

# *Althaea rosea* Cavanil and *Plantago major* L. suppress neoplastic cell transformation through the inhibition of epidermal growth factor receptor kinase

EUN-SUN CHOI<sup>1\*</sup>, SUNG-DAE CHO<sup>1\*</sup>, JI-AE SHIN<sup>1</sup>, KI HAN KWON<sup>2</sup>, NAM-PYO CHO<sup>1</sup> and JUNG-HYUN SHIM<sup>3</sup>

<sup>1</sup>Department of Oral Pathology, School of Dentistry and Institute of Oral Bioscience, Brain Korea 21 project, Chonbuk National University, Jeonju 561-756; <sup>2</sup>Department of Food Science and Nutrition, College of Health, Welfare and Education, Gwangju University, Gwangju 503-703; <sup>3</sup>Department of Biochemistry, College of Medicine, Soonchunhyang University, Cheonan 331-090, Republic of Korea

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**Abstract.** For thousands of years in Asia, *Althaea rosea* Cavanil (ARC) and *Plantago major* L. (PML) have been used as powerful non-toxic therapeutic agents that inhibit inflammation. However, the anticancer mechanisms and molecular targets of ARC and PML are poorly understood, particularly in epidermal growth factor (EGF)-induced neoplastic cell transformation. The aim of this study was to evaluate the chemopreventive effects and mechanisms of the methanol extracts from ARC (MARC) and PML (MPML) in EGF-induced neoplastic cell transformation of JB6 P+ mouse epidermal cells using an MTS assay, anchorage-independent cell transformation assay and western blotting. Our results showed that MARC and MPML significantly suppressed neoplastic cell transformation by inhibiting the kinase activity of the EGF receptor (EGFR). The activation of EGFR by EGF was suppressed by MARC and MPML treatment in EGFR<sup>+/+</sup> cells, but not in EGFR<sup>-/-</sup> cells. In addition, MARC and MPML inhibited EGF-induced cell proliferation in EGFR-expressing murine embryonic fibroblasts (EGFR<sup>+/+</sup>). These results strongly indicate

that EGFR targeting by MARC and MPML may be a good strategy for chemopreventive or chemotherapeutic applications.

## Introduction

Epidermal growth factor receptor (EGFR) is a 175-kDa trans-membrane glycoprotein in the tyrosine kinase family of growth factor receptors (1). EGFR has been reported to be overexpressed in various types of cancer, including lung, colon and head and neck cancer. Additionally, the activation of the EGFR cascades may result in various cell responses including apoptosis, development, cell proliferation, differentiation and survival (2-7). The overexpression and activation of EGFR is a common mechanism by which EGFR exerts its influence on tumorigenesis (1,8-11). Thus, EGFR is a potential target for cancer therapy due to its expression and activity in cancer tissues.

Natural products from medicinal plants have been used for a long time due to their potential chemotherapeutic activity (12,13). A recent review by Graham *et al* reported the use of *Plantago major* L. (PML) as an antitumor agent (13-15). Another plant that is used as a medicinal agent for the treatment of dry cough, catarrhal inflammation of the throat and oesophagus, as well as irregular or absent menstrual cycles is *Althaea rosea cavanil* (ARC) (16). However, the mechanisms of PML and ARC involved in the suppression of various tumors are unknown, although PML and ARC have been used as traditional medicines to treat several diseases.

In this study, methanolic extracts from *Plantago major* L. (MPML) and *Althaea rosea Cavanil* (MARC) were studied for their anti-tumorigenic effects via the inhibition of transformed normal cells. Consequently, the present study aimed to identify the molecular mechanisms and direct targets of the anti-tumorigenic effects of MARC and MPML in tumor promoter (EGF)-induced neoplastic cell transformation.

