MOLECULAR CARCINOGENESIS 47:629–637 (2008)

Fucoidan From *Laminaria cichorioides* Inhibits AP-1 Transactivation and Cell Transformation in the Mouse Epidermal JB6 Cells

Na Yeon Lee,[1] Svetlana P. Ermakova,[2] Hoo-Kyun Choi,[1] Michail I. Kusaykin,[2] Natalya M. Shevchenko,[2] Tatyana N. Zvyagintseva,[2] and Hong Seok Choi[1]*

[1]*College of Pharmacy, Chosun University, Gwangju, South Korea*
[2]*Pacific Institute of Bioorganic Chemistry, Far Eastern Branch of the Russian Academy of Sciences, Vladivostok, Russia*

Fucoidan, a sulfated polysaccharide extracted from brown seaweeds, has anticoagulant and antithrombotic activities. Unlike heparine, fucoidan is known to exhibit anticarcinogenic activities. However, the underlying molecular mechanisms of the chemopreventive activities of fucoidan are not understood. Here we report that fucoidan from *Laminaria cichorioides* inhibited the epidermal growth factor (EGF) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced neoplastic cell transformation, but had less cytotoxic effects on JB6 mouse epidermal cells. The EGF-induced phosphorylation of extracellular signal-regulated kinases 1/2 and c-Jun N-terminal kinases, and c-Jun was inhibited by fucoidan, resulting from the inhibition of phosphorylation of epidermal growth factor receptor (EGFR). Fucoidan dose-dependently attenuated the *c-fos* or *c-jun* transcriptional activity, and thereby inhibited the associated activator protein-1 (AP-1) transactivation activity. In vitro binding assay revealed that fucoidan directly interacted with EGF, suggested that antitumor promoting effect of fucoidan might be due to preventing the binding of EGF to its cell surface receptor (EGFR). These findings are the first to reveal a molecular basis for the anticarcinogenic action of fucoidan and may partially account for the reported chemopreventive effects of brown seaweeds. © 2008 Wiley-Liss, Inc.

Key words: fucoidan; AP-1; cell transformation; EGFR; *c-fos*; *c-jun*

INTRODUCTION

Brown seaweeds are known to produce different polysaccharides, namely alginates, laminarans, and fucoidans [1,2]. The latter polysaccharides usually contain large proportions of L-fucose and sulfate, together with minor amounts of other sugars like xylose, galactose, mannose, and glucuronic acid [2,3]. The structure of fucoidan, a sulfated polysaccharide present in brown seaweed, has been reported to be a heparin-like molecule [4,5]. Fucoidan has anticoagulant activities [4,6] and inhibitory effects on tube formation of human endothelial cells [7] as well as anticancer activities through the modulation of host immune systems [8–11]. It was reported that fucoidan can mobilize hematopoietic stem and progenitor cells from bone marrow into peripheral circulation, since the L-selectin on these cells is receptor for fucoidan [12,13]. Recently it also reported that fucoidan prevents the attachment of Helicobactor pylori to mucin of gastric tract [14]. Fucoidan is known to inhibit the growth of Ehrlich ascites carcinoma and lung cancer in vivo through inhibiting tumor angiogenesis [9,10]. These accumulated data represent evidence suggesting that fucoidan is a potent chemopreventive agent against carcinogenesis, but the underlying molecular mechanisms and molecular target(s) remain unclear.

Carcinogenesis is characterized as a multistage process that includes initiation, promotion, and progression stages. Cancer prevention strategies that involve intervention at the tumor promotion stage, a reversible and long-term process, seem to be more practical than those intervening at the tumor initiation stage, which is an irreversible and short-term process. Activator protein-1 (AP-1) is a well-characterized transcription factor composed of homodimers and/or heterodimers of the Fos and Jun protein families and plays a key role in ''preneoplastic-to-neoplastic transformation'' and proliferation [15]. A diverse variety of stimuli induce AP-1 binding to various genes that govern cellular processes such as transformation and proliferation [16]. In particular, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), H-Ras, and epidermal growth factor (EGF) are the most common experimental stimuli used to activate AP-1 and induce cellular transformation in many different cell types and animal models [17].

Abbreviations: AP-1, activator protein-1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MEM, minimal essential medium; FBS, fetal bovine serum; MAPK, mitogen-activated protein kinase.

*Correspondence to: College of Pharmacy, Chosun University, Gwangju 501-759, South Korea.

Received 7 September 2007; Revised 14 January 2008; Accepted 15 January 2008

DOI 10.1002/mc.20428

© 2008 WILEY-LISS, INC.

The epidermal growth factor receptor (EGFR), one of the receptor tyrosine kinases, plays a pivotal role in regulating cell proliferation, differentiation, and transformation [18]. The EGFR is an important target for cancer therapy [19]. Many carcinomas are promoted by EGFR activation, which can result from mutation of the receptor [20], its overexpression [21], or from EGFR stimulation through autocrine loops [22]. The EGFR is activated by a number of ligands known as EGF-related peptide growth factors, including EGF [23]. TPA also transactivates the EGFR and increases cell proliferation by activating the PKCd/c-Src pathway in glioblastomas [24].

In the present study, we studied the mechanism of the anticarcinogenic effects of fucoidan from *Laminaria cichorioides* on AP-1 transactivation activity and cellular transformation in mouse epidermal JB6 cells. We found that fucoidan inhibited AP-1 transactivation and cell transformation. The results of this investigation suggested a molecular mechanism that underlies the chemopreventive activity of fucoidan and might partially account for the chemopreventive effects of brown seaweeds.

MATERIALS AND METHODS

Reagents and Antibodies

Eagle's minimal essential medium (MEM), L-glutamine, gentamicin, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). EGF and TPA were purchased from Calbiochem–Novabiochem (San Diego, CA). Polyvinylidene difluoride (PVDF) membrane was from Millipore (Bedford, MA). $BaSO_4$ was from Sigma–Aldrich Co. (St. Louis, MO). The Dual-luciferase reporter assay system and CellTiter 96 non-radioactive cell proliferation assay kit were purchased from Promega (Madison, WI). Antibodies against phospho-MEK1/2, -ERK1/2, -p90RSK, -JNK, -c-Jun, -EGFR, and total GAPDH were purchased from Cell Signaling Tech., Inc. (Danvers, MA).

