Gefitinib Decreases the Synthesis of Matrix Metalloproteinase and the Adhesion to Extracellular Matrix Proteins of Colon Cancer Cells

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Abstract. *Background: Adhesion to extracellular matrix (ECM) proteins and degradation of basement membranes by matrix metalloproteinase (MMP) play important roles in cancer metastasis. In this study, the effects of gefitinib on the enzymatic activity of MMP and adhesion to ECM proteins in the HT29 colon cancer cell line were investigated. Materials and Methods: Microtiter plates, coated with ECM proteins, were used to investigate the adhesion of cancer cells to ECM proteins. The expression of MMPs was examined by zymography and semi-quantitative RT-PCR. Results: Gefitinib inhibited MMP-9 and MMP-2 secretion and mRNA expression in HT29 cells. Gefitinib also reduced the ability to adhere to laminin and type IV collagen. These effects were observed at such low doses that gefitinib had neither an antiproliferative effect nor the ability to induce apoptosis. Conclusion: Gefitinib decreased the production of MMPs and the adhesion to ECM proteins, important steps associated with cancer metastasis. These results suggest that gefitinib may have antimetastatic activity in colon cancer.*

Colorectal cancer is one of the most common types of adult malignancies in developed countries (1) and surgical excision remains the best treatment option for this cancer. However, one of the most difficult problems affecting the successful treatment of colorectal cancer is synchronous or metachronous hematogenous metastasis. Therefore, new approaches to cancer treatment and prevention of metastasis are needed. Tumor metastasis involves many steps, including adhesion of cancer cells to the extracellular matrix (ECM), cell motility and invasion. Among these, cell adhesion and degradation of ECM are the key steps in the metastatic process. Cellular adhesion is the first important step in metastasis, because cancer cells must first adhere to the ECM before they degrade ECM components. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that can degrade most ECM components and are thought to play a critical role in tumor invasion.

The epidermal growth factor receptor (EGFR) signaling pathway is associated with metastatic mechanisms, including adhesion to ECM and degradation by cancer cells (2). The EGFR is highly expressed in many human tumor cells, including colon cancer cells (3) and is frequently implicated in the pathogenesis and progression of human tumors (4). For these reasons, a blockade of the EGFR-activating pathway may serve as a potential therapeutic target for the treatment of colorectal cancer. Gefitinib (IRESSA®) is an EGFR inhibitor, which is an orally active, selective EGFR-tyrosine kinase inhibitor that blocks the signal transduction pathway implicated in the proliferation and survival of cancer cells and is currently in clinical trials with cancer patients (5). MacKenzie *et al.* reported a phase II trial of the drug in metastatic colon cancer patients (6), but they failed to show significant tumor regression. A previous study suggested that anti-EGFR MAb C225 may have an antimetastatic effect (7). Therefore, gefitinib may also exhibit antimetastatic activities by blocking the production of MMPs and adhesion to the ECM.

In this study, the effects of gefitinib on the enzymatic activity of MMPs and the adhesion to ECM proteins in a colon cancer cell line, were investigated.

Materials and Methods

Gefitinib (ZD1839). Gefitinib was kindly provided by AStraZeneca Pharmaceuticals (Macclesfield, UK) and was dissolved in DMSO before use.

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Cell line. The HT29 human colon cancer cell line was purchased from the American Type Culture Collection (ATCC) and maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS). The cell line was cultured at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air.

Cell proliferation assay. HT29 cells were seeded on 96-well plates ($2X10^4$/well) and incubated for 24 h. Gefitinib was added to the culture medium at the indicated concentrations and the cells were incubated for another 48 h. Thereafter, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) reagent (Promega, WI, USA) were added to each well and the plate was incubated for 2 h. Cell viabilities were calculated by measuring the absorbency at 490 nm using a microplate reader (Nalge Nunc International K.K., Tokyo, Japan).

Adhesion assay. Ninety-six-well plates coated with human laminin, fibronectin or type IV collagen (Becton Dickinson Co., NJ, USA) were used to evaluate the cell adhesion to the ECM proteins. Each well was filled with McCoy's 5A medium containing 0.1% bovine serum albumin (BSA) for 1 h to block non-specific binding. The HT29 cells were harvested and resuspended in serum-free medium with 0.1% BSA. Twenty thousand cells were added to the 96-well plates, with or without gefitinib. After a 1-h incubation, the non-adherent cells were removed by washing 3 times with serum-free medium. The cell adhesion rate was then calculated using the MTS reagent as indicated above.