## Materials and methods

**Reagents.** Eagle's minimal essential medium (MEM) and Dulbecco's modified essential medium (DMEM) were

**Correspondence to:** Dr Jung-Hyun Shim, Department of Biochemistry, College of Medicine, Soonchunhyang University, Ssangyong-dong, Seobuk-gu, Cheonan, Choongnam 331-090, Republic of Korea  
E-mail: sl004jh@gmail.com

\*Contributed equally

**Abbreviations:** ARC, *Althaea rosea* Cavanil; PML, *Plantago major* L.; EGF, epidermal growth factor; MARC, methanol extracts *Althaea rosea Cavanil*; MPML, methanol extracts *Plantago major* L.; EGFR, epidermal growth factor receptor; MEM, minimal essential medium; DMEM, Dulbecco's modified essential medium; MEFs, mouse embryonic fibroblasts; FBS, fetal bovine serum; MTS, (3-(4,5-dimethylthiazol-20yl)-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium); BME, basal medium Eagle

**Key words:** *Althaea rosea* Cavanil, *Plantago major* L., skin carcinogenesis, epidermal growth factor receptor

obtained from Welgene, Inc. (Korea). EGF was purchased from Invitrogen (Carlsbad, CA, USA). Antibodies to pEGFR and EGFR were obtained from Cell Signaling, Inc. (Danvers, MA, USA). Anti-actin antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). MARC and MPML were kindly supplied by Professor Ki Han Kwon (Gwangju University, Gwangju, Korea).

**Cell culture and chemical treatments.** EGFR<sup>+/+</sup> and EGFR<sup>-/-</sup> mouse embryonic fibroblasts (MEFs) were kindly provided by Professor Dong (University of Minnesota, Hormel Institute, MN, USA) (11) and the JB6 P+ (JB6 CI 41-5a) mouse epidermal cell line was obtained from the American Tissue Culture Collection (Manassas, VA, USA). The JB6 P+ cells were cultured in MEM containing 5% fetal bovine serum (FBS) and MEF cells were maintained in DMEM containing 10% FBS, each with 100 U/ml of penicillin (Welgene, Inc.), in a humidified atmosphere containing 5% CO<sub>2</sub>. An equal number of cells were seeded and allowed to attach overnight. The cells were starved in 0.1% FBS/MEM or DMEM for 24 h, incubated with MARC (30, 60 and 90 µg/ml) or MPML (20, 40 and 60 µg/ml) for 1 h and treated with 10 ng/ml of EGF.

**MTS assay.** The effects of MARC and MPML on cell viability were estimated using the CellTiter 96<sup>®</sup> Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. In brief, cells were seeded in 96-well plates and then incubated with various doses of MARC (30, 60, 90 and 120 µg/ml) and MPML (20, 40, 60 and 80 µg/ml) for 72 h. MTS (3-(4,5-dimethylthiazol-20yl)-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) solution was added to each well and incubated for 2 h at 37°C. The reaction buffer was analyzed using an ELISA microplate reader (BioTeck Instruments Inc., Winooski, VT, USA) at 490 and 690 nm (background).

**Anchorage-independent cell transformation assay.** The effect of MARC and MPML on EGF-induced cell transformation was estimated in JB6 P+ cells. The cells were treated with EGF (10 ng/ml) with or without various doses of MARC and MPML in 1 ml of 0.33% basal medium Eagle's (BME) agar over 3 ml of 0.5% BME agar containing 10% FBS. The cultures were maintained at 37°C in a 5% CO<sub>2</sub> incubator for 10 days and the cell colonies were counted using a microscope.

**Western blot analysis.** Whole cell lysates were collected in lysis buffer for the western blot analysis. The protein supernatant fractions were subjected to SDS-PAGE and then transferred on polyvinylidene difluoride membranes and blocked with 5% skimmed milk followed by hybridization with the indicated antibodies. The protein bands with horseradish peroxidase-conjugated secondary antibody were observed using a western blotting luminol reagent (Santa Cruz Biotechnology, Inc.).

**Statistical analysis.** Data were assessed as the means ± SD. of triplicate samples from at least three independent experiments. Statistical significance was evaluated using a Student's t-test or one-way ANOVA and was considered significant when  $p < 0.05$ .

