Cancer Science
The official journal of the Japanese Cancer Association

Sprouty2 protein enhances the response to gefitinib through epidermal growth factor receptor in colon cancer cells

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(Received February 16, 2010/Revised May 27, 2010/Accepted May 28, 2010/Accepted manuscript online June 7, 2010/Article first published online July 8, 2010)

Sprouty2 (Spry2) is known to increase the expression of epidermal growth factor receptors (EGFR) by conjugating with c-Casitas B-lineage lymphoma (C-Cbl) to decrease protein degradation. The effect of Spry2 on the treatment of gefitinib, a tyrosine kinase inhibitor of EGFR, with regards to colon cancer is still unclear. The half maximal inhibitory concentration (IC50) values of gefitinib in six colon cancer cell lines were assessed. HCT116 and C2BBel cells expressed lower levels of Spry2 protein and were less sensitive to gefitinib, whereas HT29 cells that expressed high levels of Spry2 protein were more sensitive to gefitinib. The sensitivity to gefitinib was increased after overexpression of Spry2 in HCT116 cells, whereas it was decreased after Spry2 knockdown in HT29 cells. The levels of both phosphorylated and total EGFR were increased when HCT116 cells ectopically overexpressed Spry2, with concomitant increase in phosphatase and tensin homolog (PTEN) expression. Inhibition of EGFR by cetuximab reduced sensitivity to gefitinib in HCT116 cells overexpressing Spry2. However, knockdown of PTEN or K-ras failed to diminish the effect of Spry2 on gefitinib sensitivity. Of note, Spry2 enhanced the antitumor effect of gefitinib in a xenograft model of HCT116 tumors, which harbored K-ras codon 13 mutation. In conclusion, Spry2 can enhance the response of colon cancer cells to gefitinib by increasing the expression of phosphorylated and total EGFR. These results suggest that Spry2 may be a potential biomarker in predicting the response to anti-EGFR treatment in colon cancer and that it is necessary to conduct clinical studies to incorporate Spry2 into the network of cancer treatment. (*Cancer Sci* 2010; 101: 2033–2038)

Colorectal cancer is the second leading cause of death from cancer in the USA. It is estimated that there will be 147 000 newly diagnosed cases of colorectal cancer and nearly 50 000 deaths associated with this disease.[1] Up to 20% of new cases present with metastatic disease. Among the patients who present with localized disease, approximately 20% will subsequently relapse with distant metastasis. Over little more than a decade, the options for systemic therapy have progressed significantly from 5-fluorouracil (5-FU) alone in 1995 to an armamentarium of several chemotherapy and biological agents.[2] Among these biological agents, epidermal growth factor receptor (EGFR)-targeted therapy is one of the important clinical strategies to apply in patients with metastatic colon cancer.

Epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane glycoprotein, with an extracellular ligand-binding domain and an intracellular region containing the tyrosine kinase domain.[3] Epidermal growth factor receptor (EGFR) activation on the cancer cell surface is believed to promote cell growth, differentiation, cell survival, drug and radiation sensitivity, and angiogenesis. Expression of a high level of EGFR has been associated with a poorer prognosis in colon cancer patients undergoing curative surgery.[4] Novel anticancer drugs targeting the EGFR family have been applied in treating various types of human cancers, including colon cancer, lung cancer, breast cancer, and squamous cell carcinoma of head and neck. These strategies targeting EGFR include monoclonal antibodies that block the extracellular ligand binding domain of the receptor such as cetuximab and low molecular weight tyrosine kinase inhibitors (TKI), such as gefitinib and erlotinib.

Gefitinib (Iressa, ZD-1839) is a synthetic anilinoquinazoline and orally active selective EGFR-TKI that blocks the signal transduction pathway implicated in the proliferation and survival of cancer cells. Disappointingly, the emerging clinical experience across a range of cancer types reveals that despite the anti-EGFR agents demonstrating some antitumor activity, there is a high level of de novo resistance to such treatment.[5–7] Several biomarkers have been demonstrated to exert a predictive value in anti-EGFR treatment. Specific mutations in the *EGFR* gene are correlated with clinical response to gefitinib in patients with non-small-cell lung cancer.[8] Phospho-EGFR level and high serum EGFR at baseline increase the sensitivity of colon cancer cells to gefitinib.[9,10] There is emerging evidence that K-ras mutation serves as a major predictor of resistance to cetuximab in colon cancer.[11,12] Furthermore, loss of phosphatase and tensin homolog (PTEN) protein has been shown to be associated with nonresponsiveness to cetuximab.[13]