Cell Culture and Transfection

JB6 Cl41 mouse epidermal cells were cultured in MEM supplemented with 5% FBS. Cells were transfected by a cationic liposome transfection method, Lipofectamine 2000 (Invirogen Co., Carlsbad, CA).

Polysaccharide Extraction

L. cichcorioides samples were collected in Troitsa Bay, Sea of Japan. The isolation and separation of water-soluble polysaccharides were carried out by the modified methods [25].

Anion Exchange Chromatography

A solution of polysaccharide in 0.1 M NaCl (2.3 g in 50 mL) was applied onto a DEAE-cellulose column (Cl^- form, 3 cm × 21 cm) equilibrated with 0.1 M NaCl. The column was then successively eluted with 0.1, 0.5, 1, and 2 M NaCl solutions, each time until the disappearance in eluate of positive reaction for carbohydrates by the phenol-sulfuric acid method [26]. The correspondent polysaccharides fractions were concentrated by ultrafiltration (1 kDa cutoff), dialyzed, and lyophilized.

Analytical Procedures

Neutral carbohydrates were quantified by the phenol-sulfuric acid method [26]; reducing carbohydrates were determined according to Nelson method [27]. Monosaccharides composition was determined by HPLC with a LC-5001 carbohydrate analyzer (a Durrum DA-X8-11 column) (Biotronik, Berlin, Germany), bicinchoninate assay, and a C-R2 AX integrating system (Shimadzu Corp., Koto, Japan) after hydrolysis by 2M TFA (6 h, 100°C).

Cell Proliferation Assay

To estimate cell cytotoxicity, JB6 Cl41 cells were seeded (1×10^4) in 96-well plates in 100 μL of 5% FBS-MEM at 37°C in a 5% CO_2 incubator. After culturing for 24 h, the various concentrations of fucoidan were treated and incubated for additional 24 h at 37°C in a 5% CO_2 incubator. After incubation, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (15 μL) were added to each well, and cells were then incubated for 4 h at 37°C in a 5% CO_2. Absorbance was measured at 570 nm.

Anchorage-Independent Transformation Assay

EGF-induced cell transformation was investigated in JB6 Cl41 cells. In brief, cells (8×10^3/mL) were exposed to EGF (1 ng/mL) in 1 mL of 0.3% basal medium Eagle (BME) agar containing 10% FBS. The cultures were maintained at 37°C, in a 5% CO_2 incubator for 10 d, and the cell colonies were scored using a microscope and the Image-Pro PLUS computer software program (Media Cybernetics, Silver Spring, MD) as described by Colburn et al. [28].

Reporter Gene Assays

The reporter gene assay for firefly luciferase activity was performed using lysates from transfected cells. In addition, the reporter gene vector phRL-SV40 (Promega) was cotransfected into each cell line and the renilla-luciferase activity generated by this vector was used to normalize the results for transfection efficiency. *c-Fos* promoter luciferase (pFos-WT GL3) and *c-jun* promoter luciferase (JC6GL3) constructs were kindly provided by Dr. Ron Prywes (Columbia University, New York). The AP-1 luciferase reporter plasmid (−73/+63 collagenase-luciferase) was kindly provided by Dr. Dong Zigang (Hormel Institute, University of Minnesota, MN).

Immunoblotting

The proteins were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto PVDF membranes. The membranes

were blocked, hybridized with the appropriate primary antibody overnight at 4°C. Protein bands were visualized by the chemiluminescence detection kit (ECL of Amersham Biosciences Corp., Piscataway, NJ) after hybridization with the horseradish peroxidase (HRP)-conjugated secondary antibody from rabbit or mouse.

Preparation of Water-Insoluble Fucoidan

Water-soluble fucoidan (1 mL, 10%, w/v) from *L. cichcorioides* was mixed with $BaSO_4$ (1 mL, 10%, w/v) and incubated for 30 min at 4°C. The mixture was precipitated with 2 volume of 95% ethanol. After washing three times with ethanol, the precipitated fucoidan, fucoidan-$BaSO_4$, was dried at room temperature.

In Vitro Fucoidan-$BaSO_4$ Pull-Down Assays

Mouse EGF (4 μg) was incubated with fucoidan-$BaSO_4$ (10 mg) in reaction buffer [50 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0,01% NP40, 2 μg/mL bovine serum albumin, 0.02 mmol/L phenylmethylsulfonyl fluoride, 1× protease inhibitors]. After incubation with gentle rocking overnight at 4°C, fucoidan-$BaSO_4$ were washed five times with washing buffer [50 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0,01% NP40, 0.02 mmol/L phenylmethylsulfonyl fluoride] and EGF bound to the fucoidan-BaSO4 was analyzed by immunoblotting with the antibody against mouse EGF.

RESULTS

Fucoidan From *Laminaria cichorioides* Has No Cytotoxic Effects on JB6 Mouse Epidermal Cells

The sulfated fucoidan from *L. cichorioides* was purified by a combination of methods referred to Materials and Methods Section (Figure 1A). Anion-exchange chromatography on DEAE-cellulose column separated the acidic polysaccharides from brown seaweed into four peaks. The peak eluted with 0.1 M NaCl contained laminaran; the peaks eluted with 0.5 M NaCl, 1 M NaCl, and 2 M NaCl contained polymannuronic acid, mixture polymannuronic acid and sulfated fucan, and sulfated fucan, correspondently [25]. Chemical analysis of the fucoidan from *L. cichorioides* revealed a high content of sulfate ester to extent of 38%. The fucoidans are heterogenic, due to variations of the contents of carbohydrate units and noncarbohydrate substances (sulfate and acetyl groups). The polysaccharide chain of the fucoidan from *L. cichorioides* is build up of 2,4-sulfated (1 → 3)-linked α-L-fucopyranose residues (type I) [29]. We used fucoidans from *L. cichorioides*, which was eluted with 2 M NaCl and was 85–90% at purity [30], to test the cytotoxic effects of fucoidan on JB6 mouse epidermal cells.