Gelatin zymography. The HT29 cells were allowed to grow to subconfluence in tissue culture dishes. The medium was subsequently replaced with new medium containing 10 ng/ml epidermal growth factor (EGF) or gefitinib, and the cells were incubated for 4 h or 24 h. The culture media were collected, concentrated, applied to 10% SDS-polyacrylamide gels copolymerized with 0.1% gelatin and electrophoresed. The gels were washed with 2.7% Triton X-100 for 30 min at room temperature and then incubated with substrate buffer (50 mM Tris base, 40 mM HCl, 200 mM NaCl, 5 mM $CaCl_2$) at 37˚C for 24 h. The gels were then stained with 0.05% Coomassie brilliant blue G-250 and decolorized. Gelatinolytic enzymes were detected as transparent bands against the blue-stained background.

Western blot analysis. HT29 cells were grown to subconfluence and then incubated with gefitinib for 24 h. The cells were harvested in lysis buffer and then clarified by centrifugation at 10,000Xg at 4˚C for 10 min. Total cell lysates were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were probed with anti-phospho-EGFR, anti-phospho-PI3K, anti-phospho-ERK and anti-phospho-Akt (Cell Signaling Technology Inc., MA, USA) antibodies.

Apoptosis assay. The HT29 cells were treated with gefitinib for 24 h and were then harvested, collected by centrifugation and prepared for examination. The cells were stained with Annexin V conjugated with FITC using the Annexin V-FIFC Apoptosis Detection Kit I (Becton Dickinson Co.) and were analyzed by FACScan (Becton Dickinson Co.).

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). The HT29 cells were incubated with gefitinib for 24 h. The total RNA was isolated using an RNeasy Mini Kit (QIAGEN GmbH., Hilden, Germany). Reverse transcription of RNA was carried out using a Super Script First-Strand Synthesis System for RT-PCR (Invitrogen, CA, USA). A thermal cycler, Gene Amp PCR system9700 (Applied Biosystems, CA, USA), was used for semi-quantitative RT-PCR. Previously reported primers (8) were used for cDNA amplification. The following primers were used; *MMP-2*: 5'-GGCCCTGTCACTCCTGAGAT-3' and 5'-GGCATCCAG GTTATCGGGGA-3'; *MMP-9*: 5'-CAACATCACCTATTGG ATCC-3' and 5'-CGGGTGTAGAGTCTCTCGCT-3'. cDNA (1 μL) was amplified by PCR in a final volume of 25 μL containing 1*TaqMan buffer, 2.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 200 μM of each primer and 1.25 units of HotStarTaq DNA Polymerase (QIAGEN GmbH). PCR conditions were as follows: 1 cycle of denaturing at 95˚C for 15 min followed by 35 cycles at 94˚C for 30 sec, 57˚C (for *MMP-2*) or 53˚C (for *MMP-9*) for 30 sec and 72˚C for 30 sec, followed by a final sequence extension incubation at 72˚C for 10 min. Five μL of each amplification reaction were analyzed electrophoretically on 2% agarose gels containing 1*TAE and ethidium bromide. Contamination was routinely checked by water blank in each RT-PCR assay.

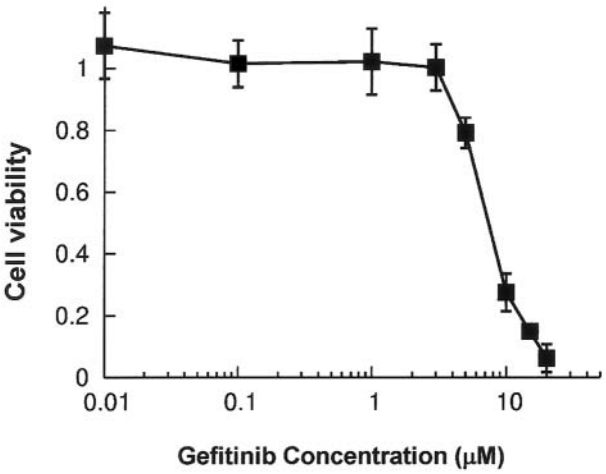

Figure 1. *Effect of gefitinib on HT29 cellular proliferation. Cells were incubated with gefitinib (0-20 μM) for 48 h. The number of viable cells in each well was counted by MTS assay. The results were expressed as the ratio of cell numbers relative to controls. Each value represents the mean of 8 determinations. Bars, SD.*

Statistical analysis. The results were statistically evaluated by the Student's *t*-test. $P<0.05$ was considered as statistically significant.