The Sprouty (Spry) protein was first described by Hacohen *et al.*[14] as an inhibitor of fibroblast growth factor-stimulated tracheal branching during *Drosophila* development. Four mammalian Spry genes have been defined based on sequence similarity with *Drosophila* Spry. Sprouty2 (Spry2) encodes 32- to 34-kDa proteins that share a highly conserved carboxyl-terminal cysteine-rich spry domain. Spry2 is known to be involved in EGFR pathway. C-Cbl is a prominent binding partner of Spry2. Cbl proteins function as ubiquitin ligases which through mono- and poly-ubiquitination of receptor tyrosine kinases (RTKs) initiate their endocytosis and proteosomal degradation. Spry2 has been shown to abrogate EGFR ubiquitylation and endocytosis, and sustain epidermal growth factor (EGF)-induced extracellular signal-regulated kinase (ERK) signaling.[15] Later, it was reported that Spry2 induces down-regulation of EGFR at the

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doi: 10.1111/j.1349-7006.2010.01637.x

Cbl/CIN85 interface and interferes with the trafficking of activated EGFR specifically at the step of progression from early to late endosomes.[16,17] However, it is still unclear whether expression of Spry2 affects the efficacy of gefitinib treatment in colon cancer. In this study, we demonstrate that expression of Spry2 increases sensitivity to gefitinib therapy in colon cancer.

Materials and Methods

Reagents. Gefitinib was provided by AstraZeneca (Wilmington, DE, USA). A 10 mmol/L working solution in DMSO was prepared and stored at −20°C. Cetuximab was obtained from Merck (Darmstadt, Germany).

Cell culture. C2BBel, LS174T, HCT116, and HT29 human colon cancer cell lines were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). SW620 and SW480 human colon cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA).

Cell viability assay. Cells were seeded at 10 000 per well in 96-well plates. They were treated with increasing doses of gefitinib for 72 h. Cell viability was assessed by the MTT assay (Sigma Aldrich, St. Louis, MO, USA). The absorbance was measured at 570 nm on the MRX Revelation microtiter plate reader (Dynex Technologies, Chantilly, VA, USA). The half maximal inhibitory concentration (IC50) was calculated with SigmaPlot 9.0 statistical software (Jandel Scientific, Corte Madera, CA, USA). Data were expressed as the ratio of cell numbers relative to the controls. Each value represents the mean ± SD of at least three determinations.

Plasmids, transfection, and stable clones. The pSpry2-EGFP-N3 plasmid encoding Spry2-EGFP fusion protein was obtained from G.F. Vande Woude (University of California, Irvine, CA, USA).[18] HCT116 cells were transfected with pSpry2-EGFP-N3 or empty vector pEGFP-N3 (Clontech, Palo Alto, CA, USA) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). G418 was used for selection of a stable clone overexpressing Spry2 (HCT116/Spry2) and a control clone (HCT116/v).

Lentivirus infection and shRNA knockdown. A set of five pLKO.1-puro-based lentiviral vectors containing stem-loop cassettes encoding shRNAs for human Spry2 (TRCN 0000007520-7524) (designated Spry2 shRNA #1–5), and similar vectors encoding shRNAs for PTEN (TRCN 0000002747), K-ras (TRCN 0000033260), and luciferase (Luc) (TRCN 0000072246) were obtained from the National RNAi Core Facility, Academia Sinica, Taiwan. Knockdown efficiencies were assessed, and two Spry2 shRNA (#1 and #2) were selected for further production of recombinant lentiviruses. Recombinant lentiviruses were produced by transient transfection of 293T cells with pLKO.1-puro constructs along with the packaging construct psPAX2 and the VSV-G expression construct pMD2G using the calcium phosphate precipitation method as previously described.[19]

Immunoblotting. Total cell lysates were harvested for immunoblot analysis using anti-Spry2 (Upstate Biotechnology, Lake Placid, NY, USA), anti-β-actin (Sigma Aldrich), anti-phospho-EGFR, anti-EGFR, and anti-PTEN (Cell Signaling Technologies, Beverly, MA, USA) antibodies as primary antibodies. Horseradish peroxidase-conjugated antirabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and antimouse IgG (Santa Cruz Biotechnology) were used as secondary antibodies where appropriate, and protein-antibody complexes were visualized using the ECL system (Amersham Biosciences, Piscataway, NJ, USA).