We examined the effects of fucoidan from *L. cichorioides* on the proliferation of JB6 mouse epidermal cells using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Fucoidan did not significantly affect cell growth at 3 d after treatment at concentration from 1 to 200 μg/mL (Figure 1B). These results indicated that fucoidan was less cytotoxic to JB6 mouse epidermal cells.

Fucoidan Suppresses EGF-Induced Phosphorylation of EGFR, MEK, ERK1/2, p90RSK, JNK, and c-Jun in JB6 Mouse Epidermal Cells

The EGFR, one of the receptor tyrosine kinases, plays a pivotal role in regulating cell transformation, differentiation, and transformation [21]. We hypothesized that the downregulation of EGFR may be an important mechanism of the antitumor activity of fucoidan. In this study, we investigated the effects of fucoidan on the EGF-induced phosphorylation of EGFR in mouse skin epidermal JB6 cells. Cells were treated with 1 ng/mL EGF for 15 min,

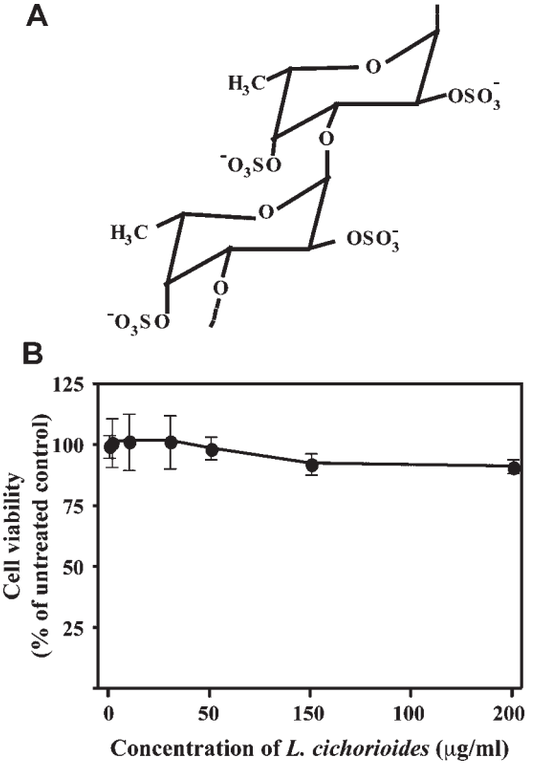

Figure 1. The Structure and cytotoxic effects of fucoidan on JB6 mouse epidermal cells. (A) Structure of the sulfated fucan isolated from the brown seaweed *L. chichorioides*. Sulfated fucan from *L. chichorioides* are linear polymer composed of disaccharide units (1 → 3)-α-L-Fuc*p*-(2,4-di-OSO_3^-). (B) JB6 Cl41 cells were seeded (1×10^4/well) in 96-well plates in 100 μL of 5% FBS-MEM, and then treated with a range of concentrations of fucoidan (1–200 μg/mL), or their vehicle, H_2O, as a negative control, for 72 h. The cell proliferation was estimated using the MTT assay as described under "Materials and Methods" Section. Data are represented as the means ± SD as determined from triplicate experiments.

in the absence or presence of various concentrations of fucoidan and assayed by immunoblotting with the antiphospho-EGFR and anti-EGFR (1005) antibodies, respectively. Results indicated that treatment of fucoidan significantly decreased the phosphorylation of EGFR, but not the EGFR total protein level (Figure 2A).

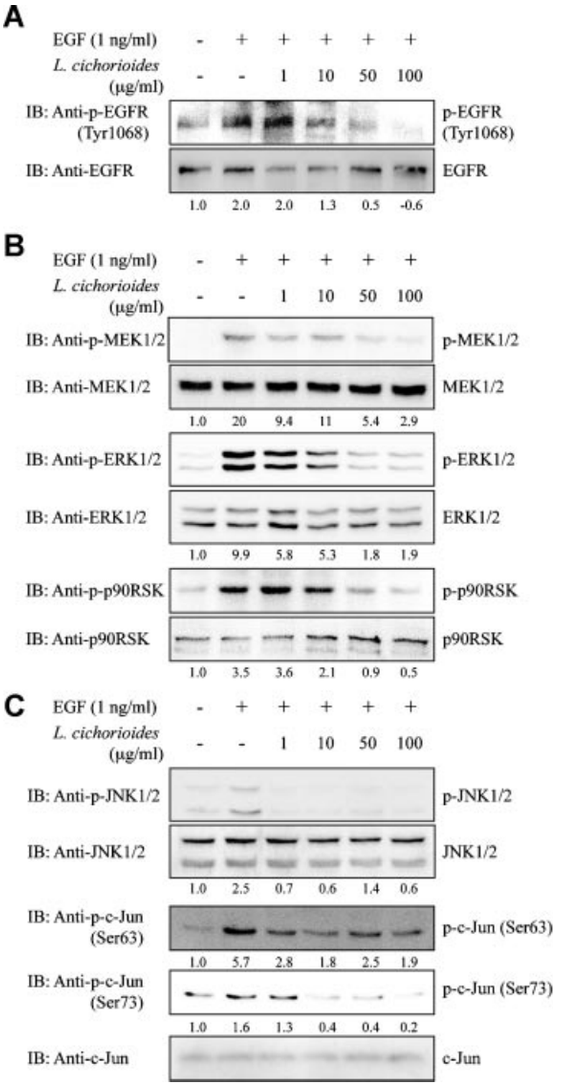

Figure 2. Effects of fucoidan on EGF-induced phosphorylation of EGFR, MEK, ERK1/2, p90RSK, JNK, and c-Jun. (A) Fucoidan inhibits EGF-induced phosphorylation of EGFR (1068). (B) Fucoidan inhibits EGF-induced phosphorylation of MEK, ERK1/2, and p90RSK. (C) Fucoidan inhibits EGF-induced phosphorylation of JNK and c-Jun. (A–C) JB6 cells were pretreated with fucoidan at the indicated concentrations (1, 10, 20, or 100 μg/mL) for 12 h, then stimulated with EGF (1 ng/mL) and harvested 15-min later. The levels of phosphorylated and total EGFR, MEK, ERK1/2, p90RSK, JNK, and c-Jun proteins in whole cell lysates were determined by Western blot analysis as described under ''Materials and Methods'' Section using specific antibodies against the corresponding phosphorylated and total proteins, respectively. Corresponding signal intensities were densitometrically determined and normalized to total protein in each lane, and is given below in each data. Data are representative of two independent experiments that gave similar results.