Results

Antiproliferative effect of gefitinib. The effects of gefitinib on the proliferation of HT29 cells were evaluated first. Gefitinib inhibited the proliferation of HT29 cells in a dose-dependent manner. The IC_{50} was estimated as 7.9 μM. As shown in Figure 1, the antiproliferative effect of gefitinib was not observed until 3 μM were applied. To evaluate the

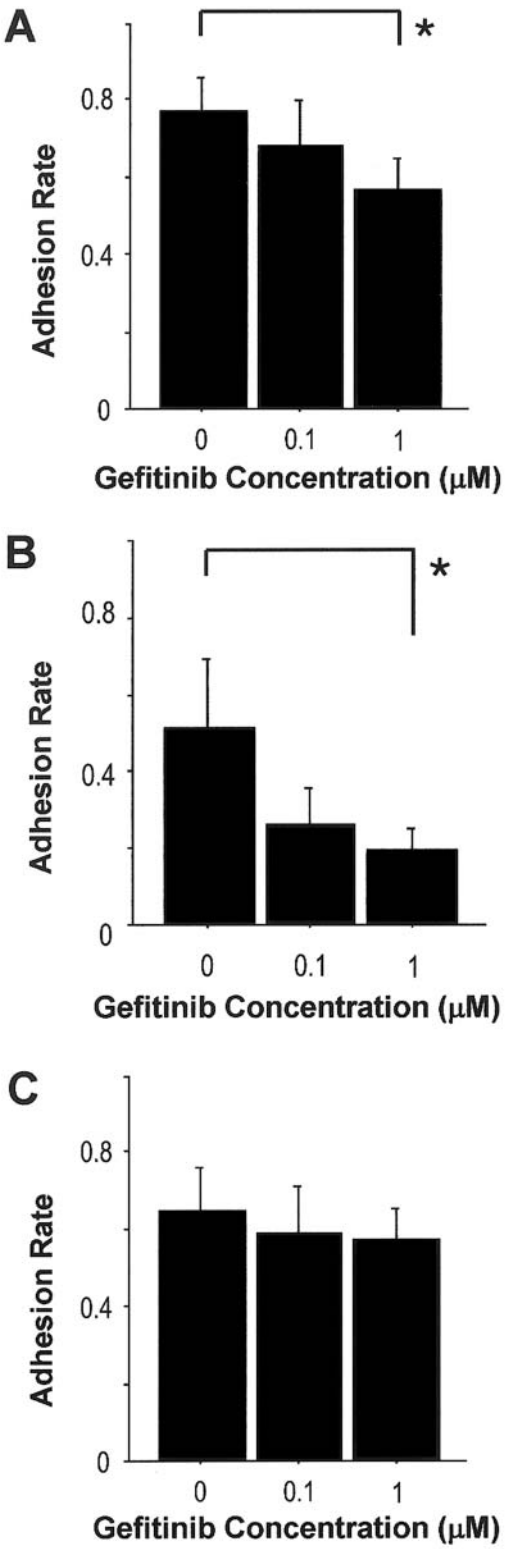

Figure 2. *Adhesion of HT29 cells to the ECM proteins. A. Type IV collagen, B. Laminin, C. Fibronectin. HT29 cells were pretreated for 24 h with 1 μM of gefitinib. The results were expressed as the optimal density relative to that in the controls (non-washed wells). Each column represents the mean of 8 determinations. Bars, SD. *, P<0.05 compared with untreated cells.*

effects of gefitinib on cellular adhesion and expression of MMPs, 1 μM of gefitinib was employed in further analyses in order to avoid the antiproliferative effect of the drug.

Adhesion assay. To investigate the effect of gefitinib on cell adhesion, the cell adhesion assay was performed. As shown in Figure 2, gefitinib significantly inhibited the adhesion of HT29 cells to type IV collagen and to laminin by 73.5% and 37.9%, respectively, of that of the control. However, no effect on the cellular adhesion to fibronectin was observed.

