EXPERIMENTAL Article

EGFR and HER2 levels are frequently elevated in colon cancer cells

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ABSTRACT

OBJECTIVE: Correlation between EGFR/HER2 protein levels and colon cancer prognosis has been suggested. However, reports on the prevalence of EGFR/HER2 overexpression have been divergent and inconclusive due to technical variations. The uncertainty of the prevalence greatly affects decisions on therapeutic interventions targeting both molecules. We aim at evaluating the prevalence and significance of EGFR/HER2 overexpression in colon cancer cell lines.

METHODS: We employed normal colon epithelial cell strain FHC and examined EGFR and HER2 levels in a series of colon carcinoma cell lines using Western blotting, and evaluated the effectiveness of siRNA targeting expression of the genes to inhibit the oncogenic properties of colon cancer cells.

RESULTS: We found that (1) as compared with normal colon epithelial cells, EGFR levels and HER2 levels were significantly increased in 7 of the 8 colon cancer cell lines examined (2) siRNA targeting EGFR or HER2 reduced colony formation in soft agar of SW480 colon cancer cells, and combined treatment further reduced the colony formation ability.

CONCLUSION: These results suggested that EGFR and/or HER2 elevation play an important role in the development of the *majority* of colon cancers, and targeting EGFR and/or HER2 could serve as an effective *common* strategy for therapeutic intervention or prevention of colon cancer.

Keywords: EGFR, HER2, Src, colon cancer, tumorigenicity, siRNA

INTRODUCTION

Colon cancer is the second leading cause of cancer death in North America. While surgery effectively removes colon cancers which are diagnosed at an early stage, relapse often occurs. In addition, over 20% of colon cancers are stage IV at the time of diagnosis. Identification of reliable tumor biomarkers and therapeutic targets is critically important in the prevention and treatment of colon cancer.

The epidermal growth factor (EGF) family of receptor tyrosine kinases consists of EGFR (ErbB1), HER2/Neu (ErbB2), ErbB3, and ErbB4. Of these members of the ErbB family, EGFR and HER2 have been implicated to play oncogenic roles in human tumors. EGFR and HER2 activate signaling

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pathways including Ras, PI3K, PLCγ, Src, mTOR, and STAT, which play important roles in cell proliferation and cell survival [1, 2], and therefore are crucial to the development of cancer. Gene amplification of the EGFR/HER2 has been detected in cancers [3]. Correlation between EGFR/HER2 protein levels and colon cancer prognosis has been suggested [4, 5]. However, reports on the prevalence of EGFR/HER2 overexpression have been divergent and inconclusive due to technical variations.

Src kinase activity was reported to be elevated in a high percentage of colon cancer cell lines and tissues [6-8]. Elevated Src kinase activity was associated with colon cancer tumorigenicity [9, 10]. Through phosphorylating substrates, the best-known of which include FAK, Ras, EGFR, and STAT3, signaling of Src in cancer cells affects cell proliferation, survival, adhesion, and motility.

Our goal was to evaluate the prevalence and significance of EGFR/HER2 overexpression in colon cancer. We employed normal colon epithelial cell strain FHC and examined EGFR and HER2 levels in a group of colon carcinoma cell lines using Western blotting. We found that as compared with normal colon epithelial cells, either EGFR or HER2 levels were significantly increased in 7 of the 8 colon cancer cell lines examined, and siRNAs targeting EGFR and HER2 could effectively inhibit the oncogenic properties of colon cancer cells. These results suggest that EGFR and/or HER2 elevation play an important role in the development of *majority* of colon cancers, and targeting EGFR and/or HER2 could serve as an effective, *common* strategy for therapeutic intervention or prevention of colon cancer.

MATERIALS AND METHODS

Cell culture

SW48, SW480, DLD-1, HCT 116, and HT-29 from The American Type Culture Collection (ATCC, USA) were grown in DMEM with 10% FBS. COLO 201 (ATCC) was grown in DMEM (ATCC) with 10% FBS (ATCC) and 1 mM sodium pyruvate (ATCC). Caco-2, LS 174T and CCD-18Co (ATCC) were grown in MEM (ATCC) with 10% FBS and 1 mM sodium pyruvate. FHC (ATCC) was grown in the growth medium according to ATCC.