Reverse transcription–polymerase chain reaction (RT-PCR). Total RNA was extracted from colon cancer cells using RNA extraction kits (Qiagen, Hilden, Germany). RNA was treated with RNase-free DNase for 30 min at room temperature. First-strand cDNA was synthesized using 2 μg of DNase-treated RNA, 1 μL of oligo dT, and SuperScript III RT with the SuperScript First-Strand Synthesis System (Invitrogen). Polymerase chain reaction (PCR) was performed with 0.3 μg of cDNA. The primers used for PCR were H-PTEN primer 1 (5′-CTCCAATTCAGGACCCACACGAC-3′), H-PTEN primer 2 (5′-AAGTACAGCTTCACCTTAAA-3′), and H-PTEN primer 3 (5′-CGGGAAGACAAGTTCATGTAC-3′). The amplification condition was used as described previously.[20] For determination of K-ras mRNA expression, the primers used for K-ras were H-K-ras primer 1 (5′-GGGTGTTGATGCCTTCC-3′) and H-K-ras primer 2 (5′-AATCAACTGCATGCACCAAA-3′). The amplification condition was 32 cycles of 1 min at 94°C for denaturation, 30 s at 55°C for annealing, and 1 min at 72°C for extension. The products were subjected to electrophoresis on a 2% agarose gel, and the DNA was visualized by ethidium bromide staining.

Animal study. Animal care in accordance with institutional guidelines and authority to perform *in vivo* work was granted by the Home Office (project license 97022902, Chi-Mei Medical Center). HCT116/Spry2 and HCT116/v xenografts were established by injection of cancer cells into 7- to 8-week-old BALB-C athymic female nude mice purchased from the National Laboratory Animal Center, Taiwan. Groups of five nude mice were inoculated subcutaneously into both flanks with HCT116/Spry2 or HCT116/v cells (2×10^6/injection site). Gefitinib dissolved in carrier solution (0.1% aqueous TWEEN 80; Sigma-Aldrich) was administered to all mice at a dose of 50 mg/kg by oral gavage on days 1–5 and 8–12.[21] Tumors were measured every 5 days and calculated as $\pi/6 \times (\text{tumor width})^2 \times \text{tumor length}$. Mice with tumors exceeding 17 mm diameter were culled in accordance with the guideline.

Immunohistochemistry. Cryostat sections (10 μm) fixed in 4% paraformaldehyde were incubated for 1 h with hSpry2 rabbit polyclonal antibodies (Upstate Biotechnology). After wash, the slides were incubated for 30 min with the DAKO EnVision System (Dako, Carpinteria, CA, USA). Antibody staining was done by treatment with diaminobenzidine. Sections were counterstained with Mayer's hematoxylin. All of the incubation and staining steps were done at room temperature. Negative control slides processed without primary antibody were included for each staining.

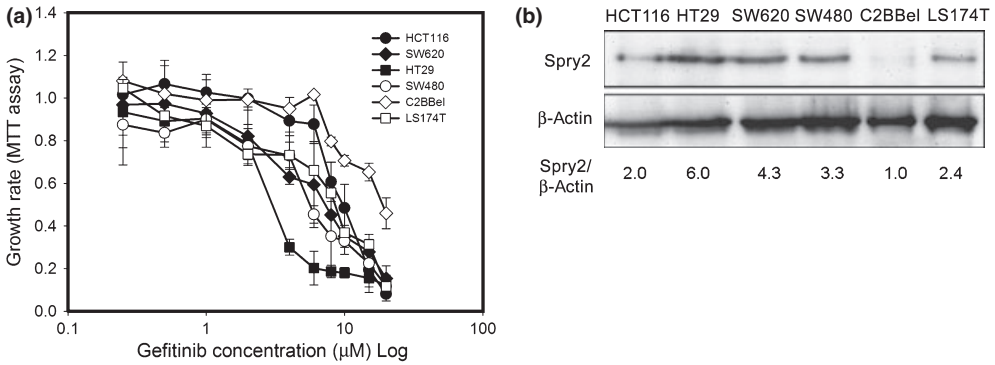
Statistics. Statistical significance between groups was assessed with the Student's *t*-test. Any *P*-value of <0.05 was regarded statistically significant.