Effects of gefitinib on MMP expression. MMP-2 and MMP-9 are major MMPs, which degrade ECM proteins and are essential for tumor invasion. The induction of MMPs by EGF was initially examined. As shown in Figure 3A, 10 ng/ml EGF induced MMP-9 and MMP-2 secretion. An investigation of the effects of gefitinib followed, and gefitinib was found to reduce secretion of MMP-9 and MMP-2 from HT29 cells at a concentration of 1 μM (Figure 3B). Furthermore, semi-quantitative RT-PCR revealed that gefitinib reduced the synthesis of both *MMP-2* and *MMP-9* mRNA (Figure 3C). Because no antiproliferative effect was observed with 1 μM gefitinib, the results suggest that the inhibition of MMPs expression by gefitinib is unrelated to its antiproliferative effect.

Western immunoblotting. To examine the signaling pathways influenced by gefitinib, the EGF-induced signal transduction through EFGR in HT29 cells was examined (Figure 4). Pretreatment with gefitinib caused inhibition of EGFR autophosphorylation. The effects of gefitinib on ERK1/2 activation were also examined, since cell adhesion and invasion are thought to be controlled through the ERKs pathway. Gefitinib blocked the phosphorylation of ERK1/2 at a dose that inhibited EGFR phosphorylation. The effects of gefitinib on PI3K and Akt activation were subsequently investigated because cell motility, invasiveness, expression of MMPs and other metastatic properties are regulated through the PI3K pathway. Gefitinib was also found to inhibit the phosphorylation of PI3K and Akt.

Apoptosis assay. To evaluate whether these effects of gefitinib contributed to apoptosis or necrosis, a double-staining method involving fluorescein isothiocyanate-conjugated Annexin V and PI was applied to HT29 cells. Flow-cytometric analysis (Figure 5) revealed that the fraction of apoptosis and necrosis in the gefitinib-treated cells did not differ from that in the control cells (7.5% and 7.6%, respectively). This result indicates that gefitinib did not induce apoptosis of HT29 cells after 24-h treatment at the tested concentrations. Therefore, the inhibitory effects

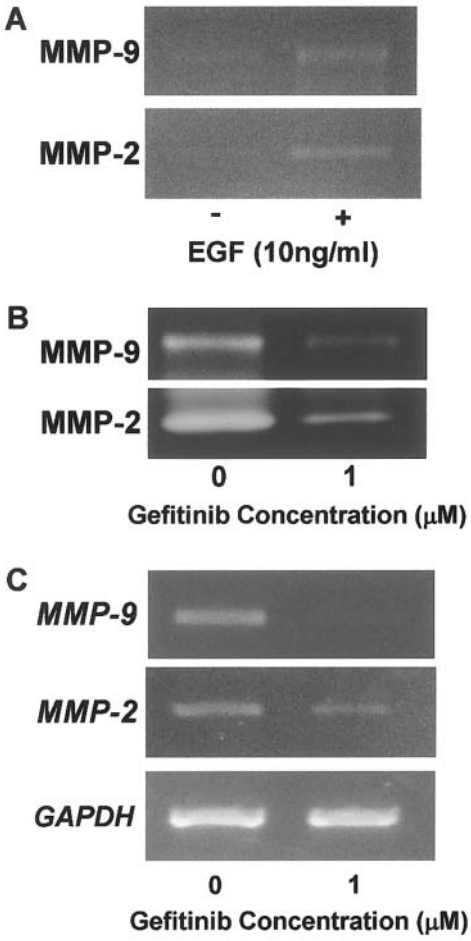

Figure 3. *Gelatin zymography assay and semi-quantitative RT-PCR analysis of MMP-9 and MMP-2. A. The HT29 cells were cultured with/without 10 ng/ml of EGF for 4 h. B. The HT29 cells were treated with serum-free medium containing 1 μM of gefitinib for 24 h. Similar results were obtained in replicate experiments. C. Semi-quantitative RT-PCR analysis of MMP-9 and MMP-2 mRNA. HT29 cells were treated for 24 h with 1 μM of gefitinib. The expression levels of the MMPs were normalized to the GAPDH level.*