Cell lysis and immunoblotting

Cell lysis and immunoblotting was as described [11]. Anti-Src monoclonal antibodies MAb2-17 (Catalogue # Ab7950), tubulin monoclonal antibody (Catalogue # Ab4074) were from Abcam Biochemicals, USA. Polyclonal anti-HER2 (C-18, Catalogue # SC-284,) was from Santa Cruz Biotechnology, USA. Monoclonal anti-EGFR (H9B4, Catalogue # 44-798G, Life Technologies, USA) has been described previously [12].

Transfection of siRNA

siRNA duplexes of human EGFR (sense, 5'-GCAGAGGAAUUAUGAUCUUUU -3'; antisense, 5'-AAGAUCAUAAUUCCUCUGCAC -3'), HER2 (sense, 5'- GGUGAAGGUGCUUGGAUCUUU -3'; antisense, 5'- AGAUCCAAGCACCUUCACCUU -3'), Src (sense, 5'-UUCGGAGGCUUCAACUCCUdTdT -3'; antisense, 5'-AGGAGUUGAAGCCUCCGAAdTdT -3') were synthesized in the DNA facility of University of Calgary. siRNA duplexes of negative control (sense, 5'-UUCUCCGAACGUGUCACGUdTdT-3'; antisense, 5'-ACGUGACACGUUCGGAGAAdTdT-3') were designed by Qiagen, and the single-stranded sequences were synthesized in the DNA facility of University of Calgary. Double-stranded siRNA was prepared by heating the complementary single strands together at 90ºC for 1 min then 37ºC for 2 h, and then stored in -80ºC.

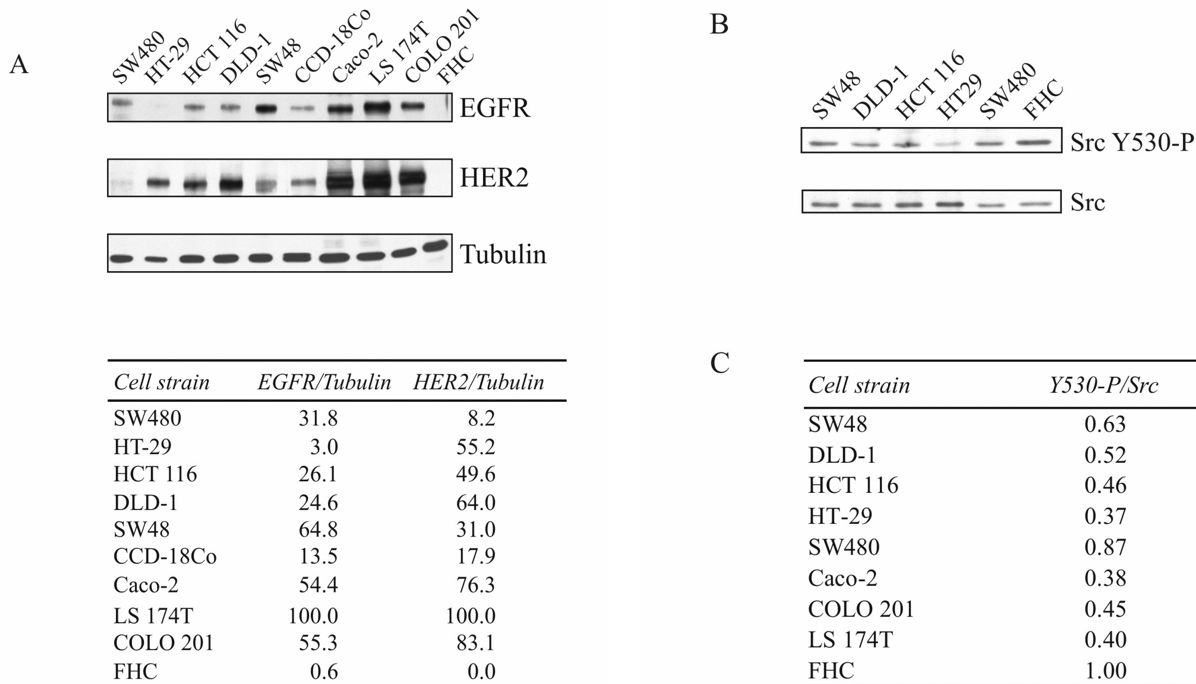
Cells grown in dishes at approximately 50% confluence were transiently transfected with 200 nM siRNA unless stated otherwise, using Oligofectamine (Life Technologies) as per the manufacturer's instructions. Cells were lysed 48 h later for western blotting.