Results

Expression of Spry2 protein positively correlates with the sensitivity of colon cancer cells to gefitinib. To test the influence of Spry2 expression on the effect of gefitinib, we assessed the expression of Spry2 protein in six colon cancer cell lines. Among these cancer cell lines, C2BBel and HCT116 cells expressed lower levels of Spry2 protein (Fig. 1b) and were highly resistant to gefitinib, with IC50 values of 14.72 ± 3.18 μM for C2BBel and 9.52 ± 0.59 μM for HCT116 (Fig. 1a). In contrast, HT29 cells that expressed high levels of Spry2 protein (Fig. 1b) were sensitive to gefitinib, with an IC50 value of 2.78 ± 0.11 μM (Fig. 1a). The remaining cell lines, including SW620, SW480, and LS174T, which expressed moderate levels of Spry2 protein (Fig. 1b), exhibited moderate resistance to gefitinib (Fig. 1a,c).

Overexpression of Spry2 enhances the sensitivity of colon cancer cells to gefitinib. We next used HCT116/Spry2 cells stably overexpressing Spry2 and HCT116/v control cells to verify the correlation between Spry2 expression and gefitinib sensitivity. Overexpression of Spry2 enhanced the sensitivity of HCT116 cells to gefitinib compared with the HCT116/v cells (Fig. 2a). The IC50 values of gefitinib were 8.34 ± 2.60 μM in HCT116/v cells and 3.22 ± 0.23 μM in HCT116/Spry2 cells.

doi: 10.1111/j.1349-7006.2010.01637.x



(c)

Cell	HCT116	HT29	SW620	SW480	C2BBel	LS174T
IC50 μM (Mean ± SD)	9.52 ± 0.59	2.78 ± 0.11	6.54 ± 1.88	6.20 ± 0.80	14.72 ± 3.18	8.21 ± 3.10

Fig. 1. Sprouty2 (Spry2) expression in colon cancer cell lines correlates with their sensitivity to gefitinib. (a) Various colon cancer cells were treated with increasing doses of gefitinib for 72 h, and cell viability was determined by the MTT assay. Each value represents the mean of three determinations. Bar, SD. (b) Expression levels of Spry2 protein in various colon cancer cells detected by immunoblot analysis. Values shown below the blot are the ratios between the intensity of the bands corresponding to Spry2 and β-actin determined by densitometry. (c) The half maximal inhibitory concentration (IC50) values of gefitinib in different colon cancer cell lines calculated from (a).

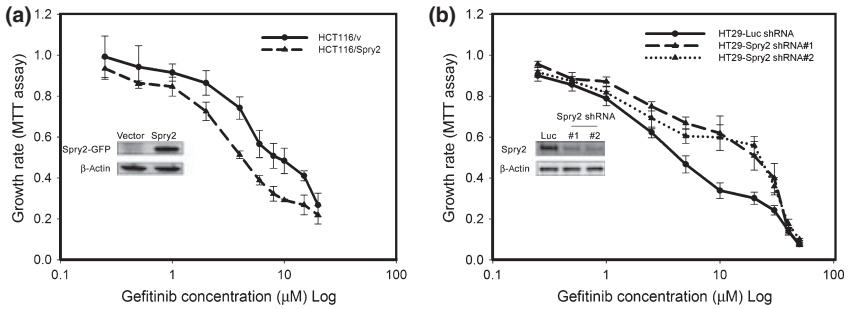


Fig. 2. Sprouty2 (Spry2) expression affects the sensitivity of colon cancer cells to gefitinib. (a) Cytotoxic effect of gefitinib on HCT116/Spry2 cells stably overexpressing Spry2. The IC50 values of gefitinib were 3.22 ± 0.23 μM in HCT116/Spry2 cells and 8.34 ± 2.60 μM in control HCT116/v cells. (b) Cytotoxic effect of gefitinib on HT29 cells after Spry2 knockdown by two Spry2 shRNA lentiviruses. Two Spry2 knockdown cells were less sensitive to gefitinib compared with Luc knockdown cells serving as the control.

Reciprocally, shRNA knockdown of Spry2 expression rendered HT29 cells more resistant to gefitinib (Fig. 2b).

Overexpression of Spry2 enhances the expression of phosphorylated EGFR, total EGFR, and PTEN in colon cancer cells. It has been reported that phosphorylated EGFR and PTEN regulate the sensitivity of colon cancer cells to anti-EGFR treatment. HCT116 cells were used to assess the effect of Spry2 on the expression of EGFR and its downstream effectors. Both phosphorylated and total EGFR as well as PTEN levels were increased in HCT116 cells overexpressing Spry2 in the absence of gefitinib (Fig. 3a). In the presence of gefitinib, overexpression of Spry2 also activated EGFR and up-regulated PTEN expression in HCT116 cells (Fig. 3b). Collectively, these results suggest that increased sensitivity of colon cancer cells to gefitinib by Spry2 overexpression might be dependent on the EGFR status and PTEN expression.

