The Effect of Combining Interferon-α and Gefitinib in Human Colon Cancer Cell Lines
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Background and Aims: Interferon-α (IFN-α) treatment is associated with up-regulation of epidermal growth factor receptor (EGFR) expression and marked growth inhibition of colon cancer cell lines. We aimed to determine the effect of combining IFN-α and gefitinib in the growth of human colon cancer cell lines.

Methods: Two human colon cancer cell lines SW480 and LOVO were treated with IFN-α alone or gefitinib alone or IFN-α plus gefitinib. Proliferation of colon cancer cells was measured by methyl thiazolyl tetrazolium (MTT) assay; the apoptosis rate was analysed by flow cytometry (FCM). The expression of XIAP, XAF1 mRNA was detected by RT-PCR and the expression of XIAP, XAF1 protein was detected by western blotting.

Results: Methyl thiazolyl tetrazolium showed that IFN-α, gefitinib and IFN-α plus gefitinib significantly inhibited SW480 and LOVO cells in a dose-dependent manner (p < 0.05). The FCM revealed that IFN-α, gefitinib and IFN-α plus gefitinib could markedly upgrade the apoptosis rate (p < 0.05). The expression of XIAP mRNA down-regulated markedly (p < 0.05) while the expression of XAF1 mRNA up-regulated significantly (p < 0.05). The expression of XIAP protein was down-regulated markedly (p < 0.05) while the expression of XAF1 protein was up-regulated significantly (p < 0.05).

Conclusion: IFN-α promotes the antiproliferative effect of gefitinib on human colon cancer cell lines and the mechanism may be related to up-regulation expression of EGFR by IFN-α.

Keywords: Apoptosis, gefitinib, human colon cancer cells, Interferon-α
INTRODUCTION
Colorectal cancer (CRC) is one of the most common malignant tumours in the world and its prevalence in China has been steadily increasing in recent years in association with changes in lifestyle and diet. Extensive molecular analyses have established that the development and progression of CRC involves multiple genetic and epigenetic alterations (1). Tumorigenesis is a loss of the balance between regulators of cell proliferation and apoptosis. Among the regulators of apoptosis, an evolutionary conserved gene family of inhibitors-of-apoptosis proteins (IAP) has been identified and implicated in caspase inhibition. Inhibitors-of-apoptosis proteins restrain cancer cell death through a mechanism involving the inhibition of the effectors caspase-3, -7 and -9 (2). The X-linked IAP is the most potent member of the IAP family and functions by direct binding and inhibition of the three caspases just mentioned (3). Recently, XIAP-associated factor 1 (XAF1) was identified as negatively regulating the caspase-inhibiting activity of XIAP. XAF1 mRNA is ubiquitously expressed in all embryonic tissue and normal adult tissues, but at low or undetectable levels in many different cancer cell lines (4). XAF1 shows priority binding with the BIR2 structure of XIAP, which indicates that it may target IAP/caspase-3 or -7. Expression of XAF1 triggers a redistribution of XIAP from the cytosol to the nucleus and thus functions as a pro-apoptotic protein (5–6). XAF1 has been defined as an IFN-stimulating gene with its expression being inducible by IFN. IFN induces XAF1 expression through either the interaction with the interferon regulatory factor1-binding element (IRF-E) or demethylation of the CpG sites within XAF1 promoter (7).

IFN-α is widely used in treatments against virus-induced diseases; it has also been used in cancer therapy for more than four decades. Multiple mechanisms are likely contributing to the anti-tumour activities of IFNs. These include modulation of immune response, anti-angiogenic activities and direct anti-proliferative and/or apoptotic effects on tumour cells (8). IFN-α activates several signalling pathways; most signalling pathways activated by anticancer drugs ultimately result in activation of caspases, a family of cysteine proteases that act as common death effector molecules in various forms of cell death (9).

Accumulating evidence suggests that the development and progression of many malignancies, including colorectal cancer, are associated with constitutive activation of multiple signalling pathways that promote proliferation, inhibit apoptosis and induce metastasis (10). A large body of evidence suggests that EGFR and/or its family members, specifically ErbB-2/HER-2 and ErbB-3/HER-3 play a crucial role in regulating several pathways that affect tumour cell survival, angiogenesis, motility and invasiveness (11).

