Epidermal Growth Factor Receptor Blockade in Combination with Conventional Chemotherapy Inhibits Soft Tissue Sarcoma Cell Growth *In vitro* and *In vivo*

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**Abstract**  
**Purpose:** The epidermal growth factor receptor (EGFR) is highly expressed in many human soft tissue sarcomas (STS). However, EGFR blockade has not apparently been used for human STS therapy; therefore, we examined the *in vitro* and *in vivo* effects and the underlying mechanisms before considering EGFR blockade as a therapy for STS patients.

**Experimental Design:** Human STS tissues and cell lines were used to study EGFR expression and activation. Western blot analysis was used to evaluate effects of EGFR activation on downstream signaling. Cell culture assays were used to assess the effect of EGF stimulation as well as EGFR blockade (using an EGFR tyrosine kinase inhibitor, Iressa; AstraZeneca) on STS cell growth, apoptosis, and chemosensitivity. An *in vivo* study (HT1080 human fibrosarcoma cell line in nude/nude mice: Iressa, doxorubicin, Iressa + doxorubicin, vehicle) was used to examine tumor growth; pEGFR, proliferating cell nuclear antigen, and terminal deoxyribonucleotidetransferase–mediated nick-end labeling staining helped assess the effect of therapy *in vivo* on STS EGFR activation, proliferation, and apoptosis.

**Results:** EGFR was expressed and activated in STS cell lines and tumors, probably due to ligand binding rather than EGFR mutation. Stimulation caused activation of AKT and mitogen-activated protein kinase pathways. EGFR blockade inhibited these effects and also caused increased apoptosis, a p53-independent G0-G1 cell cycle arrest, and decreased cyclin D1 expression. *In vivo*, Iressa + doxorubicin had markedly synergistic anti-STS effects.

**Conclusion:** EGFR blockade combined with conventional chemotherapy results in anti-human STS activity *in vitro* and *in vivo*, suggesting the possibility that combining these synergistic treatments will improve anti-STS therapy.

A static 50% 5-year overall survival rate reflects the inadequacy of available therapies and the urgent need to develop novel treatment strategies for soft tissue sarcoma (STS; ref. 1). In the current era of increasingly successful molecular targeted cancer therapy, identifying potential sarcoma molecular therapeutic targets is imperative. The success of imatinib mesylate therapy, identifying potential sarcoma molecular therapeutic current era of increasingly successful molecular targeted cancer treatment strategies for soft tissue sarcoma (STS; ref. 1). In the of available therapies and the urgent need to develop novel University of Texas M.D. Anderson Cancer Center, Houston, Texas; and 6 Department of Radiation Oncology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania

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EGFR as well as the effect of EGFR inhibition in STS cells remains to be elucidated. Preliminary observations support EGFR-mediated pathway relevance in STS: Treatment of sarcoma cells with EGF in vitro resulted in more than 5-fold increased DNA synthesis and mitogenesis, inducing proliferation and growth of sarcoma cells (17). Furthermore, transfection of sarcoma cell lines with antisense EGFR DNA significantly impaired proliferative capacity (18). Based on these findings, we sought to further consider EGFR blockade effect on STS cell growth, apoptosis, and chemoresistance in vitro and in vivo.

Materials and Methods

Cell culture and reagents. Human SKLMS1 (leiomyosarcoma), HT1080 (fibrosarcoma), RD (rhabdomyosarcoma), A204 (unclassified sarcoma), SW872 (liposarcoma), and SW684 (fibrosarcoma) STS cell lines were obtained from the American Type Culture Collection. Cells were cultured in DMEM medium (A204 in McCoy's 5A) supplemented with 10% FCS (Life Technologies). Doxorubicin (Ben Venue Lab), gemcitabine (Eli Lilly), and the EGFR inhibitor Iressa (AstraZeneca) were obtained from the MDACC Pharmacy. A 250 mg Iressa tablet was dissolved in 25 mL DMSO to obtain a stock solution. All additional dilutions were completed using the respective cell culture medium for each cell line. rhEGF (R&D Systems) was used for EGFR stimulation.

