

[42] Tsubono Y, Nishino Y, Komatsu S, *et al.* Green tea and the risk of gastric cancer in Japan. N Engl J Med 2001; 344(9): 632-6.

[43] Sun CL, Yuan JM, Lee MJ, *et al.* Urinary tea polyphenols in relation to gastric and esophageal cancers: a prospective study of men in Shanghai, China. Carcinogenesis 2002; 23(9): 1497-503.

[44] Inoue M, Sasazuki S, Wakai K, *et al.* Green tea consumption and gastric cancer in Japanese: a pooled analysis of six cohort studies. Gut 2009; 58(10): 1323-32.

[45] Myung SK, Bae WK, Oh SM, *et al.* Green tea consumption and risk of stomach cancer: a meta-analysis of epidemiologic studies. Int J Cancer 2009; 124(3): 670-7.

[46] Arab L, Il'yasova D. The epidemiology of tea consumption and colorectal cancer incidence. J Nutr 2003; 133(10): 3310S-8S.

[47] Marques-Vidal P, Ravasco P, Ermelinda Camilo M. Foodstuffs and colorectal cancer risk: a review. Clin Nutr 2006; 25(1): 14-36.

[48] Tavani A, La Vecchia C. Coffee, decaffeinated coffee, tea and cancer of the colon and rectum: a review of epidemiological studies, 1990-2003. Cancer Causes Control 2004; 15(8): 743-57.

[49] Bushman JL. Green tea and cancer in humans: a review of the literature. Nutr Cancer 1998; 31(3): 151-9.

[50] Suzuki E, Yorifuji T, Takao S, *et al.* Green tea consumption and mortality among Japanese elderly people: the prospective Shizuoka elderly cohort. Ann Epidemiol 2009; 19(10): 732-9.

[51] Sun CL, Yuan JM, Koh WP, Yu MC. Green tea, black tea and colorectal cancer risk: a meta-analysis of epidemiologic studies. Carcinogenesis 2006; 27(7): 1301-9.

[52] Yuan JM, Gao YT, Yang CS, Yu MC. Urinary biomarkers of tea polyphenols and risk of colorectal cancer in the Shanghai Cohort Study. Int J Cancer 2007; 120(6): 1344-50.

[53] Jian L, Xie LP, Lee AH, Binns CW. Protective effect of green tea against prostate cancer: a case-control study in southeast China. Int J Cancer 2004; 108(1): 130-5.

[54] Sonoda T, Nagata Y, Mori M, *et al.* A case-control study of diet and prostate cancer in Japan: possible protective effect of traditional Japanese diet. Cancer Sci 2004; 95(3): 238-42.

[55] Allen NE, Sauvaget C, Roddam AW, *et al.* A prospective study of diet and prostate cancer in Japanese men. Cancer Causes Control 2004; 15(9): 911-20.

[56] Kikuchi N, Ohmori K, Shimazu T, *et al.* No association between green tea and prostate cancer risk in Japanese men: the Ohsaki Cohort Study. Br J Cancer 2006; 95(3): 371-3.

[57] Kurahashi N, Sasazuki S, Iwasaki M, Inoue M, Tsugane S. Green tea consumption and prostate cancer risk in Japanese men: a prospective study. Am J Epidemiol 2008; 167(1): 71-7.

[58] Butt MS, Sultan MT. Green tea: nature's defense against malignancies. Crit Rev Food Sci Nutr. 2009 May; 49(5): 463-73.

[59] Lambert JD, Yang CS. Mechanisms of cancer prevention by tea constituents. J Nutr 2003; 133(10): 3262S-7S.

[60] Shukla Y. Tea and cancer chemoprevention: a comprehensive review. Asian Pac J Cancer Prev 2007; 8(2): 155-66.

[61] Sadava D, Whitlock E, Kane SE. The green tea polyphenol, epigallocatechin-3-gallate inhibits telomerase and induces apoptosis in drug-resistant lung cancer cells. Biochem Biophys Res Commun 2007; 360(1): 233-7.

[62] Milligan SA, Burke P, Coleman DT, *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(15): 4885-94.

[63] Liao J, Yang GY, Park ES, *et al.* Inhibition of lung carcinogenesis and effects on angiogenesis and apoptosis in A/J mice by oral administration of green tea. Nutr Cancer 2004; 48(1): 44-53.

[64] Lu G, Liao J, Yang G, Reuhl KR, Hao X, Yang CS. Inhibition of adenoma progression to adenocarcinoma in a 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis model in A/J mice by tea polyphenols and caffeine. Cancer Res 2006; 66(23): 11494-501.

[65] Mimoto J, Kiura K, Matsuo K, *et al.* (-)-Epigallocatechin gallate can prevent cisplatin-induced lung tumorigenesis in A/J mice. Carcinogenesis 2000; 21(5): 915-9.

[66] Wang ZY, Hong JY, Huang MT, Reuhl KR, Conney AH, Yang CS. Inhibition of N-nitrosodiethylamine- and 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone-induced tumorigenesis in A/J mice by green tea and black tea. Cancer Res 1992; 52(7): 1943-7.

[67] Masuda M, Suzui M, Lim JT, Deguchi A, Soh JW, Weinstein IB. 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(6): 350-9.

[68] Masuda M, Suzui M, Lim JT, Weinstein IB. 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(9): 3486-91.

[69] Sartippour MR, Shao ZM, Heber D, *et al.* Green tea inhibits vascular endothelial growth factor (VEGF) induction in human breast cancer cells. J Nutr 2002; 132(8): 2307-11.

[70] Thangapazham RL, Singh AK, Sharma A, Warren J, Gaddipati JP, Maheshwari RK. Green tea polyphenols and its constituent epigallocatechin gallate inhibits proliferation of human breast cancer cells *in vitro* and *in vivo*. Cancer Lett 2007; 245(1-2): 232-41.

[71] Kavanagh KT, Hafer LJ, Kim DW, *et al.* Green tea extracts decrease carcinogen-induced mammary tumor burden in rats and rate of breast cancer cell proliferation in culture. J Cell Biochem 2001; 82(3): 387-98.

[72] Lubet RA, Yang CS, Lee MJ, *et al.* Preventive effects of polyphenon E on urinary bladder and mammary cancers in rats and correlations with serum and urine levels of tea polyphenols. Mol Cancer Ther 2007; 6(7): 2022-8.