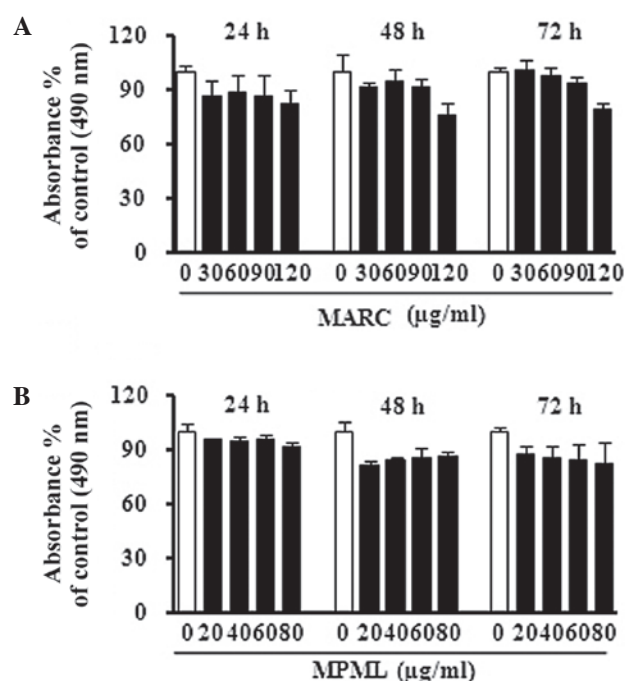


Figure 1. Effect of (A) MARC and (B) MPML on cell viability of JB6 P+ cells. JB6 P+ cells ( $1 \times 10^3$  cells/100 µl) were seeded in 96-well plates and incubated for 72 h with increasing concentrations of MARC and MPML. Cell viability was estimated using a CellTiter 96 non-radioactive cell proliferation assay, as described in Materials and methods. The graph indicates the average of triplicate samples from three independent experiments. MARC, methanol extracts of *Althaea rosea* Cavanil; MPML, methanol extracts of *Plantago major* L.

## Results

**MARC and MPML do not affect cell proliferation in JB6 P+ cells.** To determine whether MARC and MPML had cell growth inhibitory effects, we treated JB6 P+ epidermal mouse skin cells with MARC and MPML at a range of doses and assessed viability using the MTS assay. The results showed that MARC and MPML did not significantly affect cell proliferation at 24, 48 or 72 h after treatment (Fig. 1).

**MARC and MPML markedly suppress EGF-induced neoplastic transformation in JB6 P+ cells.** The JB6 P+ cell line is an excellent model to study EGF-induced cell transformation (17). We evaluated whether MARC and MPML is directly associated with EGF-induced neoplastic transformation. The results showed that MARC and MPML significantly decreased the EGF-promoted colony number and colony size in a dose-dependent manner. Colony formation and colony size induced by EGF-induced JB6 P+ cells was also significantly less than that observed in the non-treated JB6 P+ cells (Fig. 2A and B).

**MARC and MPML inhibit EGF-induced EGFR phosphorylation in JB6 P+ cells.** Numerous studies have shown that EGF promoted skin carcinogenesis through EGFR activation (6,7). To verify whether phosphorylation of EGFR was induced by EGF, we tested the expression of EGFR phosphorylation with EGF for increasing time periods. The results showed that the expression of EGFR phosphorylation significantly increased within 5-20 min after treatment with EGF (Fig. 3A). We tested whether MARC and MPML downregulated the phosphorylation