Commercially available antibodies used for Western blot analysis of EGFR, pEGF, Akt, p-Akt, S473, phosphorylated extracellular signal-regulated kinase (pERK), ERK, and cyclin D1 were purchased from Cell Signaling; p27, p53, p21, EGF, and p-actin were acquired from Santa Cruz Biotechnology. Antibodies used for immunohistochemical staining included anti-pEGFR (Tyr1173; Invitrogen), anti-EGFR (Zymed Laboratories), anti–transforming growth factor-α (TGF-α; EMD Bioscience), anti-CD31, and anti–proliferating cell nuclear antigen (Dako Cytomation). Day End Fluorometric TUNEL System (Promega) was used for terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) staining. Secondary antibodies included horseradish peroxidase–conjugated (Universal kit HRP; Biocare Medical) and fluorescent secondary antibodies (anti-rabbit Alexa488 and anti-mouse Alexa 594; Jackson Immuno Research). Other reagents included Cytochrome FC Receptor block (Innovex Bioscience), Hoechst 33342 (Polysciences, Inc.), and propyl gallate (ACROS Organics).

Immunohistochemical analysis. Immunohistochemistry was done as previously described (19). Briefly, paraffin sections were dewaxed and rehydrated before antigen retrieval. Endogenous peroxidase activity was quenched with 0.6% hydrogen peroxide before blocking with horse serum. Staining with primary antibodies (described above) was done at concentrations based on the manufacturer's recommendation. Biotinylated secondary antibodies were applied at 1:200 before ABC peroxidase system application (Vectorstain ABCComplex; Vector Laboratories, Inc.). 3,3'-diaminobenzidine color development (Sigma Chemical Co.), and Mayer's hematoxylin counterstaining. For immunofluorescence staining, fluorescence-conjugated secondary antibodies were used, followed by nuclear staining with Hoechst. Staining distribution and intensity was evaluated and scored by two independent reviewers (B.K. and A.L.).

Western blot analysis. Western blot was done by standard methods. Briefly, 50 µg of proteins extracted from cultured cells were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Millipore Co.). Membranes were then blocked and blotted with relevant antibodies. Horseradish peroxidase–conjugated secondary antibodies were detected by ECL chemiluminescence (Amersham Biosciences, Plc.). IRdye680- and IRdye800-conjugated secondary antibodies (Molecular Probes) were detected using Odyssey Imaging (LI-COR Biosciences). ELISA. EGF and TGF-α levels were measured in STS cell collected conditioned medium using ELISA, following the manufacturer's instructions (R&D).

Measurement of cell proliferation. Cell growth assays were done using CellTiter96 Aquous Non-Radioactive Cell Proliferation Assay kit (Promega), per manufacturer's instructions. The growth rate was analyzed 24, 48, 72, and 96 h after EGF (100 ng/mL) stimulation, as well as with increasing Iressa, doxorubicin, or gemcitabine doses, alone or in combination. Absorbance was measured at a wavelength of 490 nm and the absorbance values of treated cells are presented as a percentage of the absorbance of untreated cells.

Apoptosis assay. Apoptosis was measured using the Apoptosis Detection Kit 1 (BD Biosciences). As a standard, 1 × 10⁶ cells/mL per treatment condition were fixed and stained with 5 µL Annexin V–FITC (BD Pharmingen) and 5 µL propidium iodide (Sigma). Flow cytometric analysis was done for 1 × 10⁶ cells and analyzed by FACSscan (Becton Dickinson) using a single laser emitting excitation light at 488 nm. Data was analyzed by CellQuest software (Becton Dickinson).

Cell cycle analysis. STS cell monolayers were treated with relevant agents (Iressa, doxorubicin, and gemcitabine) for varying periods. Cells were harvested, washed, and fixed. Fixed cells were treated with 50 µg/mL RNase and stained with 50 µg/mL propidium iodide for 30 min. Cells were analyzed in a FACScalibur, and data were analyzed with Cell Quest and ModFitLIT v3.1 software (Verity Software House).