[73] Chen C, Shen G, Hebbar V, Hu R, Owuor ED, Kong AN. Epigallocatechin-3-gallate-induced stress signals in HT-29 human colon adenocarcinoma cells. Carcinogenesis 2003; 24(8): 1369-78.

[74] Shimizu M, Deguchi A, Lim JT, Moriwaki H, Kopelovich L, Weinstein IB. (-)-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(7): 2735-46.

[75] Shimizu M, Shirakami Y, Sakai H, *et al.* (-)-Epigallocatechin gallate inhibits growth and activation of the VEGF/VEGFR axis in human colorectal cancer cells. Chem Biol Interact 2010; 185(3): 247-52.

[76] Jia X, Han C. Effects of green tea on colonic aberrant crypt foci and proliferative indexes in rats. Nutr Cancer 2001; 39(2): 239-43.

[77] Shirakami Y, Shimizu M, Tsurumi H, Hara Y, Tanaka T, Moriwaki H. EGCG and Polyphenon E attenuate inflammation-related mouse colon carcinogenesis induced by AOM plus DDS. Mol Med Report 2008; 1(3): 355-61.

[78] Shimizu M, Shirakami Y, Sakai H, *et al.* (-)-Epigallocatechin gallate suppresses azoxymethane-induced colonic premalignant lesions in male C57BL/KsJ-db/db mice. Cancer Prev Res (Phila) 2008; 1(4): 298-304.

[79] Gupta S, Hastak K, Ahmad N, Lewin JS, Mukhtar H. Inhibition of prostate carcinogenesis in TRAMP mice by oral infusion of green tea polyphenols. Proc Natl Acad Sci USA 2001; 98(18): 10350-5.

[80] Caporali A, Davalli P, Astancolle S, *et al.* The chemopreventive action of catechins in the TRAMP mouse model of prostate carcinogenesis is accompanied by clusterin over-expression. Carcinogenesis 2004; 25(11): 2217-24.

[81] Liao S, Umekita Y, Guo J, Kokontis JM, Hiipakka RA. Growth inhibition and regression of human prostate and breast tumors in athymic mice by tea epigallocatechin gallate. Cancer Lett 1995; 96(2): 239-43.

[82] Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M. *In vivo* antitumor effect of ascorbic acid, lysine, proline and green tea extract on human prostate cancer PC-3 xenografts in nude mice: evaluation of tumor growth and immunohistochemistry. *In vivo* 2005; 19(1): 179-83.

[83] Mukhtar H, Ahmad N. Tea polyphenols: prevention of cancer and optimizing health. Am J Clin Nutr 2000; 71(6 Suppl): 1698S-702S; discussion 703S-4S.

[84] Senthil Kumaran V, Arulmathi K, Srividhya R, Kalaiselvi P. Repletion of antioxidant status by EGCG and retardation of oxidative damage induced macromolecular anomalies in aged rats. Exp Gerontol 2008; 43(3): 176-83.

[85] Srividhya R, Jyothilakshmi V, Arulmathi K, Senthilkumaran V, Kalaiselvi P. Attenuation of senescence-induced oxidative exacer bations in aged rat brain by (-)-epigallocatechin-3-gallate. Int J Dev Neurosci 2008; 26(2): 217-23.

[86] Ellinger S, Muller N, Stehle P, Ulrich-Merzenich G. Consumption of green tea or green tea products: is there an evidence for antioxidant effects from controlled interventional studies? Phytomedicine 2011; 18(11): 903-15.

[87] Shankar S, Suthakar G, Srivastava RK. Epigallocatechin-3-gallate inhibits cell cycle and induces apoptosis in pancreatic cancer. Front Biosci 2007; 12: 5039-51.

[88] Fujiki H, Suganuma M, Okabe S, *et al.* Cancer inhibition by green tea. Mutat Res 1998; 402(1-2): 307-10.

[89] Masuda M, Suzui M, Weinstein IB. 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(12): 4220-9.

[90] Smith DM, Wang Z, Kazi A, Li LH, Chan TH, Dou QP. Synthetic analogs of green tea polyphenols as proteasome inhibitors. Mol Med 2002; 8(7): 382-92.

[91] Ahmed S, Wang N, Lalonde M, Goldberg VM, Haqqi TM. Green tea polyphenol epigallocatechin-3-gallate (EGCG) differentially inhibits interleukin-1 beta-induced expression of matrix metalloproteinase-1 and -13 in human chondrocytes. J Pharmacol Exp Ther 2004; 308(2): 767-73.

[92] Dong Z, Ma W, Huang C, Yang CS. Inhibition of tumor promoter-induced activator protein 1 activation and cell transformation by tea polyphenols, (-)-epigallocatechin gallate, and theaflavins. Cancer Res 1997; 57(19): 4414-9.

[93] Chung JY, Huang C, Meng X, Dong Z, Yang CS. 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(18): 4610-7.

[94] Shimizu M, Deguchi A, Joe AK, McKoy JF, Moriwaki H, Weinstein IB. EGCG inhibits activation of HER3 and expression of cyclooxygenase-2 in human colon cancer cells. J Exp Ther Oncol 2005; 5(1): 69-78.

[95] Shimizu M, Shirakami Y, Moriwaki H. Targeting receptor tyrosine kinases for chemoprevention by green tea catechin, EGCG. Int J Mol Sci 2008; 9(6): 1034-49.

[96] Shimizu M, Weinstein IB. Modulation of signal transduction by tea catechins and related phytochemicals. Mutat Res 2005; 591(1-2): 147-60.

[97] Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell 2010; 141(7): 1117-34.

[98] Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell 2000; 103(2): 211-25.

