Combining EGFR and mTOR Blockade for the Treatment of Epithelioid Sarcoma

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Abstract

Purpose: Molecular deregulations underlying epithelioid sarcoma (ES) progression are poorly understood yet critically needed to develop new therapies. Epidermal growth factor receptor (EGFR) is overexpressed in ES; using preclinical models, we examined the ES EGFR role and assessed anti-ES EGFR blockade effects, alone and with mTOR inhibition.

Experimental Design: EGFR and mTOR expression/activation was examined via tissue microarray (n = 27 human ES specimens; immunohistochemistry) and in human ES cell lines (Western blot and quantitative reverse transcriptase PCR). Cell proliferation, survival, migration, and invasion effects of EGFR and mTOR activation treated with erlotinib (anti-EGFR small-molecule inhibitor) alone and combined with rapamycin were assessed in cell culture assays. In vivo growth effects of erlotinib alone or with rapamycin were evaluated using severe combined immunodeficient mouse ES xenograft models.

Results: EGFR was expressed and activated in ES specimens and cell lines. EGFR activation increased ES cell proliferation, motility, and invasion and induced cyclin D1, matrix metalloproteinase (MMP) 2, and MMP9 expression. EGFR blockade inhibited these processes and caused significant cytostatic ES growth inhibition in vivo. mTOR pathway activation at varying levels was identified in all tissue microarray-evaluable ES tissues; 88% of samples had no or reduced PTEN expression. Similarly, both ES cell lines showed enhanced mTOR activity; VAESBJ cells exhibited constitutive mTOR activation uncoupled from EGFR signaling. Most importantly, combined erlotinib/rapamycin resulted in synergistic anti-ES effects in vitro and induced superior tumor growth inhibition in vivo versus single agent administration.

Conclusions: EGFR and mTOR signaling pathways are deregulated in ES. Preclinical ES model–derived insights suggest that combined inhibition of these targets might be beneficial, supporting evaluations in clinical trials. Clin Cancer Res; 17(18); 5901–12. ©2011 AACR.

Introduction

First described almost 50 years ago, epithelioid sarcoma (ES) is a malignancy exhibiting both epithelial (keratins and epithelial membrane antigen) and mesenchymal (most notably vimentin and CD34) differentiation markers and remains a biological and clinical enigma (1). Both the cell lineage and cell of origin of ES are unclear. Recent insights into ES molecular underpinnings suggest that loss of INI1 expression, a component of a chromatin remodeling complex regulating transcription machinery, may be involved in disease inception (2). This distinct and uncommon soft tissue sarcoma (STS) histologic subtype is further classified into the distal and the more aggressive proximal subtypes, typically develops in young adults (third and fourth decades of life), and exhibits a propensity for local recurrence, lymphatic spread, and pulmonary metastasis (3, 4). Although complete surgical resection of localized tumors results in relatively favorable 5-year outcome rates (75%–88%), local and systemic recurrences tend to occur later in the course of disease and underlie a dismal 10-year disease-specific survival rate of less than 50% (1, 3–5). Unresectable locally advanced and metastatic ES are usually chemoresistant and consequently are generally fatal (3). No major improvements in the therapy of ES have been made since the early 1970s and novel therapeutic approaches are therefore critically needed.
Translational Relevance

Epithelioid sarcomas (ES), while uncommon, are unfavorable malignancies affecting young adults with a marked propensity for local recurrence and distant metastasis. Complete surgical resection, the only potentially curative therapy, is often not achievable, pointing to a critical need for more effective therapeutic strategies. Studies reported here suggest a role for epidermal growth factor receptor (EGFR) activation in promoting the ES aggressive/metastatic phenotype and show EGFR blockade to induce anti-ES effects including abrogated cell growth, survival, migration, and invasion. Furthermore, mTOR pathway activation was found to commonly occur in ES, possibly mediated, at least in part, by reduced or lost PTEN expression. Most importantly, dual targeting of EGFR and mTOR significantly and synergistically abrogates ES growth in vitro and in vivo. Taken together, these data offer new insights into ES molecular deregulations and support further evaluation of therapeutic combinations targeting EGFR and mTOR in the ES clinical setting.