In vivo therapeutic animal model. All animal procedures and care was approved by the Institutional Animal Care and Use Committee of the University of Texas M. D. Anderson Cancer Center. Animals received humane care as per the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals." Trypan blue staining–confirmed viable HT1080 STS cells (1 × 10⁶/0.1 mL HBSS/mouse) were injected s.c. into the flank of 6- to 8-week-old female nude/mouse mice (National Cancer Institute/NIH; n = 40). Tumors were measured twice weekly with a caliper; tumor volume was calculated as V = L × W ²/6, where V is the volume, L is the length, and W is the width. When average tumor volume reached 100 mm³, mice were assigned into four treatment groups: (a) control (vehicle only); (b) doxorubicin (1.2 mg/kg biweekly i.p.; low-dose schedule, based on our previous experience results in minimal side effects); (c) Iressa (100 mg/kg, gavage—based on previous studies using Iressa for other tumor types, this dose is lower than the MTD in mice (150-200 mg/kg) and was selected to minimize side effects when combined with chemotherapy); and (d) doxorubicin + Iressa. Mice were followed for tumor size and body weight, then sacrificed when control group tumors reached an average of 1.5 cm in largest dimension. Tumor was resected, weighed, and frozen, or fixed in formalin and paraffin embedded for further immunohistochemical studies.

Statistical analysis. Cell culture–based assays (as well as Western blot analysis) were repeated at least thrice and mean ± SD was calculated. Cell lines were examined separately. For outcomes that were measured at a single time point, two-sample t tests and two-way ANOVA models with interaction were used to assess the differences. A linear mixed model was used to assess the change of tumor volume over time. Tumor volume was first transformed using the natural logarithm scale to better fit the assumption of linear mixed model. The linear mixed model included fixed effects of doxorubicin (no treatment versus treated), Iressa (no treatment versus treated), time, and two-way and three-way interactions between three factors. The unstructured variance-covariance model was used to incorporate the correlation between assessments within animal based on Akaike's Information Criterion and Schwarz's Bayesian Criterion. Comparisons between treatment groups within each time point were made within the linear mixed models. All statistical models were done using SAS (version 9.1). Model assumptions were examined.

Results

The EGFR is expressed and activated in human STS. C consi- dering EGFR as a STS therapeutic target is rational only if the receptor is expressed, is functional, and is activated in the
clinical context. Published studies have identified increased EGFR expression in human STS specimens (17). To confirm this observation, we immunostained 22 human STS specimens of various histologic subtype, location, and stage (Fig. 1A).

Nineteen of the specimens (86%) expressed EGFR (12 high expression, 5 moderate, and 3 low); only three did not express EGFR, corroborating previous published data. To study whether STS-expressed EGFR is activated, we selected several

Fig. 1. EGFR protein is commonly expressed and phosphorylated in human STS samples. A, 19 of 22 randomly selected STS patient samples showed EGFR expression by immunohistochemistry; representative cases are shown (inset, staining control, secondary antibody only). Immunohistochemistry was done as described in Materials and Methods. a, primary alveolar soft part sarcoma (ASPS); b, primary leiomyosarcoma; c, primary liposarcoma (LPS); d, primary malignant fibrohistiocytoma (MFH); e, primary rhabdomyosarcoma (RMS); f, metastatic alveolar soft part sarcoma; g, recurrent leiomyosarcoma; h, metastatic liposarcoma; i, metastatic malignant fibrohistiocytoma; and j, recurrent rhabdomyosarcoma. B. Immunofluorescence staining for pEGFR showed pEGFR expression in STS exhibiting the EGFR (red, pEGFR; blue, Hoechst for nuclear staining; insets, negative control used exhibiting no EGFR or EGFR expression). All specimens expressed TGF-α and EGF at varying level. C. Double immunostaining (CD31, red; pEGFR, green) showed the expression of activated EGFR in STS. All original images were captured at ×400 magnification.
EGFR-expressing human STS specimens in which frozen tissues were available for further immunohistochemical studies. Using fluorescent-tagged antibodies, we identified the expression of pEGFR on tumor cells in all EGFR-expressing tumors examined (n = 6; Fig. 1B). Previous publications have shown that EGFR mutations are rare in human STS (19); therefore, activation of EGFR in studied specimens was possibly the result of appropriate ligand binding. To confirm this hypothesis, we next analyzed the expression of EGF and TGF-α, the ligands for the EGFR. All STS specimens evaluated were shown to express EGF and/or TGF-α at varying levels (Fig. 1B). In the tumor microenvironment, EGFR ligands can be secreted both by tumor cells (leading to autocrine EGFR activation) as well as by tumor-associated stromal macrophages and fibroblasts (resulting in paracrine activation of EGFR on the tumor cells). Double immunostaining for CD-31 (a marker for endothelial cells) and pEGFR identified that the STS-associated vasculature also expresses activated EGFR (Fig. 1C). Taken together, these results suggest that EGFR is both expressed and activated in human STS tumor cells as well as in tumor-associated vasculature, supporting further consideration of EGFR as a potential therapeutic target in STS.