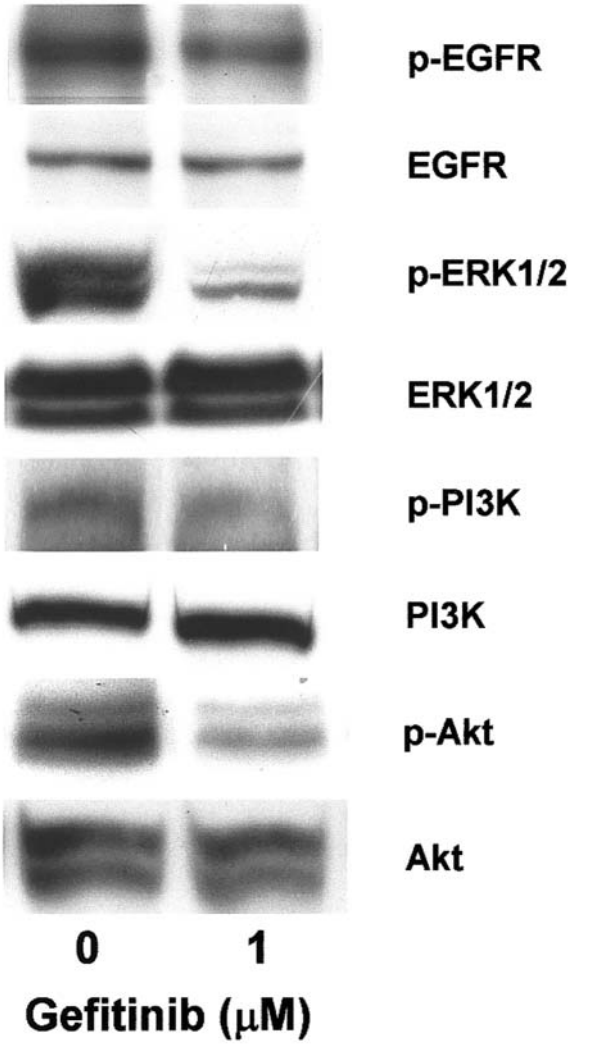

Figure 4. *Western blot analysis of EGFR and its downstream pathway. Serum-starved HT29 cells were treated for 24 h with 1 μM of gefitinib, followed by the addition of 10 ng/ml EGF for 15 min. Phospho-EGFR, phospho-ERK, phospho-PI3K, and phospho-Akt were determined by Western blotting.*

of gefitinib on cell adhesion and MMP-2 and MMP-9 production were confirmed to be independent of its direct antiproliferative effects.

Discussion

EGFR activation initiates complex signaling cascades, which result in cell proliferation, invasion, metastasis and other cellular effects (9, 10). EGFR is often expressed in human colon cancer and is implicated in the progression and poor clinical course of colon cancer patients (11). By blocking EGFR activation and/or function in cancer cells, it may be possible to reduce the metastatic potential of these cells. It has been shown that the EGFR monoclonal antibody reduced the invasive properties of cancer cells (12). In this study, we investigated the effects of gefitinib on the enzymatic activity of MMPs and the adhesion to ECM proteins in a colon cancer cell line.

Adhesion is an important event in the metastatic process, in that the cancer cells must first adhere to the ECM before they degrade these components. A previous study has suggested that EGFR and its downstream pathway may be associated with adhesion to the ECM (13). It also has been reported that the ability of cancer cells to adhere to ECM proteins is correlated with the tumorigenicity of cancer cells (14). Gefitinib significantly inhibited cell adhesion to type

IV collagen and laminin in a dose-dependent manner, even at a dose as low as 1 μM. However, gefitinib neither inhibited cell proliferation nor induced apoptosis at this dose. Thus, this effect was not due to antiproliferative or apoptotic effects. Shintani *et al.* showed that exposure of tumor cells to gefitinib reduced their ability to adhere to fibronectin and reduced the expression of integrins (15). The authors of another study reported that the expression of integrin was regulated by EGFR and its downstream pathway, MAPK/ERK (16). We also showed that the phosphorylation of MAPK/ERK was inhibited by treatment with 1 μM of gefitinib. Thus, we suggest that gefitinib effectively inhibits cancer cell adhesion, even at a minimal dose, by inhibiting EGFR and the MAPK/ERK cascade.