The epidermal growth factor receptor (EGFR) is a member of the family of transmembrane protein kinase receptors known as the erbB or HER receptor family. When activated EGFR phosphorylates and activates other intracellular proteins that affect cell signalling pathways, cellular proliferation and the control of apoptosis and angiogenesis. The EGFR is over-expressed in most gastrointestinal (GI) malignancies, and while data are not entirely consistent, EGFR over-expression often confers a poor prognosis (12). EGFR expression ranges from 72 to 82% in metastatic colorectal cancer (mCRC), 40–70% in oesophageal cancer (EC) and 30–60% in gastric cancer (GC) and is up to 90% in pancreatic cancer (PC). Therefore, due to its presence and over-expression in most tumour types, EGFR is a tempting target in GI malignancies.

Different approaches targeting the EGFR pathway are in development, but only two modalities have entered clinical trials in GI malignancies (13): low-weight molecule inhibitors of the intracellular kinase domain of EGFR (tyrosine kinase inhibitors, TKIs) and monoclonal antibodies (MAbs) designed to block the extracellular ligand-binding domain of EGFR.

Based on the hypothesis that IFN-α might improve the efficiency of EGFR-targeted treatment (14), this study aimed to determine whether combining gefitinib (an EGFR inhibitor) with IFN-α increases the anti-tumour effect over either treatment alone.
MATERIALS AND METHODS

Cell lines and cell culture
Human colon cancer LOVO and SW480 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). LOVO cells were maintained in minimum essential medium (Gibco Grand Island, NY, USA) and SW480 cells were maintained in RPMI 1640 medium (Gibco Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 100U/ml streptomycin and 100U/ml penicillin in a humidified incubator at 37°C with an atmosphere of 95% air and 5% CO₂.

Growth inhibition assay
Inhibition of cell growth in response to IFN-α (PBL Interferon Source, USA) and/or gefitinib (Tocris Bioscience, USA) was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were dispersed by trypsin-EDTA treatment and 2.5 × 10⁴ cells/ml re-suspended in medium and seeded into 96-wells culture plates with 5 replicates. After 24 hours of plating, incubation was continued for another 72 hours in the absence (control) or presence of IFN-α, gefitinib. At the end of the 72-hour incubation period, the reaction was terminated by adding 20 μl of 5 mg/ml stock of MTT to each well. The reaction was allowed to proceed for 4 hours at 37°C. The formazan crystals were then dissolved by adding 0.1 ml of dimethyl sulfoxide (DMSO). The intensity of the colour developed, which is the reflection of the number of live cells, was measured at a wave length of 570 nm. All values were compared to the corresponding controls. All assays were performed with 5 replicates.

Annexin V-FITC assay
The apoptotic cells were identified based on the binding of FITC-conjugated Annexin V to cell surface-exposed phosphatidylserine (PS), and the exclusion of PI (15). Briefly, cells were treated with IFN-α and gefitinib for 72 hours, then harvested, washed and incubated for 10 minutes at room temperature in the dark in 1×Binding Buffer containing PI and Annexin V-FITC (BD Bioscience Pharmingen, San Jose, CA, USA). After addition of 4 volumes of binding buffer, cells were immediately analysed by flow cytometry.

Western blotting analysis
Western blotting analysis was performed according to standard protocol (16). The cells were solubilized in RIPA lysis buffer phenylmethylsulfonyl fluoride (PMSF). Following clarification at 10 000 g for 15 minutes, the supernatant was used for western blot analysis. GAPDH, Goat anti-human XAF1, Goat anti-human XIAP, HRP-conjugated anti-goat IgG were purchased from Santa Cruz Biotechnology (CA, USA). All western blottings were performed three times for each experiment. Densitometric measurements of the scanned bands were performed using the digitized scientific software program UN-SCNAT. Data were normalized to GAPDH.