One of the most important protein kinase cascades activated by tumor promoters, such as EGF, are the mitogen-activated protein kinases (MAPKs) [31], following from the activation of EGFR. Thus, we next examined the effect of fucoidan on the MEK/ERK signaling pathway and found that fucoidan significantly suppressed EGF-induced phosphorylation of MEK, ERK1/2, and p90RSK, respectively in JB6 cells in a dose-dependent manner (Figure 2B).

Next, we examined whether fucoidan can inhibit the EGF-induced phosphorylation of JNK as well as c-Jun. EGF activates the *c-jun* promoter through a Ras to Rac to MEKK pathway [32]. EGF-induced MEKK1 phosphorylation can activate ERK as well as JNK [33]. Our results showed that pretreatment with fucoidan almost completely inhibited the EGF-induced phosphorylation of JNKs and c-Jun (Ser63 and Ser73) in JB6 cells (Figure 2C). Overall these results confirmed that fucoidan has an inhibitory effect of EGFR signaling pathway.

Fucoidan Suppress TPA-Induced Phosphorylation of ERK, p90RSK, and c-Jun N-Terminal Kinase

Also, TPA is a highly potent tumor promoter and is widely used for the study of the mechanism of tumor promotion. To further examine the effect of fucoidan on the TPA-induced MAPK activity, we determined the TPA-induced phosphorylation of ERK1/2 and p90RSK with/without treatment of fucoidan. Results showed that fucoidan suppressed TPA-induced phosphorylation of ERK1/2 or p90RSK (Figure 3A). Because JNK are also critical in mediating AP-1 transactivation and malignant transformation, we evaluated the ability of fucoidan to modulate TPA-induced phosphorylation of JNK. Results showed that fucoidan blocked TPA-induced phosphorylation of JNK and downstream signaling protein, c-Jun, in JB6 cells (Figure 3B). Overall results suggest that fucoidan appears to exert a strong inhibitory activity on TPA-induced phosphorylation of ERK1/2 and JNK.

Fucoidan Inhibits EGF-Induced *c-fos*, *c-jun*, and AP-1 Activation

c-Jun and c-Fos are nuclear proto-oncoproteins whose expression is stimulated by a variety of growth-promoting agents and activated oncogenes [34]. To investigate whether fucoidan suppress the EGF-induced *c-fos* or *c-jun* transcriptional activity, we took advantage of the availability of the reporter plasmid carrying the *luc* gene under the control of the murine *c-jun* or *c-fos* promoter. Twenty-four hours after transfection with these reporters in cells, cells were starved for another 24 h by incubating in serum-deprived MEM at 37°C in a 5% CO_2 atmosphere. At 12 h of starvation, cells were pretreated or not treated with fucoidan for 12 h, and then treated or not treated with EGF (1 ng/mL) for additional 12 h. As shown in Figure 4A and B, EGF-promoted *c-fos* or

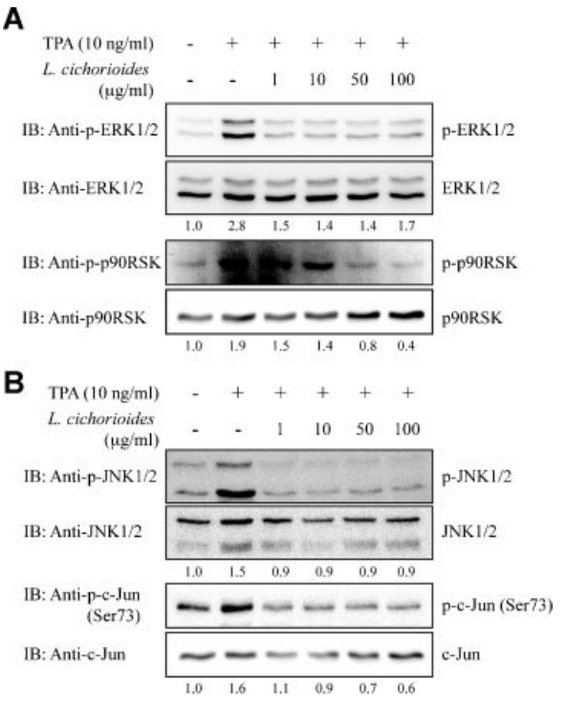

Figure 3. Effects of fucoidan on TPA-induced phosphorylation of ERK1/2, p90RSK, JNK, and c-Jun. (A) Fucoidan inhibits TPA-induced phosphorylation of ERK1/2, and p90RSK. (B) Fucoidan inhibits TPA-induced phosphorylation of JNK and c-Jun. For (A,B), JB6 cells were pretreated with fucoidan at the indicated concentrations (1, 10, 20, or 100 μg/mL) for 12 h, then stimulated with TPA (10 ng/mL) and harvested 15-min later. The levels of phosphorylated and total ERK1/2, p90RSK, JNK, and c-Jun proteins in whole cell lysates were determined by Western blot analysis as described under ''Materials and Methods'' Section using specific antibodies against the corresponding phosphorylated and total proteins, respectively. Corresponding signal intensities were densitometrically determined and normalized to total protein in each lane, and is given below in each data. Data are representative of two independent experiments that gave similar results.

c-jun transcriptional activity was significantly suppresed by fucoidan. These data support our notion that the suppression of the *c-jun* or *c-fos* promoter by EGF is one of the mechanisms explaining the antitumorigenetic effect of fucoidan.

Transcription factor AP-1 is mainly composed with Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) [35]. AP-1 is induced by several external stimuli, such as EGF, which increase MAPK activity [36]. To determine whether the antitumorigenetic effect of fucoidan is responsible for the inhibition of *AP-1* activation response to EGF, we next cotransfected the *AP-1* luciferase reporter plasmid and the *phRL-SV40* gene into cells. The EGF-induced AP-1 activation response was significantly inhibited by fucoidan similar to that observed for *c-jun* or *c-fos* activity (Figure 4C).