The rapidly increasing awareness of malignancy-related genetic and epigenetic deregulations has driven the inclusion of biologically based treatment approaches as an integral and desired component of anticancer therapeutic strategies (6). Such tumor-tailored, target-orientated approaches are aimed specifically at molecules which are involved in the initiation and maintenance of a given cancer and are easily "druggable," thereby offering hope for achieving the overarching goal of cancer therapy which is to develop drugs that eliminate tumor cells while sparing normal tissues (7). One molecular target that has attracted considerable investigative interest is the epidermal growth factor receptor (EGFR), an HER receptor family member (HER1/EGFR, HER2/neu, HER3, and HER4; ref. 8). Upon ligand binding, EGFR phosphorylation triggers the activation of downstream signaling pathways involved in critical cellular functions such as proliferation, survival, angiogenesis (9). Functional EGFR dysregulation is frequently observed in human cancers, with overexpression and activation by mutations or autocrine/paracrine growth factor loops identified in approximately 50% of epithelial malignancies, suggesting a pivotal role in tumorogenesis and disease progression (10). Similarly, increased EGFR expression has been described as occurring in several STS histologic subtypes (11). Consequently, significant efforts have been directed toward development and evaluation of anti-EGFR therapeutic approaches, specifically small molecule tyrosine kinase inhibitors and receptor-blocking monoclonal antibodies (12). Multiple preclinical and even clinical studies have shown therapeutic efficacy of EGFR inhibition especially in lung, head and neck, and colorectal carcinoma where such therapies have been integrated into the therapeutic armamentarium (12).

Although not yet extensively investigated in the context of ES, a recent report has identified enhanced EGFR expression in a cohort of human ES tumor samples (13). This initial finding has provided the impetus for the studies presented here, seeking to ascertain EGFR and activated EGFR (pEGFR) expression levels in an independent subset of human ES specimens. Most importantly, the ES-associated function of EGFR and the effects of an EGFR inhibitor on ES growth were evaluated using cellular and xenograft ES models. Finally, we sought to also identify an EGFR blockade containing therapeutic combination whose anti-ES effects were superior compared with single agent EGFR inhibition.

Materials and Methods

Cell lines and reagents

The human ES cell lines VAESBJ (American Type Culture Collection) and Epi544 (developed in our laboratory) were maintained and propagated as previously described (4). Several additional human cancer cell lines and normal cell primary cultures were used as controls (see Supplementary Information). Authentication of ES cell lines was conducted utilizing short tandem repeat DNA fingerprinting (STR; Supplementary Data). Recombinant human EGFR was purchased from R&D Systems, the EGFR inhibitor erlotinib (Tarceva) from LC Laboratories, and the mTOR inhibitor rapamycin from the University of Texas MD Anderson Cancer Center (UTMDACC) pharmacy. Commercially available antibodies were used for immunoblot or immunohistochemical detection of: pEGFR (Tyr1173), pERK (Thr202/Tyr204), ERK, pAKT (Ser473), AKT, p4EBP1 (Thr70), 4EBP1, p70S6K (Thr389), 70S6K, pSRP (Ser235/236), CDK2, CDK4, PARP, and PTEN (all from Cell Signaling); MMP2, MMP9 (R&D Systems); Ki67 (Thermo/Lab Vision); EGFR, cyclin D1, cyclin B1, p27, and β-actin (Santa Cruz Biotechnology); cyclin A1, cleaved PARP (Abcam); cleaved caspase 3 (Biocare Medical); and, INI1 (BD Transduction Laboratories).

Immunohistochemistry

A recently established tissue microarray (TMA) containing tissues retrieved from human ES surgical specimens was used to immunohistochemically evaluate biomarker expression (4). Of note, 8 breast adenocarcinoma specimens were incorporated into this TMA as controls. TMA immunostaining and xenograft-derived specimen immunohistochemistry were conducted as previously described (14). For TMA analysis, most biomarkers (see exceptions described below) were scored by 2 independent observers (A.J. Lazar and X. Xie) after excluding samples with insufficient tumor tissue. Intensity was graded as none (=0), weak/low (=1), or moderate-high (=2). This system was modified for Ki67 and cyclin D1, and the percentage of positive tumor nuclei was scored. For INI1, the tumors were scored as having nuclear expression intact or absent. As assessment of PTEN categorized tumors as: 0, no expression; 1, greatly reduced; and 2, relatively normal or increased.
expression; intratumoral blood vessel endothelium was used as an internal positive control.