Cell viability assay

Cell viability was assessed by MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation assay, Promega). Briefly, 15%-confluence cells transfected with siRNAs were grown for 48 h. Then medium were replaced with 100 µl of fresh growth medium and 20 µl of MTS were added to each well, followed by a further incubation of 45min at 37°C. Plates were analyzed on a microplate reader at 490 nm.

Soft agar colony assay

2×10^3 trypsinized and separated cells were added to 3 ml of DMEM medium containing 10% FBS, antibiotics, and 0.3% agarose. After briefly vortexing, the cells were plated on 60 mm dishes



Cell strain	*EGFR/Tubulin*	*HER2/Tubulin*
SW480	31.8	8.2
HT-29	3.0	55.2
HCT 116	26.1	49.6
DLD-1	24.6	64.0
SW48	64.8	31.0
CCD-18Co	13.5	17.9
Caco-2	54.4	76.3
LS 174T	100.0	100.0
COLO 201	55.3	83.1
FHC	0.6	0.0

Cell strain	*Y530-P/Src*
SW48	0.63
DLD-1	0.52
HCT 116	0.46
HT-29	0.37
SW480	0.87
Caco-2	0.38
COLO 201	0.45
LS 174T	0.40
FHC	1.00

Figure 1. Levels of EGFR and HER2 in human colon cancer cells
20 µg of protein lysates were separated by SDS-PAGE and analyzed by western blotting with indicated antibodies. Representative blots are shown (A, upper panel). EGFR, HER2, and tubulin protein amounts were measured using quantitative scanning of individual bands from Western blots, and levels of EGFR and HER2 were demonstrated by protein amounts of EGFR and HER2 corrected for that of tubulin (EGFR/tubulin, HER2/tubulin), and compared to the normal colon epithelial cell FHC (A, lower panel). Cell lysates were also separated by SDS-PAGE in duplicates and analyzed by western blotting with MAb2-17 antibody and Y530-P antibody. Representative blots are shown (B). Levels of Src, and Src530-P were measured using quantitative scanning of individual bands from Western blots, and then Src activation level was demonstrated by Y530 phosphorylation of Src corrected for Src protein levels (Y530-P/Src), and compared to the normal colon epithelial cell FHC (C).

over a 3 ml bottom layer of pre-hardened 0.5% agarose medium. Additional 0.3% agarose medium was added every 3 days. The cells were maintained at 37°C in a 5% CO_2 incubator for 9 days and the colonies containing over 100 cells were counted with an inverted microscope. At least 100 colonies appeared on each soft agar plate transfected with Control scrambled siRNA.

RESULTS AND DISCUSSION

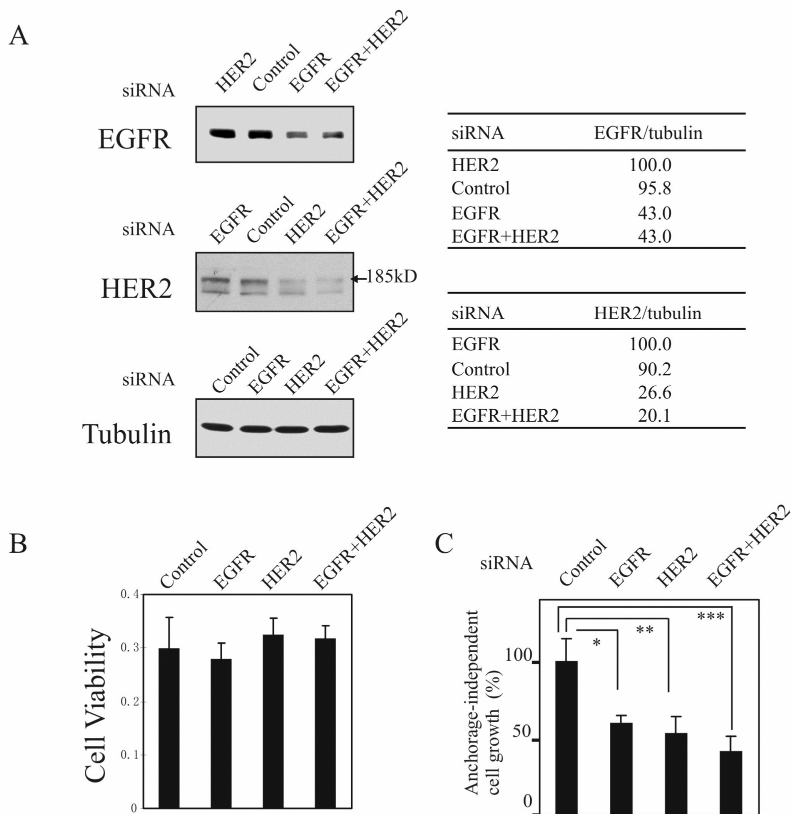
EGFR and HER2 protein levels were significantly increased in a high percentage of colon cancer cell lines