Fucoidan Suppressed EGF- or TPA Induced Neoplastic Cell Transformation

We next examined the effect of fucoidan on EGF- or TPA-induced cell transformation using our

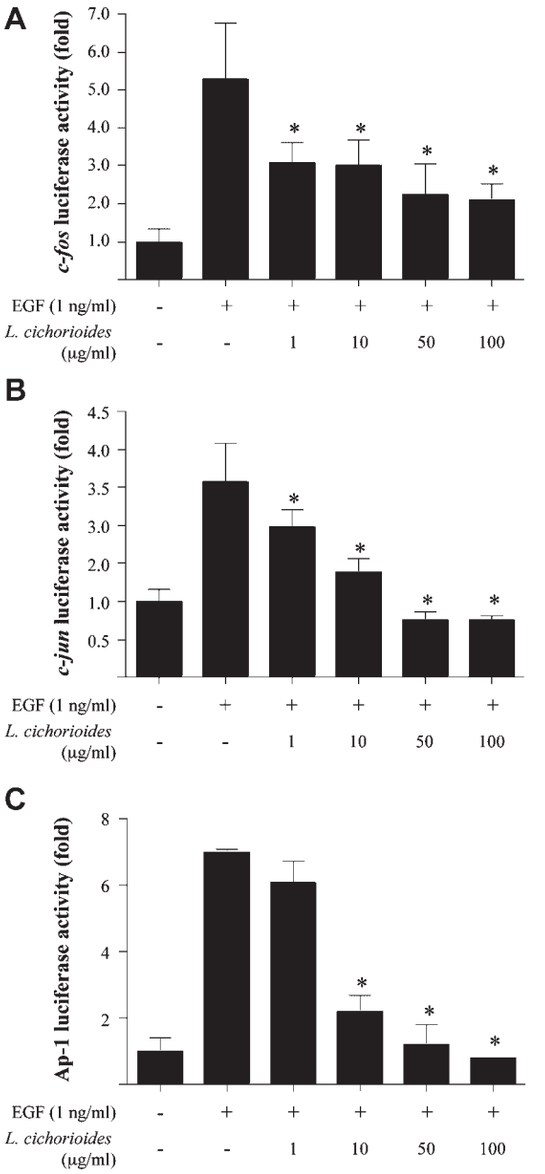

Figure 4. Effects of fucoidan on EGF-induced *c-fos*, *c-jun* or AP-1 activation. (A) Fucoidan inhibits EGF-induced *c-fos* promoter activity. (B) Fucoidan inhibits EGF-induced *c-jun* promoter activity. (C) Fucoidan inhibits EGF-induced AP-1 transactivation. For (A–C), cells were transfected with a plasmid mixture containing *c-fos* luciferase reporter gene (0.5 μg; A), the *c-jun* luciferase reporter gene (0.5 μg; B), AP-1 luciferase reporter gene (1 μg; C) each with the *phRL-SV40* gene (0.5 ng). At 24 h after transfection, cells were starved for 12 h by incubating in serum-deprived MEM at 37°C in a 5% CO_2 atmosphere, and then treated or not treated with fucoidan at the indicated concentration (1, 10, 20, or 100 μg/mL) for 12 h before they were exposed to 1 ng/mL EGF for 12 h. The *firefly* luciferase activity was determined in cell lysates and normalized against *renilla* luciferase activity, and *c-fos*-, *c-jun*-, or AP-1-luciferase activity is expressed relative to control cells without EGF treatment. Data are represented as the means ± SD of the *c-fos*-, *c-jun*-, or AP-1-luciferase activity calculated from three independent experiments, respectively. The *asterisk* (*) indicates a significant difference ($P < 0.05$) between groups treated with EGF and fucoidan and the group treated with EGF alone.