Inhibition of EGFR by cetuximab counteracts Spry2-induced gefitinib sensitivity in colon cancer cells. To confirm whether EGFR expression induced by Spry2 mediates gefitinib sensitivity, HCT116/Spry2 cells were treated with 20 μg/mL of cetuximab for 24 h as previously described.[22] Figure 4(a) shows that pretreatment with cetuximab rendered cells more resistant to gefitinib. Several downstream effectors of EGFR including K-ras and PTEN have been explored to predict for response to gefitinib. To clarify whether PTEN expression is associated with sensitivity to gefitinib in HCT116/Spry2 cells, we knocked down PTEN expression by lentivirus-mediated delivery of PTEN shRNA. Figure 4(b) shows that the IC50 values of gefitinib were similar regardless of PTEN silencing, implying that the increased sensitivity of colon cancer cells to gefitinib induced by Spry2 is independent of PTEN expression. Next, we characterized the K-ras mutations in the six colon cancer cell lines. Except for HT29 and C2BBel cells, four colon cancer cells harbored K-ras mutations, as confirmed by a K-ras mutation detection assay (Fig. S1a). DNA auto-sequencing confirmed that LS174T, SW620, and SW480 cells had codon 12, and HCT116 cells had codon 13 K-ras mutations (Fig. S1b). The impact of K-ras mutation on gefitinib sensitivity enhanced by Spry2 was assessed in SW620 cells using shRNA knockdown. Figure 4(c) reveals that knockdown of K-ras in SW620 cells did not affect their sensitivity to gefitinib.

Sprouty2 (Spry2) enhances the antitumor activity of gefitinib in the HCT116 xenograft model. To confirm the effect of Spry2 on gefitinib treatment in colon cancer, HCT116 cells were implanted in nude mice with or without ectopic expression of Spry2 accompanied with oral administration of gefitinib. As

shown in Figure 5(a), seven of 10 implants of HCT116/v cells generated tumors despite gefitinib treatment. By marked contrast, only two of 10 implants of HCT116/Spry2 cells formed tumors. Immunohistochemical analysis also confirms that tumors derived from HCT116/Spry2 cells expressed higher levels of Spry2 than did tumors derived from HCT116/v cells (Fig. 5b). Notably, HCT116/v cells formed larger tumors than HCT116/Spry2 cells, indicating that Spry2 expression may contribute to the enhanced antitumor effect of gefitinib against colon cancer in animal models.

Discussion

Gefitinib and erlotinib are EGFR-TKIs commonly used in Asian patients with lung cancer. Mutations of EGFR tyrosine kinase have been shown to predict better response to TKIs as well as to improve the survival of lung cancer patients.[23,24] Phase I/II trials in patients with metastatic colon cancer showed little activity; but preclinical studies suggested a supra-additional growth inhibitory effect of gefitinib when combined with different cytotoxic drugs, which provided the rationale for several clinical trials of gefitinib in combination with chemotherapy in patients with metastatic colon cancer.[25,26] The important mutations associated with the activity of gefitinib are EGFR tyrosine kinase mutations in exons 18, 19, and 21[8,27]; however, this seems not to be the case in metastatic colon cancer, as these mutations are not so commonly found in colon cancer. It was reported that none of the 11 colon cancer cell lines examined have mutated EGFR, and only 12% of colon cancer patients have somatic mutations of EGFR according to a Japanese study.[28] It is worth exploring the molecular network to predict the response to gefitinib in further colon cancer therapy.

Spry2 is a well-known tumor suppressor in many human cancers, such as breast, lung, prostate, and liver cancers.[28–32] Our results also demonstrated that overexpression of Spry2 reduces cell proliferation, colony formation potential, and migration of colon cancer cells, as well as suppresses cancer growth *in vivo*, which reflects the role of Spry2 as a tumor suppressor in colon cancer (Feng *et al.*, unpublished manuscript, 2010). Spry2 can enhance the expression of EGFR through decreasing EGFR degradation in cancer cells. In HeLa cells, overexpression of Spry2 increases the total amount of PTEN but decreases the amount of phosphorylated PTEN.[33] The resultant increase in PTEN activity leads to decreased activation of AKT and downstream signaling. Interestingly, Spry2 is tightly connected to the EGFR pathway which inspired our investigation to define its role in gefitinib therapy. Our data show that the expression of Spry2 is associated with a better therapeutic effect of gefitinib in colon cancer cells. These results indicate that Spry2 could be a potential target to predict or manipulate gefitinib response for colon cancer. Because K-ras mutations are also important in predicting response to anti-EGFR treatment in colon cancer, it is mandatory to clarify the status of K-ras mutations in colon cancer cells. In the present study, all of the colon cancer cell lines tested, except HT29 and C2BBel cells, had K-ras mutations. HT29 cells which do not harbor K-ras mutation became more resistant to gefitinib after their endogenous Spry2 was suppressed by shRNA knockdown (Fig. 2b). However, silencing of K-ras in SW620 cells did affect their sensitivity to gefitinib