We examined protein levels of EGFR and HER2 in a panel of eight randomly chosen colon cancer cell lines. Compared to normal colon epithelial primary cells FHC, EGFR protein levels were increased greatly in all the colon carcinoma cell lines except HT-29 (Figure 1A). Similarly, HER2 protein levels in 7 of the 8 colon cancer cell lines were greatly increased as compared with FHC cells. The increased protein levels were most notable in Caco-2, LS 174T, and COLO 201 cells. In SW480 cells, the HER2 level was also slightly increased. Our data suggest that elevation of EGFR and HER2 was a frequent occurrence in colon cancer. As a downstream target of EGFR and HER2, Src activation level represented by Y530 phosphorylation of Src per Src protein molecule (Y530-P/Src), was also enhanced compared to the normal colon epithelial cell FHC (Figure 1B and 1C), indicating that EGFR and HER2 signaling were elevated.

Reports on overexpression of EGFR have been divergent and EGFR appears to be expressed at varying levels in normal colon tissues as well as colon cancer tissues [13, 14]. Similarly, overexpression of the HER2 protein has been

reported in colon cancer samples, more often in low prevalence, but varies greatly, ranging from 4% to 83% [15, 16]. The reason for the divergence of results with colon cancer is unclear but has been attributed to the use of different research techniques by groups with different "home brews" of reagents on samples processed in different ways [14]. Our results indicate that both EGFR and HER2 protein levels were significantly increased in a *high* percentage of colon cancer cell lines, suggesting that this may be a *common* mechanism for the development of colon cancer. The difference between our results and some other results is likely due to the difference in methodologies used. Immunohistochemistry (IHC) staining has been the predominant method used for examining EGFR and HER2 levels. Ross et al. has reported that IHC can be significantly affected by technical issues, especially in archival fixed paraffin-embedded tissues [15]. Tissue fixative, specific combinations of antibodies and fixatives, prolonged storage and non-standardized antigen retrieval all have significant impacts on sensitivity and specificity of HER2 immunostaining [15].

In comparison to IHC, immunoblotting detects EGFR or HER2 protein more specifically, and is not as affected by reagents and samples. However, comparing colon tissues (normal vs. cancer) using western blotting also has its drawbacks, since colon carcinoma predominantly consists of epithelial cells, while normal colon tissue consists of multiple cell types besides epithelial cells that give rise to carcinoma. Examining epithelial normal and cancer cells in this report was made possible by the rare availability of a normal epithelial cell strain – FHC. This examination also eliminates the possibility of comparing different cell types in normal and cancer colon tissues, especially since EGFR and HER2 are expressed at much higher levels in normal colon fibroblasts (CCD-18Co) than normal colon epithelial cells (Figure 1). If compared with CCD-18Co cells, protein levels of EGFR in only four colon cancer cell lines including SW48, Caco-2, LS 174T, and COLO 201 were significantly increased, while those in the other three colon cancer cell lines including SW480, HCT 116 and DLD-1 were not changed significantly. HT-29 even displayed a lower level of EGFR as compared with CCD-18Co cells. Similarly, if compared with CCD-18Co, HER2 protein levels in only 4 of the 8 colon cancer cell lines (DLD-1, Caco-2, LS 174T, and COLO 201) were significantly increased.