[99] Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer 2005; 5(5): 341-54.
[100] Pollak MN, Schernhammer ES, Hankinson SE. Insulin-like growth factors and neoplasia. Nat Rev Cancer 2004; 4(7): 505-18.
[101] Press MF, Lenz HJ. EGFR, HER2 and VEGF pathways: validated targets for cancer treatment. Drugs 2007; 67(14): 2045-75.
[102] Adhami VM, Siddiqui IA, Ahmad N, Gupta S, Mukhtar H. 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(23): 8715-22.
[103] Shimizu M, Deguchi A, Hara Y, Moriwaki H, Weinstein IB. EGCG inhibits activation of the insulin-like growth factor-1 receptor in human colon cancer cells. Biochem Biophys Res Commun 2005; 334(3): 947-53.
[104] Shimizu M, Shirakami Y, Sakai 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(1): 10-8.
[105] Lee YK, Bone ND, Strege AK, Shanafelt TD, Jelinek DF, Kay NE. 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(3): 788-94.
[106] Shirakami Y, Shimizu M, Adachi S, *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(10): 1957-62.
[107] Adachi S, Nagao T, Ingolfsson HI, *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(13): 6493-501.
[108] Adachi S, Nagao T, To S, *et al.* (-)-Epigallocatechin gallate causes internalization of the epidermal growth factor receptor in human colon cancer cells. Carcinogenesis 2008; 29(10): 1986-93.
[109] Adachi S, Shimizu M, Shirakami Y, *et al.* (-)-Epigallocatechin gallate downregulates EGF receptor *via* phosphorylation at Ser1046/1047 by p38 MAPK in colon cancer cells. Carcinogenesis 2009; 30(9): 1544-52.
[110] Aggarwal BB, Shishodia S. Molecular targets of dietary agents for prevention and therapy of cancer. Biochem Pharmacol 2006; 71(10): 1397-421.
[111] Hussain T, Gupta S, Adhami VM, Mukhtar H. Green tea constituent epigallocatechin-3-gallate selectively inhibits COX-2 without affecting COX-1 expression in human prostate carcinoma cells. Int J Cancer 2005; 113(4): 660-9.
[112] Kundu JK, Na HK, Chun KS, *et al.* Inhibition of phorbol ester-induced COX-2 expression by epigallocatechin gallate in mouse skin and cultured human mammary epithelial cells. J Nutr 2003; 133(11 Suppl 1): 3805S-10S.
[113] Moreira L, Castells A. Cyclooxygenase as a target for colorectal cancer chemoprevention. Curr Drug Targets 2011; 12(13): 1888-94.
[114] Link A, Balaguer F, Goel A. Cancer chemoprevention by dietary polyphenols: promising role for epigenetics. Biochem Pharmacol 2010; 80(12): 1771-92.
[115] Feinberg AP, Tycko B. The history of cancer epigenetics. Nat Rev Cancer 2004; 4(2): 143-53.
[116] Choi KC, Jung MG, Lee YH, *et al.* Epigallocatechin-3-gallate, a histone acetyltransferase inhibitor, inhibits EBV-induced B lymphocyte transformation *via* suppression of RelA acetylation. Cancer Res 2009; 69(2): 583-92.
[117] Fang M, Chen D, Yang CS. Dietary polyphenols may affect DNA methylation. J Nutr 2007; 137(1 Suppl): 223S-8S.
[118] Guilleret I, Benhattar J. Unusual distribution of DNA methylation within the hTERT CpG island in tissues and cell lines. Biochem Biophys Res Commun 2004; 325(3): 1037-43.
[119] Chuang JC, Yoo CB, Kwan JM, *et al.* Comparison of biological effects of non-nucleoside DNA methylation inhibitors versus 5-aza-2'-deoxycytidine. Mol Cancer Ther 2005; 4(10): 1515-20.
[120] Stresemann C, Brueckner B, Musch T, Stopper H, Lyko F. Functional diversity of DNA methyltransferase inhibitors in human cancer cell lines. Cancer Res 2006; 66(5): 2794-800.
[121] Mittal A, Piyathilake C, Hara Y, Katiyar SK. Exceptionally high protection of photocarcinogenesis by topical application of (--)-epigallocatechin-3-gallate in hydrophilic cream in SKH-1 hairless mouse model: relationship to inhibition of UVB-induced global DNA hypomethylation. Neoplasia 2003; 5(6): 555-65.
[122] Morey Kinney SR, Zhang W, Pascual M, *et al.* Lack of evidence for green tea polyphenols as DNA methylation inhibitors in murine prostate. Cancer Prev Res (Phila) 2009; 2(12): 1065-75.
[123] Tsao AS, Liu D, Martin J, *et al.* Phase II randomized, placebo-controlled trial of green tea extract in patients with high-risk oral premalignant lesions. Cancer Prev Res (Phila) 2009; 2(11): 931-41.
[124] Little TJ, Hultmark D, Read AF. Invertebrate immunity and the limits of mechanistic immunology. Nat Immunol 2005; 6(7): 651-4.
[125] Schmid-Hempel P. Evolutionary ecology of insect immune defenses. Annu Rev Entomol 2005; 50: 529-51.
[126] Klein C, Sato T, Meguid MM, Miyata G. From food to nutritional support to specific nutraceuticals: a journey across time in the treatment of disease. J Gastroentero. 2000; 35 Suppl 12: 1-6.
[127] Yang F, de Villiers WJ, McClain CJ, Varilek GW. Green tea polyphenols block endotoxin-induced tumor necrosis factor-production and lethality in a murine model. J Nutr 1998; 128(12): 2334-40.
[128] Uyttenhove C, Pilotte L, Theate I, *et al.* Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med 2003; 9(10): 1269-74.
[129] Ogawa K, Hara T, Shimizu M, *et al.* (-)-Epigallocatechin gallate inhibits the expression of indoleamine 2,3-dioxygenase in human colorectal cancer cells. Oncology Letters 2012; 4: 546-50.
[130] Cheng CW, Shieh PC, Lin YC, *et al.* Indoleamine 2,3-dioxygenase, an immunomodulatory protein, is suppressed by (-)-epigallo catechin-3-gallate *via* blocking of gamma-interferon-induced JAK-PKC-delta-STAT1 signaling in human oral cancer cells. J Agric Food Chem 2010; 58(2): 887-94.
[131] Ogawa K, Hara T, Shimizu M, *et al.* Suppression of azoxymethane-induced colonic preneoplastic lesions in rats by 1-methyl tryptophan, an inhibitor of indoleamine 2,3-dioxygenase. Cancer Sci 2012; 103(5): 951-8.
[132] Chow HH, Cai Y, Alberts DS, *et al.* Phase I pharmacokinetic study of tea polyphenols following single-dose administration of epigallo catechin gallate and polyphenon E. Cancer Epidemiol Biomarkers Prev 2001; 10(1): 53-8.
[133] Mazzanti G, Menniti-Ippolito F, Moro PA, *et al.* Hepatotoxicity from green tea: a review of the literature and two unpublished cases. Eur J Clin Pharmacol 2009; 65(4): 331-41.
[134] 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(4): 218-24.
[135] Ahn WS, Yoo J, Huh SW, *et al.* Protective effects of green tea extracts (polyphenon E and EGCG) on human cervical lesions. Eur J Cancer Prev 2003; 12(5): 383-90.
[136] Bettuzzi S, Brausi M, Rizzi F, Castagnetti G, Peracchia G, Corti A. 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(2):1234-40.
[137] Brausi M, Rizzi F, Bettuzzi S. Chemoprevention of human prostate cancer by green tea catechins: two years later. A follow-up update. Eur Urol 2008; 54(2): 472-3.
[138] Choan E, Segal R, Jonker D, *et al.* A prospective clinical trial of green tea for hormone refractory prostate cancer: an evaluation of the complementary/alternative therapy approach. Urol Oncol 2005; 23(2): 108-13.
[139] Jatoi A, Ellison N, Burch PA, *et al.* A phase II trial of green tea in the treatment of patients with androgen independent metastatic prostate carcinoma. Cancer 2003; 97(6): 1442-6.
[140] McLarty J, Bigelow RL, Smith M, Elmajian D, Ankem M, Cardelli JA. 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 (Phila) 2009; 2(7): 673-82.
[141] Shimizu M, Fukutomi Y, Ninomiya M, *et al.* Green tea extracts for the prevention of metachronous colorectal adenomas: a pilot study. Cancer Epidemiol Biomarkers Prev 2008; 17(11): 3020-5.
[142] Stingl JC, Ettrich T, Muche R, *et al.* Protocol for minimizing the risk of metachronous adenomas of the colorectum with green tea extract (MIRACLE): a randomised controlled trial of green tea extract versus placebo for nutriprevention of metachronous colon adenomas in the elderly population. BMC Cancer 2011; 11: 360.