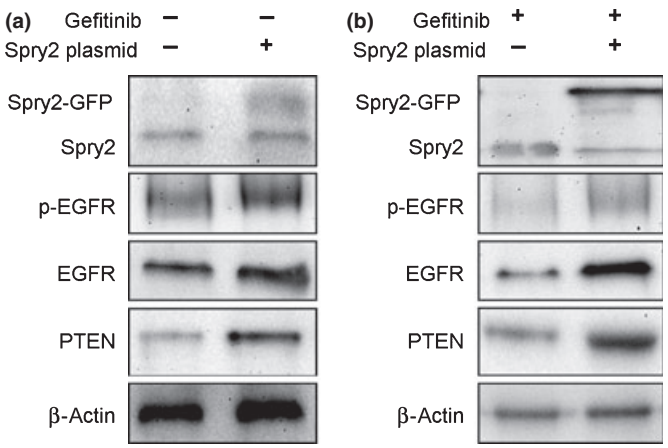


Fig. 3. Overexpression of Sprouty2 (Spry2) enhances the levels of phosphorylated and total epidermal growth factor receptor (EGFR) as well as phosphatase and tensin homolog (PTEN) in HCT116 colon cancer cells. (a) HCT116 cells were transfected with 5 μg of pSpry2-GFP-N3 plasmid, and total cell lysates were immunoblotted 48 h later for expression of phosphorylated EGFR (p-EGFR), total EGFR, PTEN, Spry2, and β-actin. Detection of Spry2-GFP and Spry2 proteins corresponds to the ectopic and endogenous expression of Spry2, respectively. (b) HCT116 cells were pretreated with 10 μM of gefitinib for 24 h after transfection with pSpry2-GFP-N3 plasmid. Total cell lysates were analyzed by immunoblotting after 48 h for expression of indicated proteins as described in (a).