**EGFR is highly expressed and functional in human STS cell lines.** To enable examination of EGFR inhibition on STS cells in vitro as well as in preclinical in vivo models, we analyzed the expression of EGFR in a panel of human STS cell lines. EGFR immunoblotting showed increased protein expression at varying levels in five of the six sarcoma cell lines tested (Fig. 2A); none of these cell lines were identified previously to harbor an EGFR mutation (see COSMIC database7). Under normal conditions, activated EGFR was found in three cell lines: HT1080, SKLMS1, and, to a lesser extent, SW872 (Fig. 2A). pEGFR expression in culture correlated with expression of the EGFR ligand TGF-α as identified by ELISA, which showed moderate concentrations of TGF-α in the supernatant collected from HT1080, SKLMS1, and SW872 cells (40 ± 1.2, 19 ± 1.4, and 12 ± 0.9 pg/mL per 10^7 cells, respectively). No TGF-α protein secretion was detected in supernatant from the other cell lines. Similarly, EGF secretion was not detected in any of the cells under these normal conditions.

Next, we asked whether the STS cell–expressed EGFR can be activated with the addition of an appropriate ligand. Figure 2B shows that EGF stimulation (but not vascular endothelial growth factor stimulation) results in EGFR phosphorylation. This phosphorylation leads to downstream activation of the AKT and the mitogen-activated protein kinase pathways. Interestingly, whereas AKT activation to a similar level was observed in all three cell lines, EGF-induced pERK expression was more pronounced in SKLMS1 cells. Functionally, EGFR activation resulted in significantly increased proliferation of the EGFR-expressing STS cells HT1080 and RD (P < 0.05) and to a lesser degree of SKLMS1, measured after 24, 48, and 72 hours of EGF stimulation (Fig. 2C). No increase in proliferation was observed in STS cells lacking the EGFR (A204). These initial findings showed that EGFR is both highly expressed and functional in human STS cell lines, justifying examining the effect of EGFR blockade, alone or with chemotherapy, on STS growth using selected STS cell lines.

**EGFR blockade inhibits STS cell proliferation and induces apoptosis.** Iressa (ZD1839, AstraZeneca) is an orally administered, small-molecule EGFR–tyrosine kinase inhibitor (20). Iressa is known to block signal transduction pathways implicated in proliferation and survival in a variety of cancer cells, as well as other host-dependent cancer growth-promoting processes (21). Iressa has shown broad effects against various solid tumors, including non–small cell lung, colorectal, breast, and head and neck cancers (22). To the best of our knowledge, the effect of Iressa (as well as other EGFR small-molecule tyrosine kinase inhibitors) on STS cell growth has not yet been considered; therefore, experiments were conducted to evaluate such possible effects. For these studies, three EGFR-expressing STS cell lines (SKLMS1, HT1080, and RD; Fig. 2A) were selected and used in all subsequent experiments.

First, we showed that Iressa blocks EGF-induced phosphorylation of EGFR in STS cells (Fig. 3A). When SKLMS1 cells were treated with incremental doses of Iressa, decreased EGFR phosphorylation was observed even at the lowest dose of 0.5 µmol/L; complete inhibition was demonstrable at 5 µmol/L (Fig. 3A). Similar results were seen with HT1080 and RD cells (data not shown). Additionally, analyzing the effect of EGFR blockade on downstream pEGFR targets showed
dose-dependent inhibition of pAKT and pERK (Fig. 3A), suggesting possible Iressa antitumor effects in our cells. To further study this possibility, we conducted cell growth assays to evaluate the effect of Iressa on STS cell proliferation. A 50% reduction in cell proliferation was seen after 5.3 ± 0.6, 6.6 ± 1.2, and 7.3 ± 1.3 μmol/L Iressa treatment of HT1080, SKLMS1, and RD cells for 96 h, respectively, consistent with an Iressa-induced dose- and time-dependent inhibition of STS proliferation. Furthermore, propidium iodide/Annexin V staining fluorescence-activated cell sorting analysis showed a significant (P < 0.05) increase in apoptosis induced by Iressa in the STS cells after 48 hours (Fig. 3B).