[143] Hakim IA, Harris RB, Brown S, *et al.* Effect of increased tea consumption on oxidative DNA damage among smokers: a randomized controlled study. J Nutr 2003; 133(10): 3303S-9S.

[144] Luo H, Tang L, Tang 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(2): 262-8.

[145] Tang L, Tang M, Xu L, *et al.* Modulation of aflatoxin biomarkers in human blood and urine by green tea polyphenols intervention. Carcinogenesis 2008; 29(2): 411-7.

[146] Nguyen MM, Ahmann FR, Nagle RB, *et al.* Randomized, double-blind, placebo-controlled trial of polyphenon E in prostate cancer patients before prostatectomy: evaluation of potential chemopreventive activities. Cancer Prev Res (Phila) 2012; 5(2): 290-8.

[147] Fujiki H, Suganuma M, Imai K, Nakachi K. Green tea: cancer preventive beverage and/or drug. Cancer Lett 2002; 188(1-2): 9-13.

[148] Fan Y, Yuan JM, Wang R, Gao YT, Yu MC. Alcohol, tobacco, and diet in relation to esophageal cancer: the Shanghai Cohort Study. Nutr Cancer 2008; 60(3): 354-63.

[149] El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epide miology and molecular carcinogenesis. Gastroenterology 2007; 132(7): 2557-76.

[150] Muto Y, Sato S, Watanabe A, *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(3): 204-14.

[151] Lee MJ, Wang ZY, Li H, *et al.* Analysis of plasma and urinary tea polyphenols in human subjects. Cancer Epidemiol Biomarkers Prev 1995; 4(4): 393-9.

[152] Yang CS, Wang X. Green tea and cancer prevention. Nutr Cancer 2010; 62(7): 931-7.

[153] Yang CS, Sang S, Lambert JD, Lee MJ. Bioavailability issues in studying the health effects of plant polyphenolic compounds. Mol Nutr Food Res 2008; 52 Suppl 1: S139-51.

[154] Ermakova S, Choi BY, Choi HS, Kang BS, Bode AM, Dong Z. The intermediate filament protein vimentin is a new target for epigallocatechin gallate. J Biol Chem 2005; 280(17): 16882-90.

[155] He Z, Tang F, Ermakova S, *et al.* Fyn is a novel target of (-)-epigallocatechin gallate in the inhibition of JB6 Cl41 cell transformation. Mol Carcinog 2008; 47(3): 172-83.

[156] Li M, He Z, Ermakova S, *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(3): 598-605.

[157] Chow HH, Cai Y, Hakim IA, *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(9): 3312-9.

[158] Bonkovsky HL. Hepatotoxicity associated with supplements containing Chinese green tea (Camellia sinensis). Ann Intern Med 2006; 144(1): 68-71.

[159] Stevens T, Qadri A, Zein NN. Two patients with acute liver injury associated with use of the herbal weight-loss supplement hydroxycut. Ann Intern Med 2005; 142(6): 477-8.

[160] Jimenez-Saenz M, Martinez-Sanchez Mdel C. Acute hepatitis associated with the use of green tea infusions. J Hepatol 2006; 44(3): 616-7.

[161] Clark J, You M. Chemoprevention of lung cancer by tea. Mol Nutr Food Res 2006; 50(2): 144-51.

[162] Suganuma M, Okabe S, Kai Y, Sueoka N, Sueoka E, Fujiki H. Synergistic effects of (--)-epigallocatechin gallate with (--)-epicatechin, sulindac, or tamoxifen on cancer-preventive activity in the human lung cancer cell line PC-9. Cancer Res 1999; 59(1): 44-7.

[163] Hirose M, Mizoguchi Y, Yaono M, Tanaka H, Yamaguchi T, Shirai T. Effects of green tea catechins on the progression or late promotion stage of mammary gland carcinogenesis in female Sprague-Dawley rats pretreated with 7,12-dimethylbenz(a) anthracene. Cancer Lett 1997; 112(2): 141-7.

[164] Yang CS, Maliakal P, Meng X. Inhibition of carcinogenesis by tea. Annu Rev Pharmacol Toxicol 2002; 42: 25-54.

[165] Suganuma M, Ohkura Y, Okabe S, Fujiki H. Combination cancer chemoprevention with green tea extract and sulindac shown in intestinal tumor formation in Min mice. J Cancer Res Clin Oncol 2001; 127(1): 69-72.

[166] Suganuma M, Saha A, Fujiki H. New cancer treatment strategy using combination of green tea catechins and anticancer drugs. Cancer Sci 2011; 102(2): 317-23.