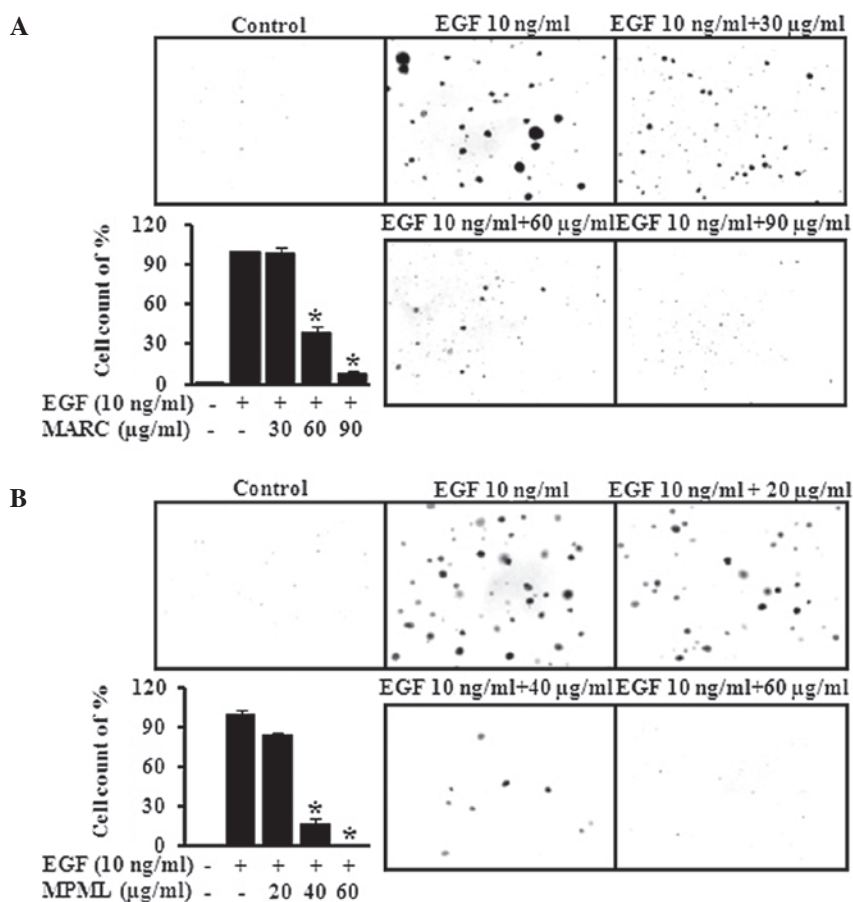


Figure 2. Effect of (A) MARC and (B) MPML on EGF-induced neoplastic cell transformation in JB6 P+ cells. JB6 P+ cells were treated with or without MARC and MPML, and exposed to EGF (10 ng/ml) in 1 ml of 0.3% basal medium Eagle's agar containing 10% FBS. The culture was maintained at 37°C in a 5% CO<sub>2</sub> atmosphere for 10 days, after which colonies were counted automatically. MARC, methanol extracts of *Althaea rosea Cavanil*; MPML, methanol extracts of *Plantago major L.*; EGF, epidermal growth factor; FBS, fetal bovine serum. The asterisk indicates a significant difference (P<0.05) between groups treated with EGF and MARC or MPML, and the group treated with EGF alone.

of EGFR activated by EGF in JB6 P+ cells. Our results indicated that MARC (90 µg/ml) and MPML (60 µg/ml) suppressed the EGF-induced phosphorylation of EGFR (Fig. 3B and C). These results suggested that the inactivation of EGFR by MARC and MPML leads to the suppression of neoplastic transformation.

*MARC and MPML inhibit the EGF-induced phosphorylation of EGFR and suppress the EGF-induced cell proliferation in an MEF cell line.* MARC and MPML strongly inhibited the EGF-induced phosphorylation of EGFR in JB6 P+ cells (Fig. 3). Thus, we investigated the direct effect of MARC and MPML on the phosphorylation of EGFR in EGF knockout MEF cells (EGFR<sup>-/-</sup>) and EGFR-expressing MEF cells (EGFR<sup>+/+</sup>). MARC and MPML decreased the EGF-induced phosphorylation of EGFR in EGFR/WT MEF cells, but not in EGFR KO MEF cells (Fig. 4A and B). To further explore the inhibitory effect of MARC and MPML on EGF-induced cell proliferation, EGFR<sup>+/+</sup> cells were cultured for 24 h in 96-well culture plates with MARC (90 µg/ml) and MPML (60 µg/ml) and cell proliferation was measured using the MTS assay. Treatment with EGF (10 ng/ml) resulted in an ~60% increase compared to the DMSO control in the EGFR<sup>+/+</sup> cells, but MARC (90 µg/ml) and MPML (60 µg/ml) inhibited EGF-induced cell proliferation in EGFR<sup>+/+</sup> cells. These results indicated that EGFR<sup>+/+</sup> MEF proliferation affects the inhibition of EGFR activation by MARC and MPML.

## Discussion

Cancer is a major public health problem. Numerous studies have indicated that chemotherapeutic drugs cannot successfully treat cancer as they have severe side effects including various types of rash, hair loss, painful paronychia and xerosis cutis (18). Plants as medicinal agents have been used in the treatment of various types of diseases in humans (19-23) and a number of investigators have also reported that natural products may be safe and free from side effects (24-27). Particularly, PML as a natural product, has been reported to exert its anticancer effect via DNA damage in cancer cells (15). Wang *et al* also reported that ARC inhibited inflammation in rat paw edema (28). However, no study currently exists on the anti-cancer activity and related molecular targets of ARC and PML.