These results suggest that EGFR blockade in EGFR-expressing STS cells results in downstream signaling inhibition, decreased cell proliferation, and increased tumor cell apoptosis. Additionally, because p53 mutations are the most common genetic alteration in STS and p53-mutated STS are more therapeutically resistant, it is important that no significant differences in response to Iressa could be found when comparing STS cells bearing wt p53 (HT1080) versus mutated p53 genes (SKLMS1, RD).

**EGFR blockade induces G1 cell cycle arrest in STS cells.** To examine whether antiproliferative Iressa effects on STS cell lines were partly mediated via specific cell cycle arrest, we investigated cell cycle phase distributions of Iressa-treated cells using flow cytometric analysis (Fig. 4A). Results revealed that Iressa caused accumulation of cells in the G0-G1 fraction for all three cell lines. For example, after exposure to Iressa (10 μmol/L) for 48 hours, the percentage of G0-G1 phase cells increased from 60 ± 1.2% to 71 ± 2.1%, 62 ± 2.3% to 76 ± 3.3%, and 43 ± 1.7% to 63 ± 2.5% in HT1080, SKLMS1, and RD cells, respectively (P < 0.05), with a proportionate reduction in S and G2-M phase fractions. Additionally, the G0-G1 cell cycle arrest induced by Iressa was found to be dose and time dependent for all three cell lines (data not shown). These results are consistent with other tumor models (23) and suggest that EGFR blockade induces G1 cell cycle arrest in STS cells.

To investigate possible molecular mechanisms involved in the Iressa-mediated G0-G1 cell cycle arrest, several key molecules regulating transition from the G1 to the S cell cycle phase were examined in both HT1080 (harboring wt p53) and SKLMS1 (harboring mutated p53) STS cell lines after 24 hours of Iressa therapy, including cyclin D1, p27, and p21 (Fig. 4B). Results showed major reductions in cyclin D1 protein levels and increases in the p21 protein levels in both cell lines independent of p53 mutational status. An increase in p27 protein was seen in SKLMS1 cells only. No increase was observed in p53 protein levels, even in wt p53 cells (HT1080). It is possible that the observed changes in expression of these EGFR down-regulated molecules is at least partially responsible for the pronounced G1 arrest occurring after Iressa therapy in STS cell lines.

**Combined EGFR blockade and low-dose chemotherapy results in superior tumor cell growth inhibition and apoptosis in vitro and in vivo.** Conventional chemotherapy in STS (even in combinations of drugs) remains problematic due to modest response rates coupled with significant toxicities, especially in high-dose regimens. Even doxorubicin, the single most active agent in STS, has a disappointing overall 30% response rate, typified by break through tumor progression and frequent recurrence after initial sarcoma chemoresponsiveness (24). Studies in many epithelial malignancies suggest superior tumor responses when chemotherapy is used in combination with EGFR blockade (25–30); therefore, we investigated whether this approach might be useful for STS. Two chemotherapies were studied including doxorubicin, the most commonly used first-line agent for treatment of STS, and gemcitabine, often used as second-line chemotherapy after doxorubicin failure.

We first evaluated the effect of combined EGFR blockade/chemotherapy on tumor cell proliferation and apoptosis in vitro. STS cell lines were treated for 48 hours with Iressa (5 μmol/L) alone, doxorubicin (0.1 μmol/L) alone, or a combination of both agents. When Iressa was used alone, only a limited effect (~20% inhibition for HT1080 and RD and 5% for SKLMS1) on cell proliferation rate could be observed (Fig. 5A). However, combined therapy resulted in significant synergistically decreased STS cell proliferation in all three cell lines studied (Fig. 5A; P for the interaction between doxorubicin and Iressa <0.001 for three cell lines). Similar results were observed with the use of combined EGFR blockade and
gemcitabine (data not shown). A similar therapeutic protocol was also used to study the effect of combination therapy on STS cell apoptosis. Gemcitabine induced only minimal apoptosis in HT1080 and RD cells, whereas SKLMS1 cells were more sensitive (Fig. 5B). Iressa alone induced apoptosis in all three STS cells examined. Most importantly, the combination of Iressa and gemcitabine exhibited superior rates of apoptosis in all three cell lines (Fig. 5B). The synergism between Iressa and gemcitabine was statistically significant ($P < 0.0001$ for all cell lines). Similar results were found when combining Iressa and doxorubicin (data not shown).