[167] Fujiki H, Imai K, Nakachi K, Shimizu M, Moriwaki H, Suganuma M. Challenging the effectiveness of green tea in primary and tertiary cancer prevention. J Cancer Res Clin Oncol 2012; 138(8): 1259-70.

[168] Okabe S, Fujimoto N, Sueoka N, Suganuma M, Fujiki H. Modulation of gene expression by (-)-epigallocatechin gallate in PC-9 cells using a cDNA expression array. Biol Pharm Bull 2001; 24(8): 883-6.

[169] Suganuma M, Kurusu M, Suzuki K, Tasaki E, Fujiki H. Green tea polyphenol stimulates cancer preventive effects of celecoxib in human lung cancer cells by upregulation of GADD153 gene. Int J Cancer 2006; 119(1): 33-40.

Received: August 14, 2012 **Revised: October 01, 2012** **Accepted: November 03, 2012**

PMID: 23140294

Cancer Letters 323 (2012) 215–222

Contents lists available at SciVerse ScienceDirect

Cancer Letters

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

Synergistic growth inhibition of human hepatocellular carcinoma cells by acyclic retinoid and GW4064, a farnesoid X receptor ligand

Tomohiko Ohno, Yohei Shirakami, Masahito Shimizu *, Masaya Kubota, Hiroyasu Sakai, Yoichi Yasuda, Takahiro Kochi, Hisashi Tsurumi, Hisataka Moriwaki

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

ARTICLE INFO

Article history:
Received 4 November 2011
Received in revised form 29 March 2012
Accepted 20 April 2012

Keywords:
Acyclic retinoid
GW4064
Hepatocellular carcinoma
RXRα
FXR
Synergism

ABSTRACT

Abnormalities in the expression and function of retinoid X receptor (RXR), a master regulator of the nuclear receptor superfamily, are associated with the development of hepatocellular carcinoma (HCC). Dysfunction of farnesoid X receptor (FXR), one of the nuclear receptors that forms a heterodimer with RXR, also plays a role in liver carcinogenesis. In the present study, we examined the effects of acyclic retinoid (ACR), a synthetic retinoid targeting RXRα, plus GW4064, a ligand for FXR, on the growth of human HCC cells. We found that ACR and GW4064 preferentially inhibited the growth of HLE, HLF, and Huh7 human HCC cells in comparison with Hc normal hepatocytes. The combination of 1 μM ACR plus 1 μM GW4064 synergistically inhibited the growth of HLE cells by inducing apoptosis. The combined treatment with these agents acted cooperatively to induce cell cycle arrest in the G_0/G_1 phase and inhibit the phosphorylation of RXRα, which is regarded as a critical factor for liver carcinogenesis, through inhibition of ERK and Stat3 phosphorylation. This combination also increased the expression levels of $p21^{CIP1}$ and SHP mRNA, while decreasing the levels of c-*myc* and cyclin D1 mRNA in HLE cells. In addition, a reporter assay indicated that the FXRE promoter activity was significantly increased by treatment with ACR plus GW4064. Our results suggest that ACR and GW4064 cooperatively inhibit RXRα phosphorylation, modulate the expression of FXR-regulated genes, thus resulting in the induction of apoptosis and the inhibition of growth in HCC cells. This combination might therefore be effective for the chemoprevention and chemotherapy of HCC.

1. Introduction

Nuclear receptors are ligand-dependent transcription factors that are involved in various physiological processes. Retinoid X receptors (RXRs) are regarded as master regulators of nuclear receptors because they play an essential role in controlling normal cell proliferation and metabolism by forming a heterodimer with other nuclear receptors [1,2]. Therefore, abnormalities in the expression and function of RXRs are closely associated with the development of various disorders, including cancer, whereas using a retinoid might be an effective strategy for the prevention and treatment of human malignancies [3]. A malfunction of RXRα, one of the subtypes of RXR, due to phosphorylation by the Ras/MAPK signaling pathway is profoundly associated with liver carcinogenesis [4–8]. On the other hand, administration of acyclic retinoid (ACR), a synthetic retinoid which targets RXRα, reduced the incidence of post-therapeutic recurrence of hepatocellular carcinoma (HCC) and improved the survival rate of patients with this malignancy [9,10]. ACR also inhibits the growth of HCC-derived cells by inducing apoptosis and cell cycle arrest in the G_0/G_1 phase [11,12]. These findings suggest that nuclear receptors, especially RXRα, are critical targets for the prevention and treatment of HCC.

Farnesoid X receptor (FXR), which has been characterized as a bile acid receptor, is also a member of the nuclear receptor superfamily of ligand-dependent transcription factors that form heterodimers with RXR [13]. FXR has been shown to be essential in controlling bile acid, lipid, and glucose homeostasis [13]. It also plays a critical role in normal liver regeneration and promotes liver repair after injury by mediating its related signaling pathways [14].

Abbreviations: ACR, acyclic retinoid; CI, combination index; DAPI, 4',6-diamidino-2-phenylindole; ERK, extracellular signal-regulated kinase; FXR, farnesoid X receptor; FXRE, farnesoid X receptor response element; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; IFN, interferon; MAPK, mitogen-activated protein kinase; PARP, poly (ADP-ribose) polymerase; RAR, retinoic acid receptor; RARE, retinoic acid response element; RTK, receptor tyrosine kinase; RT-PCR, reverse transcription PCR; RXR, retinoid X receptor; SHP, small heterodimer partner; Stat3, signal transducer and activator of transcription 3; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

* Corresponding author. Address: Department of Internal Medicine, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan. Tel.: +81 58 230 6313; fax: +81 58 230 6310.

E-mail address: shimim-gif@umin.ac.jp (M. Shimizu).

0304-3835/$ - see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.canlet.2012.04.015

In addition, recent studies have revealed that aberrations in FXR are involved in liver carcinogenesis. FXR deficiency in mice leads to the development of neoplasms in the liver, including hepatic adenoma, HCC, and hepatocholangiocellular carcinoma [15,16]. A significant decrease in FXR expression and activity is also observed in human HCC samples [17]. Therefore, targeting FXR and improving its function might be a promising strategy for the prevention and treatment of HCC.