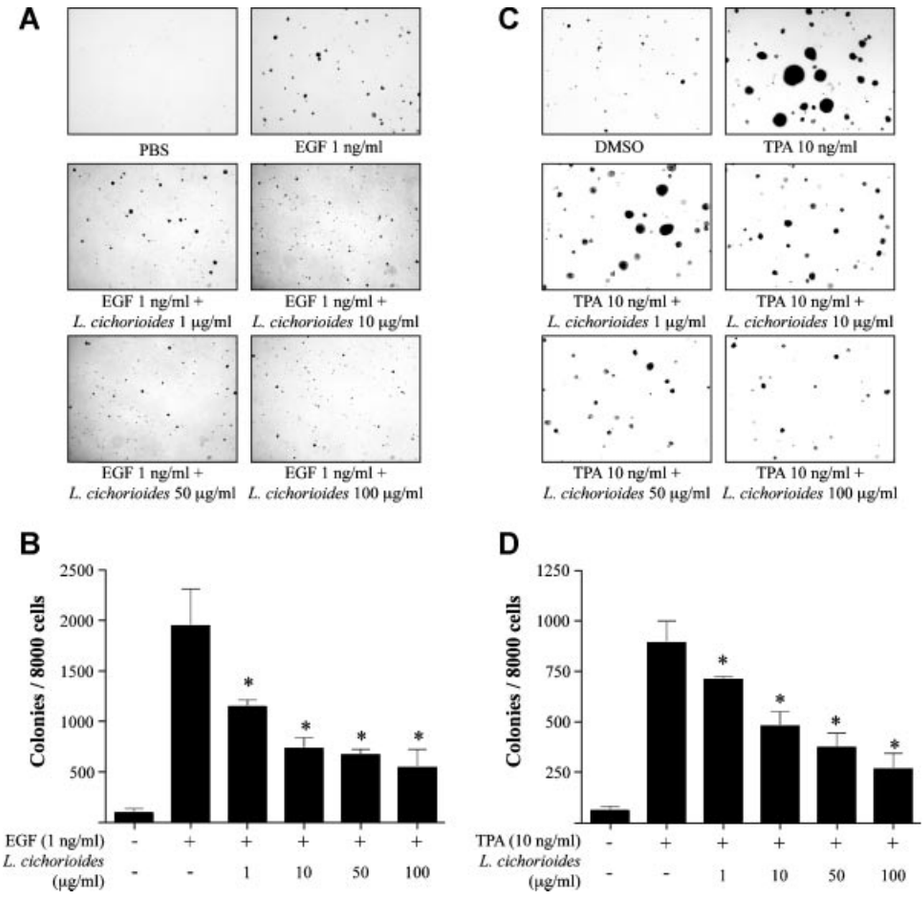

Figure 5. The inhibitory effects of fucoidan on EGF- or TPA-induced neoplastic cell transformation in JB6 mouse epidermal cells. (A,B) The effect of fucoidan on EGF-induced cell transformation comparing untreated control cells. (C,D) The effect of fucoidan on TPA-induced cell transformation comparing untreated control cells. For (A,C), cells (8×10^3/mL) treated with/without fucoidan (1, 10, 20, or 100 μg/mL) were exposed to EGF (0 or 1 ng/mL) or TPA (0 or 10 ng/mL) in 1 mL of 0.3% BME's agar containing 10% FBS. The culture was maintained at 37°C in a 5% CO_2 atmosphere for 10–14 d. The colonies were counted under a microscope with the aid of the Image-Pro Plus soft ware (Version 4) program. For (B,D), data are represented as the means ± SD of the number of colonies are determined from three independent experiments. The *asterisk* (*) indicates a significant difference ($P < 0.05$) between groups treated with EGF (or TPA) and fucoidan and the group treated with EGF (or TPA) alone.

previously developed methods [37]. JB6 mouse epidermal cells were treated separately with EGF (1.0 ng/mL) or TPA (10 ng/mL) in the absence or presence of various concentrations of fucoidan in a soft agar matrix and incubated at 37°C in a 5% CO_2 incubator for 10–14 d. Our results showed that fucoidan significantly inhibited the formation of EGF- or TPA promoted neoplastic cell transformation of JB6 cells in a dose-dependent manner (Figure 5A and C). Taken together, these data strongly support the idea that fucoidan plays an inhibitory role of neoplastic cell transformation in epidermal mouse skin cells stimulated with EGF or TPA.

Fucoidan Binds With Mouse EGF

The results above indicated that the inhibition of EGF-induced cell transformation by fucoidan is associated with the suppression of EGFR activity and its downstream signaling pathway. To further confirm whether fucoidan directly interacts with EGF, we used an in vitro fucoidan pull-down assay. Our results revealed that EGF bound to fucoidan-$BaSO_4$ (Figure 6, lane 3), but not to $BaSO_4$ alone

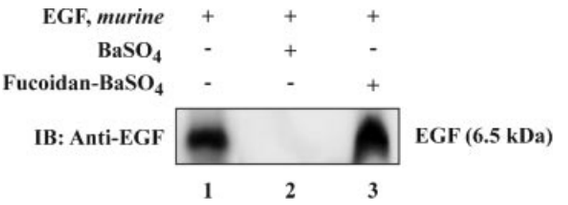

Figure 6. Fucoidan specifically binds with EGF. Mouse EGF was incubated with fucoidan-$BaSO_4$ or $BaSO_4$ overnight at 4°C, respectively. The EGF-fucoidan binding in vitro was confirmed by immunoblotting analysis using an antibody against EGF. *First lane* (input control), EGF protein standard (0.20 μg); *second lane* (control), $BaSO_4$ was used to pull down mouse EGF; *third lane*, mouse EGF was pulled down using Fucoidan-$BaSO_4$ as described under "Material and Methods" Section.

(Figure 6, lane 2). This result suggests that EGF appears to be an important target molecule of fucoidan for inhibiting the phosphorylation of EGFR and its downstream signaling.

DISCUSSION

Fucoidans have a wide spectrum of activity in biological systems, and it seems to be determined by their high degree of sulfatation [11], although these activities depend on the fine structure and molecular weight [38]. Fucoidans represent the family sulfated homo- and heteropolysaccharides mainly build up of α-L-fucose with sulfate and acetate units. Besides, D-galactose, D-mannose, D-xylose, L-rhamnose, D-glucuronic acid residues and acetyl groups were found to be the constituents of fucoidans [1]. Accumulating research evidence suggested that fucoidan have a chemoprventive or chemotherapeutic effects to prevent or treat cancer [911]. Furthermore, previous studies showed that oversulfatation of fucoidan effectively enhanced its antiangiogenic and antitumor activities [11]. However, the underlying molecular mechanisms and molecular target remain unclear. The present study demonstrated that fucoidan from *L. cichorioides* exhibited a strong inhibitory effect EGF- or TPA-induced neoplastic cell transformation (Figure 5). Also, fucoidan blocked EGF-induced AP-1, *c-fos*, and *c-jun* activation in JB6 mouse epidermal cells (Figure 4). These results suggested that fucoidan might play an important role in the cancer-preventive activity by targeting the AP-1-signaling pathway.

As a sequence-specific transcriptional activator, AP-1 mediates a broad range of external stimuli that lead to gene transcription. Many stimuli, including TPA, EGF, and UV radiation that induce AP-1, are associated with tumorigenesis [16]. Neoplastic tansformation is often associated with a dramatic increase in AP-1 activity [39], and this transient induction of AP-1 has been shown to be involved in the promotion of epidermal tumors [40]. Constitutive AP-1 activity has been associated with the malignant conversion of papillomas to carcinomas as well [41]. Chemopreventive agents or modification of AP-1 proteins that inhibit AP-1 activation are effective in preventing cell transformation or tumorigenesis [39,42,43].

Many mechanisms are involved in the up- and down-regulation of AP-1 activity [16]. The MAP kinase signaling pathways are critical for AP-1 activation [44]. It was reported that EGF and TPA induce high levels of AP-1 activation and a high frequency of neoplastic transformation in JB6 P+ cells but have no effect on P− cells [15]. The lack of response was shown to be directly attributable to a low level of both TPA- and EGF-induced phosphorylation of ERK and total ERK protein levels [15]. Our results demonstrated that fucoidan from *L. cichorioides* was able to block EGF- or TPA-stimulated ERK activities (Figures 2B and 3B). Although both ERKs and JNKs of the MAPK family have been reported to be able to induce AP-1 activity, each of the kinases may activate different AP-1 components resulting in the transcription of different genes [44]. Many reports indicated that JNKs are critical in mediating AP-1 transactivation and malignant transformation [45]. For instant, it was reported that TNF-α induced cell transformation requires activation of JNKs [46]. It was also reported that TPA-induced skin tumorigenesis was strikingly suppressed in JNK-2-deficient mice [47]. In our experiments, the inhibition of EGF- or TPA-induced JNK activity by fucoidan agreed well with the inhibitory effects of it on EGF-induced AP-1 activity and cell transformation (Figures 2C and 3C).