Based on the above findings, we next sought to evaluate whether the effect of EGFR alone and in combination with chemotherapy observed in vitro was also observable in vivo. Using an animal model of fibrosarcoma, we conducted a four-armed therapeutic study comparing the effect of low-dose doxorubicin, Iressa, and combinations of both agents on human STS growth in nude mice. Therapy was initiated after tumor establishment (100 mm$^3$), thereby seeking to mimic a clinical therapeutic trial rather than a less clinically relevant prophylactic regimen. Mice in all groups were followed for tumor size and toxicity; treatment was terminated when tumors in control group (vehicle only) reached an average of 1.5 cm in largest dimension. Treatment with low-dose doxorubicin alone did not significantly affect growth of HT1080 xenografts (Fig. 6A); average tumor volume (analyzed as log values) was similar to that of control mice at each time point of follow up. Iressa alone induced significant inhibition of tumor growth (Fig. 6A) compared with control tumors and doxorubicin alone–treated tumors, an effect that could be observed as early as after the first 7 days of treatment ($P = 0.0234$ compared with doxorubicin alone group and $P < 0.0001$ compared with control group) and continued throughout the entire treatment period ($P = 0.0001$). Combined low-dose doxorubicin and Iressa was markedly inhibitory (Fig. 6A) compared with control, doxorubicin alone, or Iressa alone–treated tumor groups ($P < 0.0001$). Average tumor weights recorded at termination of the study were as follows: control group, 1.41 ± 0.04 g; doxorubicin group, 1.32 ± 0.037 g; Iressa group, 0.7182 ± 0.035 g; and combination group, 0.3625 ± 0.041 g (Fig. 6B). Statistical analysis indicates that there seemed to be a significant antitumor synergistic effect between doxorubicin and Iressa in vivo ($P < 0.0012$).

H&E staining of tumor specimens from the different treatment arms revealed pronounced tumor necrosis in both the Iressa and combination treatment groups (Fig. 6C). Consequently, sections containing viable tumor cells were selected for further immunohistochemical studies. To confirm that Iressa blocked EGFR phosphorylation in vivo, xenograft

![Fig. 4. EGFR blockade induced G1 cell cycle arrest in STS cells. A, propidium iodide staining shows a significant increase ($P < 0.05$) in the proportion of STS cells in the G1 cell cycle phase after 48 h of Iressa treatment (10 μmol/L). B, Iressa (10 μmol/L) treatment for 24 h results in changes in key G1 cell cycle regulators in both mutp53 STS cells (SKLMS1) and wtp53 cells (HT1080).](image-url)
sections from tumors of each of the treatment groups were immunostained for pEGFR. Figure 6C shows the marked inhibition of EGFR activation in Iressa-treated groups without demonstrable effect on total EGFR expression level. Next, we evaluated the effect of the different therapies on STS cell proliferation and apoptosis. Scoring of immunohistochemical preparations for proliferating cell nuclear antigen levels (a nuclear marker for proliferation) and TUNEL assay staining levels (marker for apoptosis) revealed the following values: control group, 80 ± 5 and 8 ± 2; doxorubicin group, 65 ± 15 and 8 ± 3; Iressa group, 18 ± 5 and 15 ± 5; and combination group, 10 ± 5 and 20 ± 4, respectively. Based on these findings, we concluded that Iressa treatment resulted in a significant decrease in tumor cell proliferation and a significant increase in apoptosis \( (P < 0.001) \). Moreover, combination therapy resulted in the most marked antiproliferation and apoptosis-inducing effects. The test of interaction effects between doxorubicin and Iressa on apoptosis and antiproliferation were marginally significant \( (P = 0.0577 \text{ and } P = 0.067, \text{ respectively}) \). Taken together, these data suggest that EGFR blockade combined with low-dose conventional chemotherapy results in significant STS tumor growth inhibition and apoptosis, an observation of potential clinical utility.