Recently, combination therapy and prevention have garnered much interest in the cancer field because they can synergistically inhibit growth and induce apoptosis in cancer cells. In human HCC-derived cells, ACR acts synergistically with other agents, such as interferon (IFN)-β, OSI-461, vitamin K_2, valproic acid, and trastuzumab, in suppressing growth and inducing apoptosis [11,18–21]. The agents that inhibit RXRα phosphorylation are among the most promising agents to use in combination with ACR [11,20,21]. In addition, the induction of nuclear receptors that dimerize with RXR, such as retinoic acid receptor (RAR)-β, and activation of these receptors by their ligands may also lead to synergistic growth inhibition in HCC cells when combined with ACR [11,19]. GW4064, a synthetic ligand for FXR, is known to induce the expression of genes involved in the transport of bile acids in the liver and intestines [22,23]. GW4064 also inhibits the growth of breast and prostate cancer cell lines [24–26], whereas the anticancer effects of this agent on HCC cells have not been evaluated. In the present study, we examined the effects of GW4064 on the growth of human HCC cells. We also investigated whether the combination of ACR plus GW4064 exerts synergistic growth inhibitory effects on HCC cells and examined the possible mechanisms responsible for such synergy.

2. Materials and methods

2.1. Materials

ACR (NIK-333) was supplied by Kowa Pharmaceutical Co. Ltd., (Tokyo, Japan). GW4064 was purchased from Sigma–Aldrich (St. Louis, MO, USA). The anti-RXRα antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibodies for ERK, phosphorylated ERK (p-ERK), Stat3, phosphorylated Stat3 (p-Stat3), PARP, and GAPDH were from Cell Signaling Technology (Beverly, MA, USA).

2.2. Cell lines and cell culture conditions

HLE, HLF, and Huh7 human HCC cell lines were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). HLE and HLF cells were maintained in DMEM and Huh7 cells were in RPMI1640 media, respectively. All media were supplemented with 10% FCS and 1% Penicillin/Streptomycin. Hc human normal hepatocyte cell line was purchased from Cell Systems (Kirkland, WA, USA) and maintained in a CS-S complete medium (Cell Systems). These cells were cultured in an incubator with humidified air with 5% CO_2 at 37 °C.

2.3. Cell proliferation assays

One thousand of HCC (HLE, HLF, and Huh7) or Hc cells were seeded on 96-well plates. The following day, the medium was changed to serum free medium and the cells were treated with the indicated concentrations of ACR or GW4064 for 48 h. Cell proliferation assays were performed using a MTS assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. To determine whether the combined effects of ACR plus GW4064 were synergistic, HCC cells were treated with combinations of the indicated concentrations of ACR and GW4064 for 48 h and the combination index (CI)-isobologram was calculated. Variable ratios of drug concentrations were used in the studies, and mutually exclusive equations were used to determine the CIs. Each CI was calculated from the mean affected fraction at each drug ratio concentration (triplicate), as described previously [11,19,27].

2.4. Apoptosis assays

TUNEL, caspase-3 activity, and Annexin V assays are conducted to evaluate apoptosis. For TUNEL assay, HLE cells (1×10^6) were treated with 1 μM ACR alone, 1 μM GW4064 alone, or the combination of these agents for 48 h on glass bottom culture dishes. The cells were then fixed with 4% paraformaldehyde at room temperature for 10 min, permeabilized with 0.3% Triton X-100 in TBS (pH 7.4), and stained with both 4',6-diamidino-2-phenylindole (DAPI) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) methods using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany) [11].

Caspase-3 activity and Annexin V assays were performed using HLE cells that were treated with the same concentration of test drugs for 72 h. The cell lysates were prepared and the caspase-3 activity assay was done using the Apoalert Caspase Fluorescent Assay Kit (Clontech Laboratories, Mountain View, CA, USA). The Annexin V-binding capacity of treated cells was investigated by flow cytometry using the Annexin V-FITC apoptosis detection kit I (BD, Franklin Lakes, NJ, USA). Cultured cells were washed with cold phosphate-buffered saline before incubation with Annexin V-FITC in a buffer containing propidium iodine (PI). Stained cells were analyzed by flow cytometry using the FACScan (BD). Annexin V-FITC-positive and PI negative cells were considered to be populations undergoing apoptosis.

2.5. Cell cycle assays

HLE cells were treated with 1 μM ACR alone, 1 μM GW4064 alone, or the combination of these agents for 72 h in DMEM medium with 1% FCS. The harvested cells were stained with PI using Cell Cycle Phase Determination Kit (Cayman, Ann Arbor, MI, USA), and the samples were then analyzed for DNA histograms and cell cycle phase distribution using a FACScan flow cytometer. The data were analyzed by using the CellQuest computer program (BD) [11].

2.6. Protein extraction and Western blot analysis

Equivalent amounts of extracted protein were examined by a Western blot analysis using specific antibodies [21]. To detect the expression level of phosphorylated RXRα (p-RXRα) protein, total phosphoprotein was affinity-purified from the total cell extracts using a PhosphoProtein Purification Column (QIAGEN, Valencia, CA, USA) and then was subjected to the Western blot analyses using an anti-RXRα antibody. GAPDH expression served as a loading control. The intensities of protein bands were quantified using NIH image software version 1.45.

2.7. RNA extraction and quantitative RT-PCR analysis

Total RNA was isolated from the HLE cells using the RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX, USA) and cDNA was amplified from 0.2 μg of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) [28]. Quantitative real-time reverse transcription-PCR (RT-PCR) analysis was performed using specific primers that amplify the *c-myc*, small heterodimer partner (SHP), $p21^{CIP1}$, cyclin D1, and β-actin genes. The specific primer sets for $p21^{CIP1}$, cyclin D1, and β-actin were used as described elsewhere [12,29]. The sequences for *c-myc*- and SHP-specific primers were as follows: FMYC (5'-CCC TGA GCG ATT CAG ATG AT-3') and RMYC (5'-GCT CCA GGA TGT TGT GGT TT-3'), and FSHP (5'-GCT GTC TGG AGT CCT TCT GG-3') and RSHP (5'-ACC TGA GCA AAA GCA TGT CC-3'), respectively.

2.8. FXRE reporter assays

HLE cells were transfected with FXR response element (FXRE) reporter plasmids (100 ng/well in 96-well dish), which were kindly provided by Dr. T. Nishimaki-Mogami (National Institute of Health Sciences, Tokyo, Japan), along with *pRL*-CMV (*Renilla* luciferase, 10 ng/well in 96-well dish; Promega) as an internal standard to normalize the transfection efficiency. Transfections were done using Lipofectamine LTX Reagent (Invitrogen). After exposure of the cells to the transfection mixture for 24 h, the cells were treated with 1 μM ACR alone, 1 μM GW4064 alone, or the combination of these agents for 24 h. The cell lysates were then prepared, and the luciferase activity of each cell lysate was determined using a dual-luciferase reporter assay system (Promega) [11].