EGFR a 170 kDa single-pass transmembrane tyrosine kinase receptor. Ligand, EGF, binding to this receptor results in receptor dimerization, autophosphorylation, and activation of various downstream signaling molecules [48]. The traditional view of growth factor receptors in general is that a specific ligand directly recognizes a highly specific binding site on its cognates receptor, and thereby activates receptor-dependent signaling and biological responses [49]. Since overexpression of EGFR has been associated with an overall poor prognosis in patients with cancer, a number of strategies to block or downregulate EGFR have been developed to inhibit tumor proliferation and improve overall clinical outcome. These include monoclonal antibodies to the EGFR, tyrosine inhibitors, ligand-linked toxins, and antisense [50–52]. Recently, it was reported that natural and oversulfated fucoidans significantly suppressed the mitogenic and chemotactic actions of $VEGF_{165}$ on HUVEC by preventing the binding of $VEGF_{165}$ to its cell surface receptor [11]. Therefore, we hypothesized that fucoidan may bind with EGF, resulting to inhibit EGF-induced cell transformation. The present study illustrated that fucoidan exerted a strong inhibitory activity on EGF-induced phosphorylation of EGFR (Figure 2A). Additionally, fucoidan directly bind with EGF in vitro to prevent the binding of EGF to its receptor (Figure 6). These results suggested that fucoidan has potent anticancer-promoting activity and mainly targets the EGFR-signaling pathway, which may contribute to the chemopreventive potential.

In summary, fucoidan from *L. cichorioides* was effective in inhibiting cell transformation induced by EGF or TPA in the mouse epidermal JB6 Cl41 cells. The inhibition was found to be associated with the inhibitory effects of these compounds on AP-1 activity. Fucoidan interacted with EGF to suppress EGF-induced phosphorylation of EGFR and the downstream signaling to the ERK/p90RSK/AP-1 pathway as wells as JNK/c-Jun/AP-1 pathway. This represents the first report related to the molecular

mechanism of the cancer-preventive action of fucoidan and a significant step forward in our understanding of the chemopreventive potential of brown seaweeds.

ACKNOWLEDGMENTS

This study was supported by the Korean Ministry of Science and Technology and by the Korean Science and Engineering Foundation through the Research Center for Resistant Cells.

REFERENCES

1. Painter T. Algal polysaccharides. The polysaccharides, Vol. 2. New York: Academic Press; 1983. pp. 195–285.
2. Percival E. Chemistry and enzymology of marine algae polysaccharides. New York: Academic Press; 1967. pp. 53–71.
3. Duarte ME, Cardoso MA, Noseda MD, Cerezo AS. Structural studies on fucoidans from the brown seaweed *Sargassum stenophyllum*. Carbohydr Res 2001;333:281–293.
4. Durig J, Bruhn T, Zurborn KH, Gutensohn K, Bruhn HD, Beress L. Anticoagulant fucoidan fractions from Fucus vesiculosus induce platelet activation in vitro. Thromb Res 1997;85:479–491.
5. Patankar MS, Oehninger S, Barnett T, Williams RL, Clark GF. A revised structure for fucoidan may explain some of its biological activities. J Biol Chem 1993;268:21770–21776.
6. Chevolot L, Mulloy B, Ratiskol J, Foucault A, Colliec-Jouault S. A disaccharide repeat unit is the major structure in fucoidans from two species of brown algae. Carbohydr Res 2001;330: 529–535.
7. Soeda S, Kozako T, Iwata K, Shimeno H. Oversulfated fucoidan inhibits the basic fibroblast growth factor-induced tube formation by human umbilical vein endothelial cells: Its possible mechanism of action. Biochim Biophys Acta 2000; 1497:127–134.
8. Riou D, Colliec-Jouault S, Pinczon du Sel D, et al. Antitumor and antiproliferative effects of a fucan extracted from ascophyllum nodosum against a non-small-cell bronchopulmonary carcinoma line. Anticancer Res 1996;16:1213–1218.
9. Itoh H, Noda H, Amano H, Zhuaug C, Mizuno T, Ito H. Antitumor activity and immunological properties of marine algal polysaccharides, especially fucoidan, prepared from *Sargassum thunbergii* of Phaeophyceae. Anticancer Res 1993;13:2045–2052.
10. Zhuang C, Itoh H, Mizuno T, Ito H. Antitumor active fucoidan from the brown seaweed, umitoranoo (*Sargassum thunbergii*). Biosci Biotechnol Biochem 1995;59:563–567.
11. Koyanagi S, Tanigawa N, Nakagawa H, Soeda S, Shimeno H. Oversulfation of fucoidan enhances its anti-angiogenic and antitumor activities. Biochem Pharmacol 2003;65:173–179.
12. Frenette PS, Weiss L. Sulfated glycans induce rapid hematopoietic progenitor cell mobilization: Evidence for selectin-dependent and independent mechanisms. Blood 2000;96: 2460–2468.
13. Sweeney EA, Lortat-Jacob H, Priestley GV, Nakamoto B, Papayannopoulou T. Sulfated polysaccharides increase plasma levels of SDF-1 in monkeys and mice: Involvement in mobilization of stem/progenitor cells. Blood 2002;99: 44–51.
14. Shibata H, Iimuro M, Uchiya N, et al. Preventive effects of Cladosiphon fucoidan against Helicobacter pylori infection in Mongolian gerbils. Helicobacter 2003;8:59–65.
15. Dong Z, Birrer MJ, Watts RG, Matrisian LM, Colburn NH. Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells. Proc Natl Acad Sci USA 1994;91:609–613.
16. Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta 1991;1072:129–157.
17. Hunter T, Karin M. The regulation of transcription by phosphorylation. Cell 1992;70:375–387.
18. Chen WS, Lazar CS, Poenie M, Tsien RY, Gill GN, Rosenfeld MG. Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. Nature 1987;328:820–823.
19. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2001;2:127–137.
20. Humphrey PA, Wong AJ, Vogelstein B, et al. Anti-synthetic peptide antibody reacting at the fusion junction of deletion-mutant epidermal growth factor receptors in human glioblastoma. Proc Natl Acad Sci USA 1990;87:4207–4211.
21. Gorgoulis V, Aninos D, Mikou P, et al. Expression of EGF, TGF-alpha and EGFR in squamous cell lung carcinomas. Anticancer Res 1992;12:1183–1187.
22. Sizeland AM, Burgess AW. Anti-sense transforming growth factor alpha oligonucleotides inhibit autocrine stimulated proliferation of a colon carcinoma cell line. Mol Biol Cell 1992;3:1235–1243.
23. Riese DJ, II., Stern DF. Specificity within the EGF family/ErbB receptor family signaling network. Bioessays 1998;20:41–48.
24. Amos S, Martin PM, Polar GA, Parsons SJ, Hussaini IM. Phorbol 12-myristate 13-acetate induces epidermal growth factor receptor transactivation via protein kinase Cdelta/c-Src pathways in glioblastoma cells. J Biol Chem 2005;280: 7729–7738.
25. Zvyagintseva TN, Shevchenko NM, Popivnich IB, et al. A new procedure for the separation of water-soluble polysaccharides from brown seaweeds. Carbohydr Res 1999;322:32–39.
26. Dubois M, Gilles K, Hamilton J, Roberts P, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350–356.
27. Nelson TE. A photometric adaptation of the Somogy method for determination of glucose. J Biol Chem 1944;153:375–381.
28. Colburn NH, Wendel EJ, Abruzzo G. Dissociation of mitogenesis and late-stage promotion of tumor cell phenotype by phorbol esters: Mitogen-resistant variants are sensitive to promotion. Proc Natl Acad Sci USA 1981;78:6912–6916.
29. Zvyagintseva TN, Shevchenko NM, Nazarenko EL, et al. Water-soluble polysaccharides of some brown algae of the Russian Far-East. Structure and biological action of low-molecular mass polyuronans. J Exp Mar Biol Ecol 2005;320: 123–131.
30. Zvyagintseva TN, Shevchenko NM, Nazarenko EL, et al. Water-soluble polysaccharides of some far-eastern brown seaweeds. Distribution, structure, and their dependence on the developmental conditions. J Exp Mar Biol Ecol 2003;294: 1–13.
31. Bode AM, Dong Z. Mitogen-activated protein kinase activation in UV-induced signal transduction. Sci STKE 2003;2003:RE2.
32. Clarke N, Arenzana N, Hai T, Minden A, Prywes R. Epidermal growth factor induction of the c-jun promoter by a Rac pathway. Mol Cell Biol 1998;18:1065–1073.
33. Gupta P, Prywes R. ATF1 phosphorylation by the ERK MAPK pathway is required for epidermal growth factor-induced c-jun expression. J Biol Chem 2002;277:50550–50556.
34. Herschman HR. Primary response genes induced by growth factors and tumor promoters. Annu Rev Biochem 1991;60: 281–319.
35. Curran T, Franza BR Jr. Fos and Jun: The AP-1 connection. Cell 1988;55:395–397.
36. Tyagi A, Agarwal R, Agarwal C. Grape seed extract inhibits EGF-induced and constitutively active mitogenic signaling but activates JNK in human prostate carcinoma DU145 cells: Possible role in antiproliferation and apoptosis. Oncogene 2003;22:1302–1316.