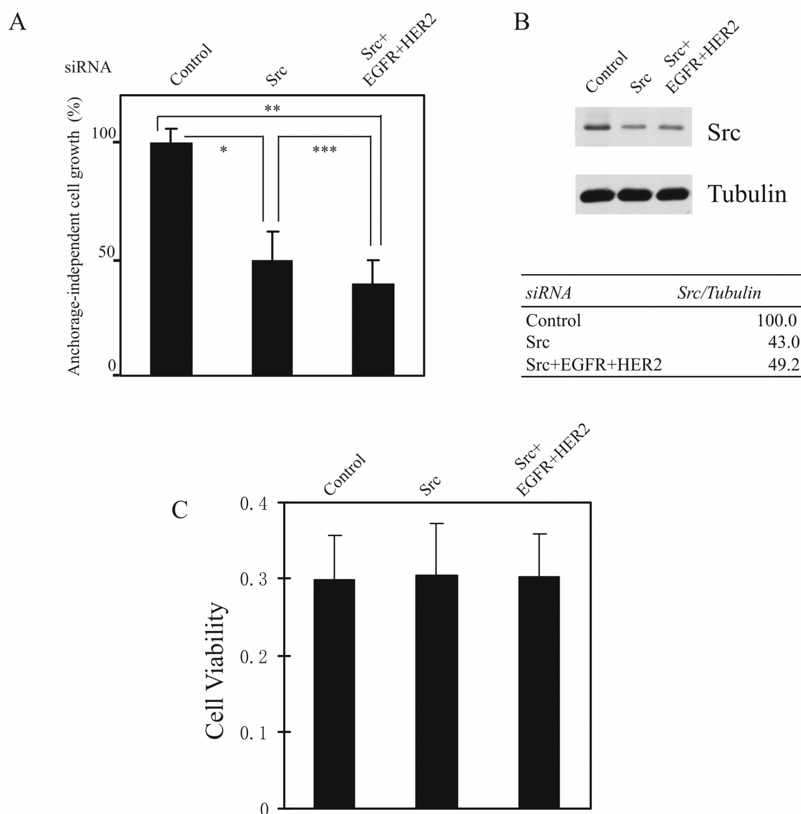
siRNA	EGFR/tubulin
HER2	100.0
Control	95.8
EGFR	43.0
EGFR+HER2	43.0

siRNA	HER2/tubulin
EGFR	100.0
Control	90.2
HER2	26.6
EGFR+HER2	20.1

Figure 2. Suppression of colony formation in soft agar by EGFR/HER2 siRNAs. SW480 cells were transfected with 200 nM siRNAs individual or combined (EGFR 100 nmole +HER2 100 nmole) as indicated**.** After 24 h, one set of the cells was trypsinized and seeded in soft agar medium. The number of colonies formed in soft agar after 9 days was counted and expressed relative to Control (C). Cell viability was assessed by MTS assay after cells had been transfected with siRNAs for 48 h (B). At 48 h after transfection, another set of the cells was lysed and 20 μg of protein lysates were separated on SDS-PAGE for Western blot analysis with indicated antibodies. Representative blots are shown (A). EGFR, HER2, and tubulin protein amounts were measured using quantitative scanning of individual bands from Western blots, and levels of EGFR and HER2 were demonstrated by protein amounts of EGFR and HER2 corrected for that of tubulin (EGFR/tubulin, HER2/tubulin). *,**, ***, $P < 0.05$, unpaired, one-tailed t test.

siRNA targeting EGFR or HER2 reduced anchorage-independent growth of colon cancer cells