Degradation of the ECM by cancer cells is another important step in cancer metastasis. MMPs, which are highly induced by cancer cells, are a key factor in this process. Interestingly, the expression of MMPs was shown to be correlated with the clinical course and prognosis of colon cancer patients (17). Matsuyama *et al.* reported that the expression levels of MMP-2 and MMP-9 in colon and pancreas carcinomas with liver metastasis were higher than those in cases without liver metastasis (18). *MMP* mRNA expression and the secretion of MMPs have been reported to be associated with tumor invasiveness (19). Thus, inhibition of their expression or secretion may be a potential treatment strategy for tumor metastasis. In this study, we showed that gefitinib, even at a low dose, inhibited the secretion of MMP-2 and MMP-9 into the culture medium, although the growth of HT29 cells remained unaffected. RT-PCR examination revealed that gefitinib reduced *MMP-2* and *MMP-9* mRNA expressions. These results indicate that gefitinib inhibits the expressions of MMP-2 and MMP-9 at the transcriptional level, while having no effects on cell proliferation or apoptosis. Previous evidence suggests that MMP-9 secretion and mRNA expression might be controlled *via* PI3K and its downstream pathway (20). In this study, gefitinib was found to inhibit the phosphorylation of PI3K at a dose which inhibited the secretion of MMPs. Gefitinib may reduce the synthesis of MMPs through inhibition of these signaling pathways. Matsuo *et al.* reported that gefitinib inhibited the formation of metastatic lesions and the production of MMP-9 in murine hepatocellular carcinoma (21). In addition, Zhiwen *et al.* showed that gefitinib reduced cell migration and MMP-9 production in malignant mesothelioma (22). These reports, together with our results, suggest that gefitinib may be a potent agent which prevents tumor metastasis at lower doses.

As reported in a previous study, gefitinib did not contribute to tumor reduction in patients with metastatic colorectal cancer (6). The results of another study indicated that the maximum plasma concentrations of gefitinib resulting from clinically relevant doses are 0.5-1 μM or more

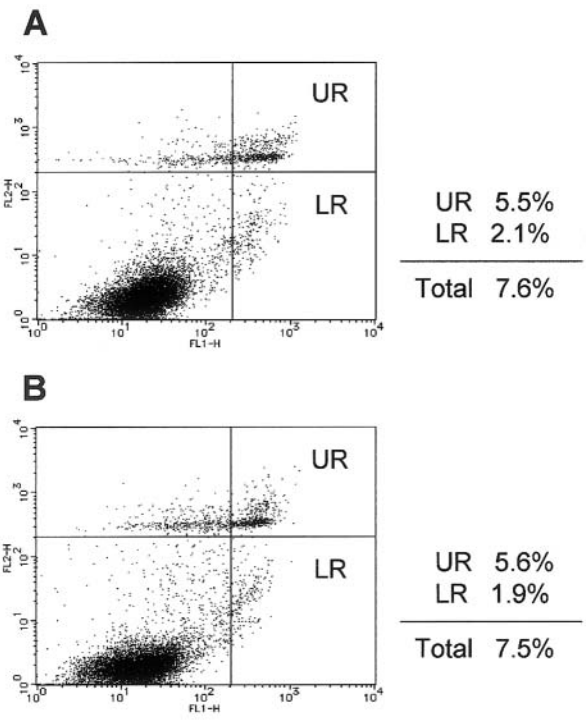

Figure 5. *Induction of apoptosis by gefitinib treatment. The HT29 cells were treated with 1 μM of gefitinib for 24 h. Apoptosis was quantified by the Annexin-V /propidium FACS assay. The percentage of early apoptotic (lower right: LR) and late apoptostic/ necrotic (upper right: UR) cells are shown at the right of the figure.*

(23). Our results suggest that gefitinib may exert an antimetastatic effect at these concentrations, while it was not seen to exert antiproliferative or apoptotic effects. Therefore, gefitinib may be a promising agent for controlling metastatic lesions or preventing metastasis after curative surgery in patients with colon cancer.

In conclusion, gefitinib was shown, even at a minimal dose, to decrease the production of MMPs and adhesion to the ECM in a colon cancer cell line, both important steps associated with cancer metastasis. These results suggest that gefitinib may have an antimetastatic activity and may be useful in preventing metastasis after curative resection of colon cancers.

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