Reverse transcription–polymerase chain reaction (RT-PCR)
Total RNA was extracted from cells using TRizol Reagent (Invitrogen). Polymerase chain reaction (PCR) was performed using 2 μl of resulting cDNA, 0.5 U Hotstart DNA polymerase, primers and deoxynucleoside triphosphates in a final volume of 25 μl. The primer sequences were as follows: XAF1, forward: 5’-GAGCTCCACGAGTCCTACTG-3’, reverse: 5’-AAACTCTGAGCTGGACAA-3’ (product size: 238 bp); XIAP, forward: 5’-CAACACTGGCAGGAGCGGTT-3’, reverse: 5’-ACATGGCAGGGTTCTCGG-3’ (product size: 348 bp); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward: 5’-GACCACAGTCCATGCCATCAC-3’, reverse: 5’-TCCACACCCTGTGTTGTA-3’ (product size: 453 bp). Hot-start PCR was performed for 30 cycles with 95°C denaturation for 30 minutes (first cycle), 94°C denaturation for 45 seconds (s) 55°C annealing for 30 seconds and 72°C elongation for 30 seconds and then 10 minutes (final cycle). Amplified PCR products were electrophoresed onto 1.5% agarose gels and visualised by 1.0% ethidium bromide (EB) staining. The results of electrophoresis were analysed by the Gel Image System Version 3.73 (Bio-Tanon Corp, Shanghai, China).

Statistical analysis
Data are represented as the mean ± SEM. Statistics were done by SPSS programme 10.5 versions. The statistical significance of differential findings between experimental groups and control groups was determined by unpaired t test (one way ANOVA). Each experiment included at least triplicate measurements for each condition tested. p < 0.05 was considered statistically significant.

RESULT
The effect of INF-α and/or gefitinib on cell proliferation
We studied the effect of INF-α, gefitinib on the proliferation of SW480 and LOVO cells. INF-α inhibited cell proliferation in both cell lines in a dose-dependent manner (Figs. 1a, 2a). Gefitinib inhibited cell proliferation in a dose-dependent manner in both cell lines (Figs. 1b, 2b). The effect of the combination of the tested agents on SW480 and LOVO proliferation was also studied (Figs. 2c). To avoid an eclipse effect, we chose lower doses for combining INF-α (50 IU/ml) with gefitinib (1.0 µM/L). Combining INF-α and gefitinib had additional effect on both SW480 and LOVO cells.
The effect of IFN-α and/or gefitinib on cell apoptosis
SW480 and LOVO cells were treated with the tested agents at low dose concentrations and effect on cell proliferation noted. Apoptosis was evaluated by the Annexin V binding assay 72 hours later (Figs. 3a, 3b, 3c).

Fig. 1: The effect of INF-α (a) and gefitinib (b) on SW480 proliferation. Different doses of INF-α and gefitinib were applied on SW480 cells. Results are expressed as mean ± SEM of the survival rate of cells. The letter 'a' denotes a statistically significant difference compared to untreated cells. \( p < 0.05 \).

Fig. 2: The effect of IFN-α (a) and gefitinib (b) on LOVO proliferation, and their combination (c) on SW480 and LOVO proliferation. Different doses of IFN-α and gefitinib were applied on SW480 cells. Results are expressed as mean ± SEM of the survival rate of cells. The letter 'a' denotes a statistically significant difference compared to untreated cells. \( p < 0.05 \).

Fig. 3a: Effect of IFN-α and gefitinib on apoptosis of SW480 cells.

Fig. 3b: Untreated LOVO cells; G: LOVO cells treated with 1.0 µM/L gefitinib; I: LOVO cells treated with 50 IU/ml IFN-α; I+G: LOVO cells treated with 1.0 µM/L gefitinib and 50 IU/ml IFN-α.
The present study sought to investigate the effect of IFN-α, gefitinib on two human colon cancer cell lines, SW480 and LOVO, by evaluating changes in cell proliferation, apoptosis, and expression of XIAP and XAF1. After using IFN-α, gefitinib and IFN-α plus gefitinib, the SW480 and LOVO cells growth were significantly inhibited in a dose-dependent way, and the apoptosis rate markedly increased; the expression of XIAP mRNA and XIAP protein down-regulated markedly, while the expression of XAF1 mRNA and XAF1 protein up-regulated significantly. The effect of combining Interferon-α and gefitinib is better than either alone in human colon cancer cell lines. It means that IFN-α can promote the anti-proliferative effect of gefitinib on human colon cancer cell lines, and the mechanism may be related to up-regulation of EGFR by IFN-α (9, 14).