**Discussion**

Despite intensive applications of multimodality treatment, overall STS survival rates remain unsatisfactory \( (31, 32) \).
suggesting the need for better targeted therapies. In our study, the administration of Iressa, a small-molecule inhibitor of the EGFR tyrosine kinase, significantly inhibited STS growth in vitro and in vivo. Although EGFR inhibition has been investigated in several epithelial malignancies where it has shown promise, not much is known about its effect on mesenchymal-origin tumors. To the best of our knowledge, this is the first report of the effect of EGFR blockade in STS, which is especially intriguing because EGFR overexpression in STS was initially reported more than 20 years ago: Gusterson et al. (10) identified overexpressed EGFR in 18 of 35 sarcoma specimens by immunohistochemistry and Nielsen et al. (12) showed that 20 of 40 STS exhibited positive immunoreactivity. Since then, other studies have reported increased EGFR

Fig. 6. Combining Iressa (orally, 100 mg/kg/d) with low-dose doxorubicin (i.p., 1.2 mg/kg biweekly) results in significant reduction of HT1080 xenograft tumor growth (A) and weight (B) when compared with treatment with each agent alone. C, immunohistochemical analysis of HT1080 xenograft specimens reveals inhibition of EGFR phosphorylation in Iressa-treated tumors with no effect on total EGFR expression levels. A decrease in tumor cell proliferation (proliferating cell nuclear antigen [PCNA]) as well as an increase in apoptosis (TUNEL) was seen in both groups treated with Iressa, but was more pronounced in the combination therapy group (insets, staining controls, secondary antibody only).
expression in several STS histologic subtypes (13, 33); the largest contemporary series showed positive EGFR staining in 60% of 281 human adult STS. Specifically, malignant fibrous histiocytoma, myxofibrosarcoma, synovial sarcoma, malignant peripheral nerve sheath tumor, and leiomyosarcoma exhibited strong, diffuse positivity for EGFR in a substantial proportion of tumors (13). Our results presented here support these previous findings, with 55% of human STS specimens exhibiting strong expression of EGFR in more than 50% of tumor cells. Additionally, *EGFR* mRNA overexpression was observed in STS (17, 34). In a high-throughput cDNA array study of human STS, Baird et al. (34) identified high *EGFR* mRNA expression in several STS, particularly synovial sarcoma; on weighted analysis, *EGFR* was the 25th most highly weighted gene, further supported by correspondingly intense immunohistochemistry antibody-mediated staining. *EGFR* gene amplification was identified in only 3.5% to 7% of STS (35, 36) and thus cannot solely account for the observed increased *EGFR* mRNA and protein; we are currently examining additional molecular mechanisms driving STS *EGFR* expression. Most importantly, as shown in our study and published data (13), phosphorylation of *EGFR* is demonstrable, suggesting *EGFR* activation in STS. Stabilizing mutations in *EGFR* were previously found to result in autoactivation and phosphorylation of the *EGFR* protein (37). When a large panel of 275 sarcoma DNAs was screened for mutations in all 28 *EGFR* exons, no mutations were identified (34). It is therefore likely that human STS *EGFR* phosphorylation is the result of autocrine/paracrine ligand activation. Overproduction of HER1/EGFR ligands (e.g., TGF-α) by tumor cells and/or tumor-associated normal cells can cause autocrine/paracrine receptor activation, respectively.

Our current results show increased TGF-α and *EGFR* expression in human STS.

Most importantly, we found that ligand stimulation of *EGFR* results in receptor phosphorylation, downstream target activation, and increased proliferation of STS cells, suggesting a functional role for the *EGFR* phosphorylation observed in human samples and a rationale to further explore the effect of *EGFR* inhibition on STS cells in *vitro* and *in vivo*. To investigate *EGFR* blockade in STS, we used Iressa; although not shown, most experiments presented here have also been repeated using a second *EGFR* tyrosine kinase inhibitor, Traceva (OSI-774; OSI Pharmaceuticals), demonstrating similar effects. This suggests that the observed results are due to *EGFR* blockade per se rather than being dependent on a certain drug. Cell proliferation assays showed that Iressa inhibited the cell growth of all three studied STS cell lines in a dose- and time-dependent manner. These antitumor effects did not directly correlate with the level of *EGFR* protein expressed by these cells, consistent with other reports showing that Iressa-induced growth inhibition of several cancer cells was dose dependent but did not correlate with *EGFR* expression level (25).

Complete blockade of *EGFR* phosphorylation was seen after treatment of cells with 5 μmol/L of Iressa, inducing inhibition of *EGFR* downstream AKT and ERK signaling. *EGFR* blockade–induced STS growth inhibition was found to be partially due to the result of G1 cell cycle arrest, consistent with findings in other tumor systems (38). This Iressa-induced cell cycle effect was independent of p53 mutational status and was observed in both wt/p53 STS cells (HT1080) and STS cells harboring mutations in the p53 DNA core binding domain (SKLMS1 and RD). This observation is of major importance in STS because p53 dysregulation is very common, and STS cells harboring p53 mutations are more resistant to current therapeutic strategies (39).