37. Dong Z, Cmarik JL. Harvesting cells under anchorage-independent cell transformation conditions for biochemical analyses. Sci STKE 2002;2002:PL7.
38. Berteau O, Mulloy B. Sulfated fucans, fresh perspectives: Structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. Glycobiology 2003;13:29R–40R.
39. Vallone D, Battista S, Pierantoni GM, et al. Neoplastic transformation of rat thyroid cells requires the junB and fra-1 gene induction which is dependent on the HMGI-C gene product. EMBO J 1997;16:5310–5321.
40. Domann FE Jr, Levy JP, Finch JS, Bowden GT. Constitutive AP-1 DNA binding and transactivating ability of malignant but not benign mouse epidermal cells. Mol Carcinog 1994; 9:61–66.
41. Yuspa SH. The pathogenesis of squamous cell cancer: Lessons learned from studies of skin carcinogenesis. J Dermatol Sci 1998;17:1–7.
42. Barthelman M, Chen W, Gensler HL, Huang C, Dong Z, Bowden GT. Inhibitory effects of perillyl alcohol on UVB-induced murine skin cancer and AP-1 transactivation. Cancer Res 1998;58:711–716.
43. Dong Z, Crawford HC, Lavrovsky V, et al. A dominant negative mutant of jun blocking 12-*O*-tetradecanoylphorbol-13-acetate-induced invasion in mouse keratinocytes. Mol Carcinog 1997;19:204–212.
44. Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem 1995;270:16483–16486.
45. 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;57:4414–4419.
46. Huang C, Li J, Ma WY, Dong Z. JNK activation is required for JB6 cell transformation induced by tumor necrosis factor-alpha but not by 12-*O*-tetradecanoylphorbol-13-acetate. J Biol Chem 1999;274:29672–29676.
47. Chen N, Nomura M, She QB, et al. Suppression of skin tumorigenesis in c-Jun NH(2)-terminal kinase-2-deficient mice. Cancer Res 2001;61:3908–3912.
48. Wells A. EGF receptor. Int J Biochem Cell Biol 1999;31:637–643.
49. Gschwind A, Zwick E, Prenzel N, Leserer M, Ullrich A. Cell communication networks: Epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. Oncogene 2001;20:1594–1600.
50. Ciardiello F. Epidermal growth factor receptor tyrosine kinase inhibitors as anticancer agents. Drugs 2000;60:25–32; discussion 41–22.
51. Mendelsohn J, Baselga J. The EGF receptor family as targets for cancer therapy. Oncogene 2000;19:6550–6565.
52. Kong AN, Yu R, Hebbar V, et al. Signal transduction events elicited by cancer prevention compounds. Mutat Res 2001; 480–481:231–241.