2.9. Statistical analysis

The data are expressed as the means ± SD. Statistical significance of the differences in the mean values was assessed with a one-way ANOVA, followed by Tukey–Kramer's multiple comparison tests. Values of $P < 0.05$ were considered to be significant.

3. Results

3.1. ACR and GW4064 cause preferential inhibition of the growth of human HCC cells in comparison with Hc normal hepatocytes

In our initial study, we examined the growth inhibitory effect of ACR and GW4064 on HLE, HLF, and Huh7 human HCC cells and on Hc hepatocytes. ACR inhibited the growth of HCC cells with an IC_{50} value of less than 4 μM. The HLF cells were most susceptible to ACR

because the IC_{50} value with this agent was 2 μM (Fig. 1A). GW4064 also inhibited the growth of this series of HCC cells with an IC_{50} value of about 1.4 μM (Fig. 1B). On the other hand, Hc cells were resistant to these agents up to 5 μM (Fig. 1). These results suggest that ACR and GW4064 preferentially inhibit the growth of HCC cells compared with that of normal hepatocytes.

3.2. ACR plus GW4064 cause synergistic inhibition of the growth of HCC cells

Next, the effects of combined treatment were examined with a range of concentrations of ACR plus GW4064 to determine whether they synergistically inhibited the growth of HLE (Fig. 2A), HLF (Fig. 2B), and Huh7 (Fig. 2C) HCC cells. We found that the CI indices for less than 1 μM ACR (0.5 or 1 μM) plus less than 0.5 μM GW4064 (0.1 or 0.5 μM) were 1+(slight synergism), 2+(moderate synergism), or 3+(synergism), respectively, in this series of HCC cells (Fig. 2D and Table 1). These findings suggest that ACR plus GW4064 might be an effective combination for the inhibition of HCC cell growth due to their synergistic activity. The combination of 1 μM ACR (about IC_{25} value) and 1 μM GW4064 (about IC_{30} value) in HLE cells (Fig. 2A and D, and Table 1) was used for the following experiments because a CI-isobologram analysis gave this combination a CI index of 1+(0.88), indicating slight synergism.

3.3. ACR plus GW4064 cooperatively induce apoptosis in HLE cells

We next examined whether the synergistic growth inhibition in HLE cells induced by treatment with ACR plus GW4064 might be associated with the induction of apoptosis. In TUNEL assays, the treatment of HLE cells with either 1 μM ACR or 1 μM GW4064 alone induced TUNEL-positive cells in approximately 19.3% or 11.9% of the total viable cells, respectively. However, the combination of these agents markedly enhanced the induction of apoptosis, with 51.6% of the total viable cells being TUNEL-positive (Fig. 3A). Similar results were also observed in the Western blot analysis for PARP expression; the combination of ACR plus GW4064 markedly enhanced PARP cleavage, indicating the induction of apoptosis (Fig. 3B). We also found an increase in the levels of caspase-3 activity in ACR alone- and GW4064 alone-treated cells, and this was significantly enhanced when the cells were treated with a combination of these agents (Fig. 3C). In addition, the percentage of Annexin V-positive cells, which was increased by treatment with GW4064 alone, was substantially increased by the combined treatment with ACR plus GW4064 (Fig. 3D). These findings suggest that the combination with ACR plus GW4064 synergistically inhibited growth of HLE human HCC cells, mainly, through the induction of apoptosis.

3.4. ACR plus GW4064 cooperatively induce G_0/G_1 cell cycle arrest in HLE cells

A cell cycle analysis was performed using DNA flow cytometry to determine whether the synergistic effects on growth inhibition caused by combined treatment with ACR plus GW4064 were associated with specific changes in cell cycle distribution. As shown in Fig. 4, the combined treatment with 1 μM ACR plus 1 μM GW4064 significantly increased the percentage of cells in the G_0/G_1 phase in comparison to that of untreated cells (76.1 ± 4.3% vs. 57.3 ± 5.8%, $P < 0.05$), whereas the population of cells in this phase was not significantly increased by treatment with ACR alone (63.6 ± 3.0%) or GW4064 alone (65.3 ± 4.5%). These findings suggest that the combination of ACR plus GW4064 cooperatively induced G_0/G_1 phase cell cycle arrest in HLE human HCC cells.

3.5. ACR plus GW4064 additively suppress the phosphorylation of RXRα, ERK, and Stat3 proteins in HLE cells

RXRα phosphorylation plays a critical role in the development of HCC and might be a promising target for HCC chemoprevention [4–8]. Therefore, the effects of the combination of ACR plus GW4064 on the phosphorylation of this nuclear receptor and related signaling molecules were investigated in HLE cells. As shown in Fig. 5, when the cells were treated with 1 μM ACR, there was a marked decrease in the expression levels of p-RXRα and p-Stat3 proteins. Treatment with 1 μM GW4064 alone also decreased the expression levels of p-ERK and p-Stat3 protein. Moreover, the expression levels of p-RXRα, p-ERK and p-Stat3 proteins were markedly decreased when the cells were treated with the combination of these agents.

3.6. ACR plus GW4064 cooperatively affect the expression levels of $p21^{CIP1}$, c-myc, cyclin D1, and SHP mRNA in HLE cells

We next examined the combined effects of ACR plus GW4064 on the expression levels of $p21^{CIP1}$, *c-myc*, and cyclin D1 mRNA in HLE cells because these genes control cell proliferation and cell cycle progression. The quantitative RT-PCR analyses revealed that treatment with neither 1 μM ACR nor 1 μM GW4064 alone had any apparent effect on the expression levels of $p21^{CIP1}$, *c-myc*, and cyclin D1 mRNA. However, when the cells were treated with the combination of these agents, there was a significant increase