Gefitinib decreased cell proliferation in a dose-dependent manner and increased apoptosis in colon cancer cells. Previous studies have shown that gefitinib induces apoptosis in PCa, a prostate cancer cell line by down-regulating the PI3K pathway through PTEN expression. It is known that EGFR can activate three critical signalling cascades: the Ras-Raf-MEK-ERK pathway, PI3K-Akt pathway and the JAK-STAT pathway (17). Inhibition of these pathways results in a decrease in cell proliferation and an increase in apoptosis, respectively.

IFN-α binds to cell membrane receptors, IFN-α R1 and IFN-α R2 (18), leading to activation of IFN-α-associated Janus kinases (JAK), Jak1 and Tyk2. These are non-receptor tyrosine kinases that mediate cytokine-induced signal transduction. The activated JAKs phosphorylate and activate cytoplasmic signalling proteins including STAT (signalling transducers and activators of transcription) factors (19). IFN-α via the JAK-STAT pathway regulates target gene expression leading to cell growth inhibition. This same pathway is
involved in the mediation of downstream signalling by EGF and is enhanced in cells over-expressing EGF (20), raising the potential of a downstream interaction between the two as a mechanism for the enhanced anti-proliferative activity (21).

In the current search for new therapeutic targets in cancer, growth factors and their receptors represent a field of active investigation both at preclinical and clinical levels. The EGFR receptor is significantly overexpressed in solid tumours and constitutes an important target for the development of the new targeted anti-cancer drugs. There are two different categories of compounds in the current area of active drugs targeting EGFR with monoclonal antibodies and TKIs. Gefitinib is a small molecule that binds competitively to the ATP-binding pocket of the HER1/EGFR tyrosine kinases, thus inhibiting phosphorylation of the receptor. The inhibitory effect of gefitinib has been previously shown in human, lung, breast, prostate and several colon cancer cell lines.

A plausible hypothesis is that IFN-α may induce a similar ‘gain-of-function’ phenotype that enhances EGF dependence, thus increasing susceptibility to EGFR inhibition. Gefitinib inhibits activation of the HER1/EGFR intracellular tyrosine kinase and downstream signalling pathways such as PI3/Akt and Ras/Raf/MAPK, a downstream interaction may enhance inhibition of cell proliferation (22). The downstream effect of IFN-α in such a setting may enhance the signalling of the JAK/STAT and PI3K/Akt pathways, sensitising the cells to the EGFR inhibitor and reducing the dose required for effective blockade of relevant shared pathways of growth stimulation in the absence of mutations.

Regardless of the mechanism, the IFN-α-associated up-regulation of EGFR may indicate that EGFR signalling can be modulated and it may be an alternative to positive expression of the receptor as a predictor for identifying tumour cells that may be susceptible to enhanced growth inhibition by combining EGFR inhibitors with IFN.

Abnormal activity of EGFR has been associated with the development and progression of many malignancies, including colon cancer. In particular, over-expression of EGFR and HER2 in colorectal cancer correlates with an extremely poor clinical prognosis (23). The majority of solid tumours, including colon cancer, over-express one or more members of the EGFR family and co-expression of EGFR with HER-2 or HER-3 results in the development of enhanced drug resistance (24). We know that the expression of XAF1 was down-regulated in colon cancers and they correlated with advanced stage and high grade tumour (25).

Our study supports that gefitinib is an active drug in vitro against human colon cancer cell lines. Of importance, is the finding that combining IFN-α treatment with gefitinib, can have synergistic inhibitory effect on the growth of colon cancer cells. The anti-proliferative effects of both IFN-α and EGFR blockers are ultimately mediated via increased apoptosis using a caspase pathway (26). Studies of potential common pathways may identify the source of synergy and ways to further enhance the effect. The development and combination of new agents that target members of the ErbB family or downstream effectors will lead to a more comprehensive approach in using targeted therapies in the future.

References