EGFR or HER2 signaling initiates intracellular signal transduction cascades by recruiting/activating Ras, PI3K, and PLCγ promoting cell proliferation, survival and other properties of cancer cells. Since EGFR and HER2 protein levels were increased in many colon cancer cell lines, targeting either of the two signaling molecules could be a common strategy to reduce tumorigenicity in colon cancers. EGFR siRNA knocked down EGFR protein levels by over 60% in SW480 colon cancer cells, and caused a reduction of approximately 40% in the anchorage-independent cell growth, a characteristic of transformed or cancer cells that is highly correlated with tumorigenicity [17] (Figure 2). HER2 protein (upper band, 185 kDa) levels in SW480 were knocked down by over 70%, and the anchorage-independent cell growth of SW480 cells were reduced by 47% (Figure 2). (The lower molecular weight band (about 155 kDa) is assumed to be an immuno-cross-reacting protein or a precursor of the 185 kDa HER2 protein [18]). This result demonstrated that both EGFR and HER2 could be effective targets to decrease colon cancer tumorigenicity. In order to prevent potential mutual compensation between EGFR and HER2 [19, 20], we also co-transfected SW480 cells with siRNAs targeting both EGFR and HER2. Results show that although neither EGFR nor HER2 level was affected by specific knocking down of the other, the colony formation ability was slightly further decreased by up to 60% in the presence of both siRNAs (Figure 2), suggesting partial additive effects of the two siRNAs in reducing tumorigenicity. This is consistent with recent preclinical use of drugs in breast cancer cells, which supports that dual inhibition of EGFR/HER2 enhances anti-tumor activity [21]. siRNA targeting was more specific in downregulating EGFR and HER2 signaling than many clinical drugs. On the other hand, it is interesting that the tumorigenicity was not more substantially reduced when both EGFR and HER2 were targeted (Figure 2). EGFR and HER2 form heterodimers leading to autophosphorylation and downstream signaling cascades [22]. It is possible that the remaining levels of EGFR and HER2 after knock down were sufficient to perform the synergistically pro-oncogenic role, as early work showed that minimal dimer formation is sufficient for enzyme activation [23]. It's also possible that the merely slight further reduction in tumorigenicity by dual targeting was



siRNA	*Src/Tubulin*
Control	100.0
Src	43.0
Src+EGFR+HER2	49.2

Figure 3. Suppression of colony formation in soft agar by Src and EGFR/HER2 siRNAs. SW480 cells were transfected with 300 nM siRNAs. After 24 h, one set of the cells was trypsinized and seeded in soft agar medium. The number of colonies formed in soft agar after 9 days was counted and expressed relative to Control (A). Cell viability was assessed by MTS assay after cells had been transfected with siRNAs for 48 h (C). At 48 h after transfection, another set of the cells was lysed and 20 μg of protein lysates were separated on SDS-PAGE for Western blot analysis with indicated antibodies; protein bands were measured using quantitative scanning of individual bands from Western blots, and then Src level was demonstrated by amount of Src band corrected for that of tubulin band (Src/tubulin), and compared to the Control (B). Control, 300 nM control siRNA; Src, 100 nM Src siRNA + 200 nM control; Src+EGFR+HER2, 100 nM Src siRNA + 100 nM EGFR siRNA + 100 nM HER2 siRNA. *,**, $P < 0.001$, ***, $P < 0.05$, unpaired, one-tailed t test.

due to that EGFR and HER2 overlap in the same signaling pathway to a great extent, in presence of other parallel pathways which are also important for tumorigenicity.

Effects of Src siRNA and combined siRNAs of Src and EGFR/HER2

Src kinase activity has been reported to play an important role in colon cancer samples and cell lines [6-8].

When Src levels were knocked down in SW480 cells (~60%, Figure 3B), the anchorage-independent cell growth was also decreased correspondingly, as reflected by approximately 50% reduction in the colony formation (Figure 3A). EGFR and HER2 were knocked down at the same level as in Figure 2 (data not shown). Combination of siRNAs for Src with siRNAs for EGFR and HER2 further reduced the colony formation by approximately 10% (Figure 3). However, the effect of this combination of siRNAs on the colony formation appeared to be similar to that of the combination of siRNAs targeting EGFR and HER2 (Figure 2). This is likely due to the fact that EGFR/HER2 signaling and Src signaling overlap to a great extent - EGFR and HER2 recruit Src and activate Src signaling pathways and other signaling pathways including Ras, PI3K, and STATs; Src phosphorylates and activates EGFR/HER2 as well as other substrates including Ras, caspase 8, and STAT3. A cooperation between EGFR and Src was recently reported in the in vitro cell proliferation of a lung cancer cell line [24]. The difference in results is probably due to the difference in the cellular contexts, and difference between anchorage-independent cell growth and cell proliferation. As in Figure 2, we have not observed mutual effects on expression levels between Src and EGFR/HER2 whether using siRNA separately or in combination (data not shown).