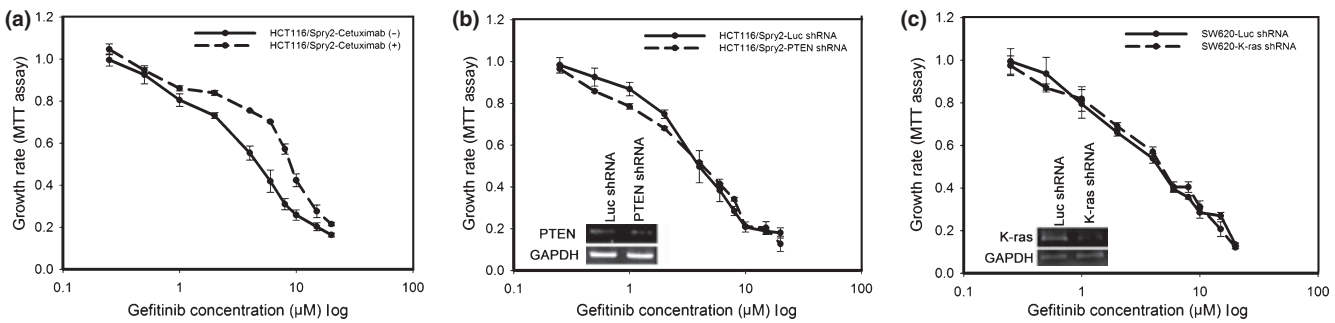


Fig. 4. Inhibition of epidermal growth factor receptor (EGFR) by cetuximab reduces the sensitivity of HCT116 colon cancer cells to gefitinib enhanced by Sprouty2 (Spry2). (a) HCT116/Spry2 cells that had been treated with or without 20 μg/mL of cetuximab for 24 h were exposed to different doses of gefitinib for 72 h. Cell viability was determined by the MTT assay. The IC50 values of gefitinib were 9.17 ± 2.00 μM in cetuximab-treated cells and 4.31 ± 0.49 μM in cells without cetuximab treatment. (b) HCT116/Spry2 cells were infected with lentiviral vectors encoding phosphatase and tensin homolog (PTEN) shRNA or Luc shRNA serving as the control for 24 h. Cells were then treated with different doses of gefitinib for 72 h. Cell viability was determined by the MTT assay. The IC50 values of gefitinib were 3.84 ± 0.68 μM for PTEN knockdown cells and 3.23 ± 0.24 μM for Luc knockdown cells. (c) SW620 cells were infected with lentiviral vectors encoding K-ras shRNA or Luc shRNA serving as the control for 24 h. Cells were then treated with different doses of gefitinib for 72 h. Cell viability was determined by the MTT assay. The IC50 values of gefitinib were 3.86 ± 0.71 μM for K-ras knockdown cells and 3.56 ± 0.76 μM for Luc knockdown cells. The efficiency of PTEN (b) and K-ras (c) knockdown was assessed by RT-PCR analysis for the expression of PTEN (b) and K-ras (c) as well as GAPDH serving as the loading control (b,c).