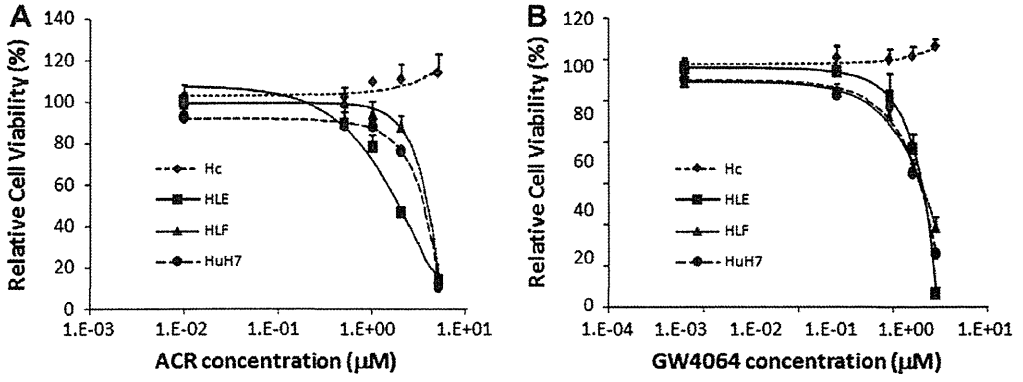


Fig. 1. Inhibition of cell growth by ACR and GW4064 in HLE, HLF, and Huh7 human HCC cells and Hc normal hepatocytes. HLE, HLF, Huh7, and Hc cells were treated with the indicated concentrations of ACR (A) or GW4064 (B) for 48 h. Cell viability was determined by MTS assay and was expressed as a percentage of the control value. *Error Bars*, SD of triplicate assays.

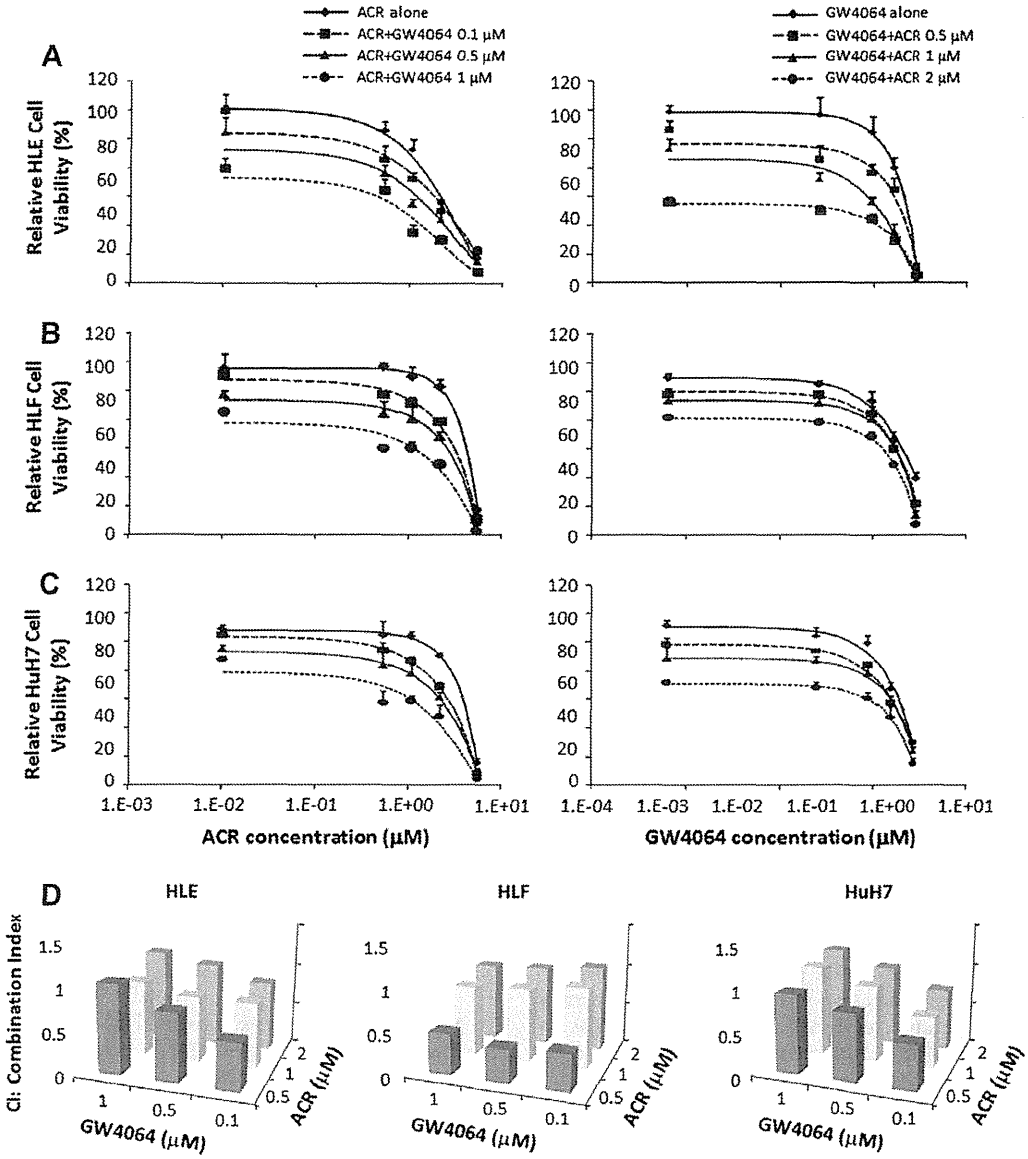


Fig. 2. Inhibition of cell growth by ACR alone, GW4064 alone, and various combinations of these agents in HCC cells. HLE (A), HLF (B), and Huh7 (C) cells were treated with the indicated concentrations of ACR alone, GW4064 alone, and various combinations of these agents for 48 h. Cell viability was determined by MTS assay and expressed as a percentage of the control value. *Error Bars*, SD of triplicate assays. (D) The data obtained in (A), (B), and (C) was used to calculate the combination index.

Table 1
Combined effects of ACR and GW4064 on HCC cells.

	HLE ACR concentration (μM)			HLF ACR concentration (μM)			HuH7 ACR concentration (μM)		
GW4064 concentration (μM)	0.5	1	2	0.5	1	2	0.5	1	2
0.1	+++	++	+	+++	+	±	+++	++	++
0.5	++	++	±	+++	++	±	++	+	±
1	±	+	±	+++	+	±	±	±	±

Note: "±", CI 0.9–1.1 additive effect; "+", CI 0.8–0.9 slight synergism; "++", CI 0.6–0.8 moderate synergism; "+++", CI 0.4–0.6 synergism;
Abbreviations: CI, combination index; ACR, acyclic retinoid.