Cells overexpressing EGFR appear to be more sensitive to EGFR inhibition than cells expressing normal levels of EGFR [25]. Therefore, our observation that both EGFR and HER2 were overexpressed in a *high* percentage of colon cancer cell lines suggested that targeting EGFR and/or HER2 could be an *common* strategy in colon cancer treatment. It is also expected that targeting of EGFR/HER2 in conjunction with other key pathways may be more beneficial in therapeutic prevention and intervention of colon cancer [26].

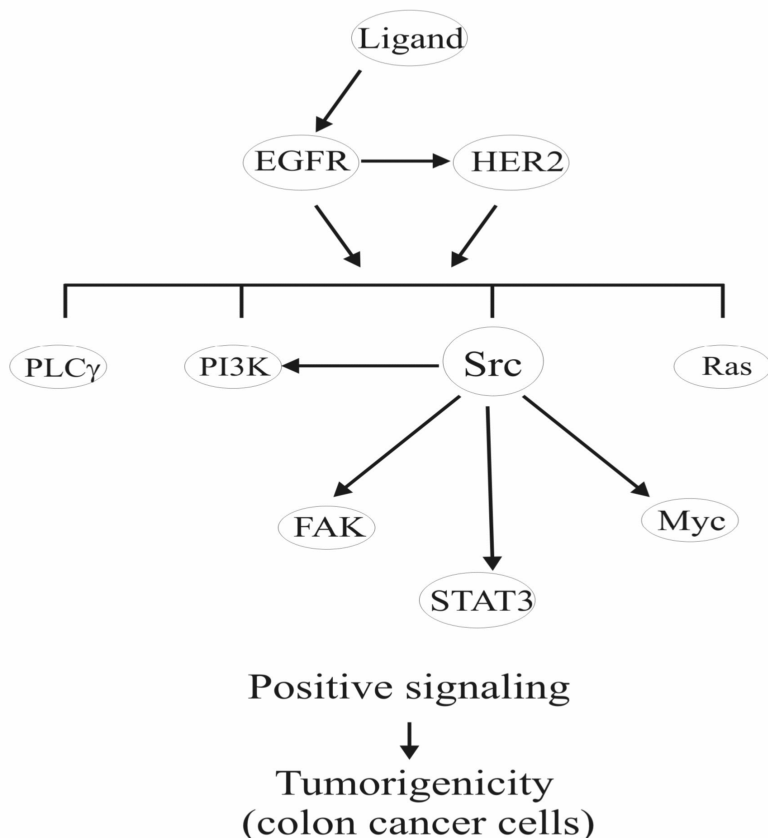


Figure 4. Cooperation of EGFR, HER2 and Src in colon cancer cells.
Upon stimuli to EGFR, EGFR and HER2 become activated, leading to subsequent activation of PLCγ, PI3K, Ras, and Src, which further enhances PI3K signaling, to initiates positive signaling pathways [1, 2]. We show that EGFR cooperates with HER2 and Src in promoting anchorage-indepen-dent cell growth of colon cancer cells. Signaling affecting anchorage-independant cell growth may play a significant role in contributing to the tumorigenicuity of colon cancer cells.

Through employing appropriate normal colon epithelial cell control, we found that EGFR and HER2 protein levels were significantly increased in 7 of the 8 colon cancer cell lines examined, suggesting that EGFR and/or HER2 elevation play an important role in the development of a *high percentage* of colon cancers. Thus, targeting EGFR and/or HER2 could serve as an effective *common* strategy for therapeutic intervention or prevention in colon cancer.

CONCLUSIONS

In summary, the current study demonstrates that EGFR and/or HER2 were elevated in a *high* percentage of colon cancer cell lines, and suggests that EGFR and/or HER2 elevation play important roles in the development of the *majority* of colon cancers. Therefore, targeting EGFR and/or HER2 could serve as an effective *common* strategy for therapeutic intervention or prevention of colon cancer.

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Conflict of interest

The authors have declared no conflict of interest

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