In this study, we focused on three primary objectives in relation to the chemopreventive effect of ARC and PML in EGF-induced neoplastic cell transformation. The first was to examine the effect of ARC and PML on the cell toxicity of mouse epidermal JB6 P+ cells. The second was to determine the anti-tumorigenic effect of ARC and PML on EGF-induced neoplastic cell transformation. The third was to identify critical key molecules in mouse skin anti-cancer activities by ARC and PML. For this study, ARC and PML were extracted with methanol. We investigated whether MARC and MPML were

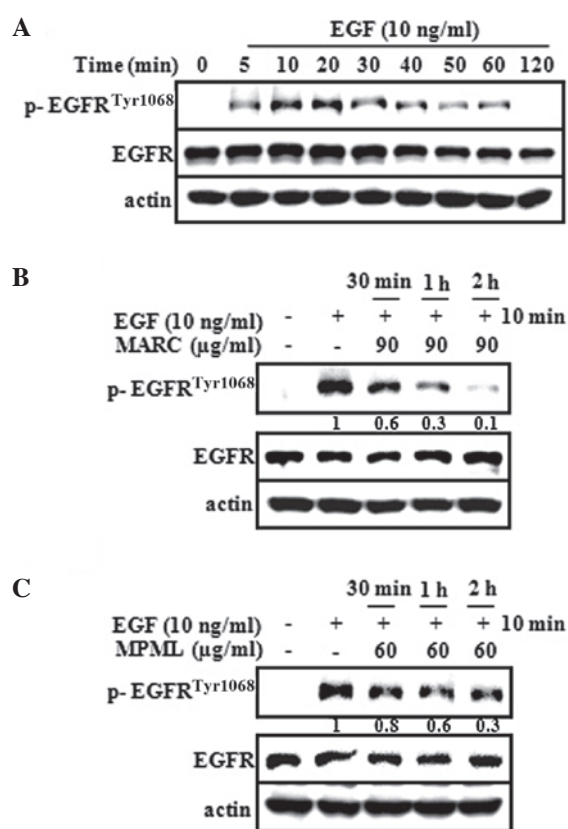


Figure 3. Inhibitory effect of MARC and MPML on EGF-induced EGFR phosphorylation. JB6 P+ cells were starved for 24 h via incubation in serum-deprived MEM at 37°C in a 5% CO<sub>2</sub> atmosphere, treated with MARC and MPML for 30 min, 1 and 2 h and then exposed to EGF (10 ng/ml) for 10 min. Whole cell lysates were collected for the western blotting with antibodies detecting phosphorylated EGFR as described in Materials and methods. (A) Time-course expression of EGFR phosphorylation was detected by EGF-stimulation. (B and C) MARC and MPML inhibit EGFR phosphorylation induced by EGF. MARC, methanol extracts of *Althaea rosea* Cavanil; MPML, methanol extracts of *Plantago major* L.; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor.

cytotoxic in the mouse epidermal JB6 P+ cells. The results showed that MARC and MPML did not have any effect on the viability of JB6 P+ cells when treated at various doses.

The irregular biological process of tumorigenesis is associated with the abnormal regulation of growth signaling. EGF is one of the tumor promoters that is capable of causing the induction of abnormal cell growth (29) and the EGF-EGFR transduction system has been confirmed to be significantly increased in human cancer cells of the skin, breast, colon, lung and prostate to stimulate cell proliferation, invasiveness and angiogenesis (11,30,31). The JB6 P+ cell line is a well-established system extensively used as an *in vitro* model for the study of tumor promotion (32,33) and provides an ideal model for the investigation of the molecular mechanisms involved in neoplastic transformation, promotion and progression (11,34-36). Notably, it has been reported that EGF induced the formation of anchorage-independent colonies in JB6 P+ cells using a soft agar assay (11,33,37). Therefore, we investigated whether MARC and MPML are capable of suppressing the EGF-induced neoplastic cell transformation. We showed that MARC and MPML clearly inhibited EGF-induced neoplastic cell transformation as well as colony number and