To further evaluate mechanisms underlying *EGFR* blockade–induced cell cycle G1 arrest, we examined the effect of Iressa on the expression of key G1-S checkpoint regulatory proteins. Progression through the early to mid-G1 cell cycle phase is largely regulated by D-type cyclins. Our results showed that 24 hours of Iressa treatment decreased cyclin D1 levels in STS cells. Additionally, an increase in cyclin-dependent kinase inhibitors p27(KIP1) and p21(CIP1/WAF1) was concomitantly observed. Whereas increased p27 was observed in one of the two cell lines (SKLMS1), p21 was increased in both. These changes were all independent of p53 mutational status and/or protein expression as they occurred in both wild-type and mutp53 cells. Furthermore, no increase in p53 protein expression was induced by *EGFR* blockade in wt/p53 cells.

There are various possible transcriptional and posttranscriptional mechanisms by which *EGFR* blockade could induce altered expression levels of G1 cell cycle regulators observed in STS cells, and these should be further explored. In several experiments not presented here, we identified that ERK inhibition in STS cells results in G1 cell cycle arrest, whereas AKT inhibition leads to G2 cell cycle arrest. It is possible that ERK inhibition is the dominant *EGFR* blockade–induced cell cycle regulatory pathway in STS. *EGFR* blockade–induced G1 cell cycle arrest has been shown in cancer types (38, 40, 41). However, the identified molecular derangements leading to the G1 cell cycle block are varied. Decreased cyclin D1 levels as well as increases in p27 and p21 after *EGFR* blockade have been shown in various tumors, whereas not in other cell types where changes in other G1 phase regulators, such as cyclin A and E and the Rb protein, have been identified (42–46). The underlying mechanisms causing these inter–cell type differences remain for future studies; however, these initial findings suggest complex and heterogeneous tumor-specific and perhaps even cell-specific *EGFR*-induced downstream signaling.

Current therapy of locally advanced and metastatic STS is significantly limited by the modest antitumor effects of conventional chemotherapy. STS initially responsive to chemotherapy frequently become chemo-resistant during therapy or after recurrence. Given the temporal, financial, and significant toxicity costs of STS chemotherapy, it is critical to develop new therapeutic and more effective therapeutic approaches. Based on our results suggesting that *EGFR* blockade has anti-STS effects and previous reports showing synergism between the molecular inhibition of *EGFR* and DNA-damaging agents, including doxorubicin, in several epithelial-origin tumors (25–30), we investigated the combination of *EGFR* blockade with chemotherapy. Coadministration of Iressa and doxorubicin or gemcitabine to STS cells in culture resulted in superior inhibition of tumor cell proliferation and increased rates of apoptosis compared with each agent alone. *In vivo* studies using human STS xenografts in nude mice show that Iressa alone (100 mg/kg/d) inhibited tumor growth, whereas low-dose doxorubicin (1.2 mg/kg/biweekly), as expected, exhibited only minimal anti-STS effects. Importantly, the combination of both agents led to
significantly reduced xenograft proliferation and increased apoptosis.

If such regimens were applicable in the human STS clinical context, it might help reduce doxorubicin-related side effects, currently a critically limiting factor in STS therapy. Our results are encouraging and justify consideration of EGFR blockade in the clinical STS context. However, it is relevant that although antitumor effects of EGFR inhibition have been shown in several epithelial tumors in vitro and in vivo, the results of clinical trials have frequently been disappointing (47–49). This reality shows the limitations of xenograft animal experiments to consistently predict actual therapeutic responses in humans. Construction of STS EGFR blockade clinical trials will require appropriate patient selection, drug scheduling, and combination therapies. It is possible that EGFR inhibition in combination with blockade of additional STS-relevant molecular targets will emerge as a better therapeutic strategy, taking into account the significant heterogeneity of these tumors. Multiple target inhibitors are currently available.

In summary, we have shown that EGFR tyrosine kinase inhibition results in significant antitumor activity against human STS in vitro and in vivo. Our results suggest the possibility of EGFR blockade in combination with conventional chemotherapy as a promising therapeutic intervention for the treatment of STS.

Disclosure of Potential Conflicts of Interest

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