doi: 10.1111/j.1349-7006.2010.01637.x

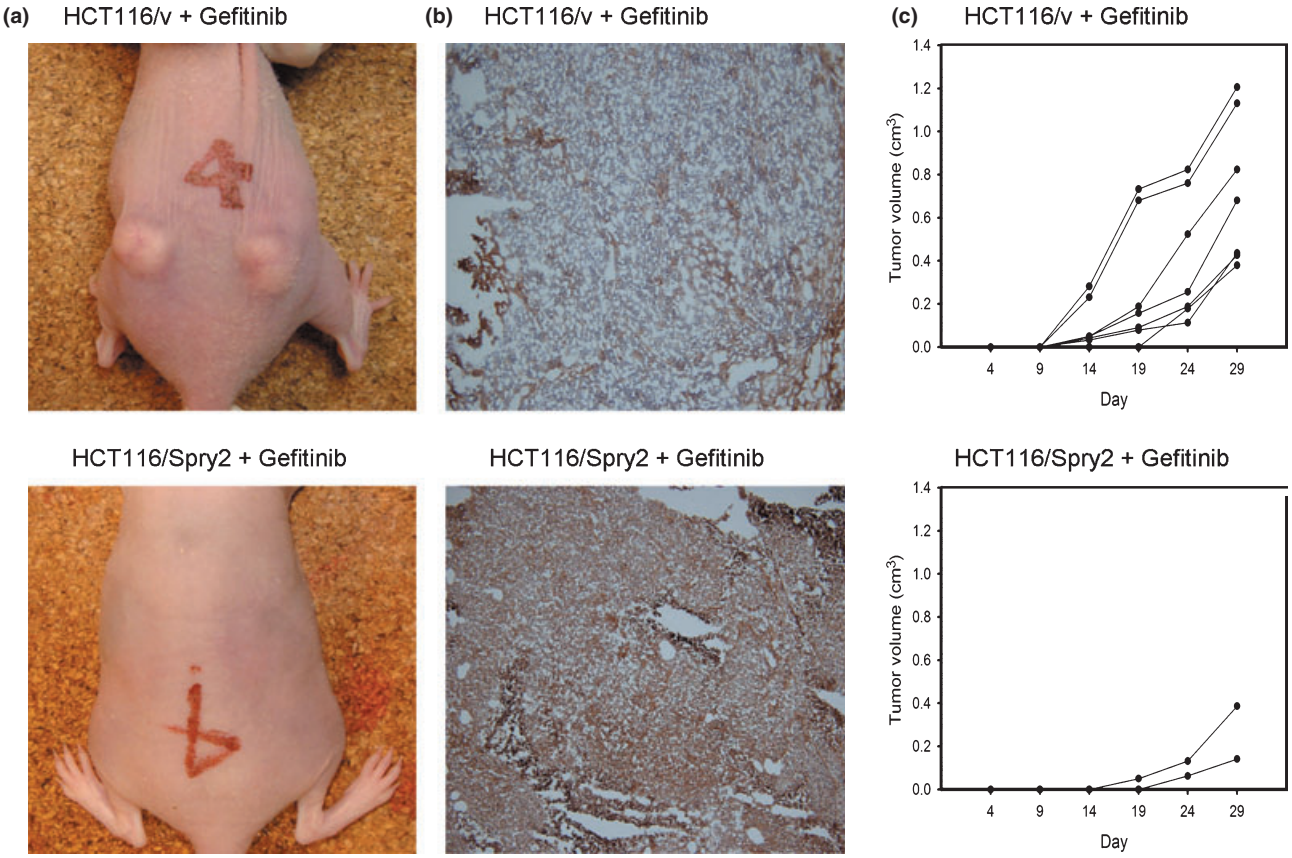


Fig. 5. Sprouty2 (Spry2) enhances the effect of gefitinib on suppressing colon cancer growth in a xenograft model. Groups of five nude mice were inoculated subcutaneously with HCT116/Spry2 or HCT116/v cells into both flanks with 2×10^6 cells/injection site. Mice were administered with gefitinib (50 mg/kg/day) by oral gavage at days 1–5 and 8–12. They were sacrificed at 29 days after tumor cell inoculation or when their tumors exceeded 17 mm in diameter. (a) Representative HCT116/v and HCT116/Spry2 tumor-bearing mice after gefitinib treatment at day 29. (b) Representative immunostaining of Spry2 in HCT116/v and HCT116/Spry2 tumor sections (original magnification, ×100). (c) Tumor volume was measured at indicated days from each implant. Seven of 10 implants from HCT116/v cells developed palpable tumors, whereas only two of 10 implants from HCT116/Spry2 cells formed tumor masses. HCT116/v tumors were significantly larger than HCT116/Spry2 tumors ($P = 0.006$).

(Fig. 4c). The findings of an insignificant role of K-ras in gefitinib treatment of colon cancer cells are consistent with the results from clinical trials in lung cancer.[34,35] This implies that Spry2 affects gefitinib sensitivity in a K-ras mutation-independent manner. Reduction of PTEN protein is associated with natural resistance to gefitinib,[36] and restoration of PTEN induces significant growth inhibition in gefitinib-resistant lung cancer cells.[37] Phosphatase and tensin homolog (PTEN) expression was enhanced after HCT116 cells were forced to express Spry2 in our study. However, we failed to show that knockdown of PTEN could reduce the effect of Spry2 on gefitinib sensitivity in colon cancer cells. Further investigations are warranted to define the association between PTEN and Spry2 in gefitinib treatment. Our failure to identify any association between K-ras mutation or PTEN expression and gefitinib sensitivity in colon cancer using the RNA interference approach does not exclude the possibility that such a correlation may exist.

In summary, we have found that the overexpression of Spry2 enhances the sensitivity of colon cancer cells to gefitinib *in vitro*. For further validation of this correlation, we have also shown that overexpression of Spry2 in HCT116 cells leads to dramatic decreases in both tumor incidence and size in comparison to the control HCT116 cells in nude mice. These results suggest that Spry2 may play a role in gefitinib sensitivity in addition to EGFR mutations. However, more clinical studies involving the correlation of expression of Spry2 and EGFR as well as gefitinib sensitivity in colon cancer are necessary to guide the clinical implications.

Acknowledgments

This work was supported by grants from National Cheng Kung University Hospital and Chi-Mei Medical Center, Liouying, Taiwan (CLFHR 9724 and CLFHR 9833).

Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. K-ras mutation of colon cancer cell lines. (a) Multiplex PCR and extension analysis of mutations in K-ras codons 12 and 13 were done in six colon cancer cell lines. A 289-bp fragment was detected in SW620, SW480, and LS174T cells, indicating the mutation of K-ras codon 12. HCT116 cells were found to have a 109-bp fragment representing K-ras codon 13 mutation. (b) DNA auto-sequencing of K-ras confirmed that HT29 and C2BBel cells harbored wild-type K-ras and that the remaining four cell lines had mutations in either codon 12 or 13. K-ras mutation analysis: We used multiplex PCR and primer extension analysis of mutation in K-ras codons 12 and 13 as previously described.[38,39] For direct sequencing of the *K-ras* gene, the extracted DNA was subjected to PCR amplification using forward 5′-CCT TAT GTG TGA CAT GTT CT-3′ and reverse 5′-GTC CTG CAC CAG TAA TAT GC-3′ primers. Subsequently, PCR fragments were purified using the PCR clean-up system (Promega, Madison, WI, USA) and subjected to cycle sequence reactions using BigDye Terminators (Applied Biosystems, Foster City, CA, USA). The sequence fragments were precipitated and analyzed using an automated sequencer (ABI 3130XL; Applied Biosystems).

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doi: 10.1111/j.1349-7006.2010.01637.x