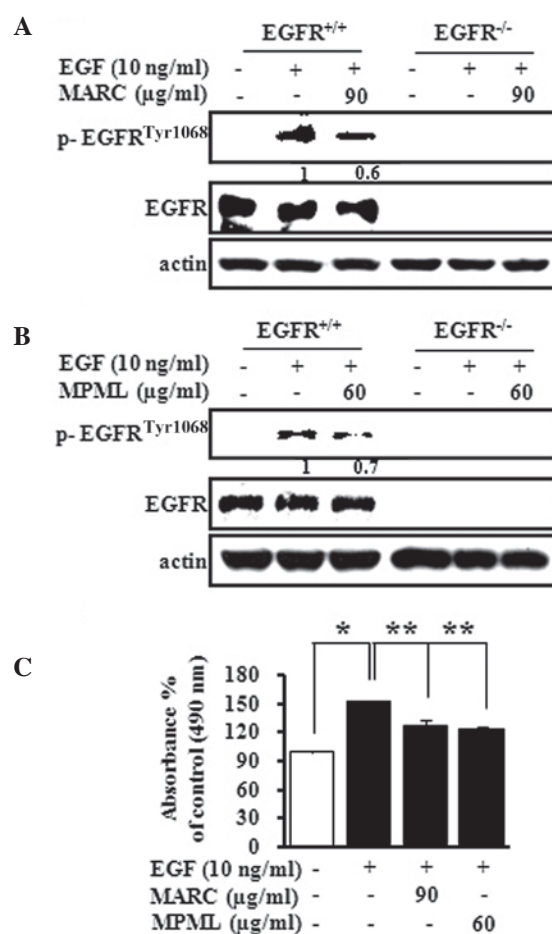


Figure 4. MARC and MPML inhibited EGF-induced proliferation and EGFR phosphorylation in EGFR wild-type (WT) (+/+) and knockout (-/-) MEFs. (A and B) Treatment with MARC and MPML inhibited EGFR phosphorylation only in EGFR/WT MEFs. After EGFR/WT and KO MEFs were starved for 24 h via incubation in serum-deprived MEM at 37°C in a 5% CO<sub>2</sub> atmosphere, cells were treated with MARC 90 µg/ml and MPML 60 µg/ml for 1 h, followed by EGF (10 ng/ml) for 10 min. Cells were lysed and EGFR phosphorylation expression was analyzed by western blotting. (C) MARC and MPML significantly decreased EGF-induced cell proliferation only in EGFR/WT MEFs. Cell proliferation was estimated using the CellTiter 96 non-radioactive cell proliferation assay at an absorbance of 490 nm. The graph is representative for three independent experiments and bars show the mean ± SD. \*P<0.05 versus control group. \*\*P<0.05 versus EGF-treated group. MARC, methanol extracts of *Althaea rosea* Cavanil; MPML, methanol extracts of *Plantago major* L.; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MEFs, mouse embryonic fibroblasts.

size in EGF-treated JB6 P+ cells, indicating that MARC and MPML could be acting as anti-cancer agents. EGFR has been recognized as a convergence point for diverse signal transduction pathways (37-39). After EGF binds with the ectodomain of the EGFR, which exists as homodimers or heterodimers, this leads to the autophosphorylation of tyrosine residues in the cytoplasmic domain and activation of the receptor's intrinsic kinase activity (11,40). Notably, EGFR is frequently overexpressed and abnormally activated in many types of cancer. Thus, we examined whether MARC and MPML inhibited EGF-induced EGFR phosphorylation to suppress neoplastic cell transformation in EGFR-expressing cells (JB6 P+, EGFR<sup>+/+</sup>). These results suggested that MARC and MPML significantly inhibited the phosphorylation of EGFR by EGF on JB6 P+ and EGFR<sup>+/+</sup> cells, but not EGFR<sup>-/-</sup> cells. MARC and MPML effectively



inhibited the EGF-induced proliferation of EGFR<sup>+/+</sup> cell lines. Our data suggest that the dephosphorylation of EGFR may be associated with MARC- and MPML-suppressed neoplastic cell transformation and cell proliferation in JB6 P+ cells.

In conclusion, MARC and MPML have suppressed EGF-induced transformation and proliferation through the dephosphorylation of EGFR. Thus, MARC and MPML are capable of inhibiting the growth of cancer cells through EGFR and may be developed into effective anticancer agents against various types of cancer that highly express EGFR.

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