**Cellular assays**

A panel of *in vitro* cell culture–based assays was used. These included the following: MTS and clonogenicity assays to determine cell growth; propidium iodide (PI) staining and PI/Annexin V staining fluorescence-activated cell-sorting (FACS) analyses to evaluate cell-cycle progression and rate of apoptosis, respectively; and migration and invasion assays to assess these respective cellular phenotypes. Western blot analyses were used to evaluate levels of protein expression and phosphorylation, and quantitative real time PCR (qRT-PCR) was used to determine matrix metalloproteinase (MMP) 2 and MMP9 mRNA expression levels. All these experiments were carried out as we have previously described (15); further information is available as Supplementary Data.

**In vivo animal models**

All animal procedures/care were approved by UTM-DACC Institutional Animal Care and Usage Committee. Animals received humane care as per the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals." Animal models were used as previously described (16). Information about the xenograft model and therapeutic schemas are provided in Supplementary Data.

**Statistics**

Cell culture–based assays were repeated at least twice; mean ± SD was calculated. Cell lines were examined separately. For outcomes that were measured at a single time point, 2-sample Student’s t tests were used to assess the differences. To determine whether the cytotoxic interactions of erlotinib and rapamycin in ES cells were synergistic, additive, or antagonistic, drug effects were examined using the combination index (CI) method of Chou and Talalay (17–19). Briefly, the fraction affected ($F_a$) was calculated from cell viability assays, and CIs were generated using CalcuSyn software (Biosoft). Differences in xenograft size, weight, and lung weight *in vivo* were assessed using a 2-tailed Student’s t test. Significance was set at $P \leq 0.05$.

**Results**

**EGFR is highly expressed and activated in human ES**

Before evaluating the potential utility of EGFR as an ES therapeutic target, we sought to expand previous observations of enhanced EGFR expression in this STS histologic subtype (13). A preconstructed TMA containing human ES specimens retrieved from 27 patients was used for immunohistochemical analysis (Fig. 1A and Supplementary Table S2). Twenty of the evaluable specimens (77%) expressed EGFR (11 moderate to high expression and 9 low); only 6 did not express EGFR, corroborating the above described previously published observation. Of potential importance, only 2 of the breast cancer samples included on the TMA exhibited EGFR expression. To further determine whether ES-expressed EGFR is activated we evaluated the expression of pEGFR in ES samples: 95% (19, 20) of EGFR-expressing specimens exhibited positive staining at varying levels whereas no EGFR phosphorylation was observed in only 1 sample (Fig. 1A). Taken together, these results suggest that the EGFR is both expressed and activated in human ES.
Next, we analyzed the expression of EGFR in 2 human ES cell lines available to us. Immunoblotting showed loss of INI1 expression in both of these cell lines as compared with normal human cells (NHDF and HC-SMC) as well as a panel of randomly selected cancer cell lines representing both sarcomas (SW872, SKLMS1, and MESA) and carcinomas (A549), a finding which supports their ES origin (Fig. 1B). Increased EGFR expression was noted in ES cells as compared with normal controls at levels comparable with those observed in several other cancer cell lines (Fig. 1B). Increased EGFR expression was similarly noted in 2 human ES primary cultures (early passages; Supplementary Fig. S1); both these cell strains exhibit INI1 protein loss confirming their ES origin. Under serum-containing growth conditions, pEGFR expression was found in both ES cell lines although it was more pronounced in VAESBJ as compared with Epi544. We evaluated whether ES cell–expressed EGFR can be further activated with the addition of an appropriate ligand. Toward that end, cells were cultured under serum-free conditions overnight and were then stimulated with EGF; a marked increase in EGFR phosphorylation was observed and a consequent activation of the downstream extracellular signal–regulated kinase (ERK) signaling pathways was found in both cell lines (Fig. 1C). Interestingly, even under serum-free conditions, we noted a high basal level of pAKT in VAESBJ cells and EGF stimulation induced only a minimal additional phosphorylation in contrast to the marked pAKT induction observed in Epi544 cells. These initial findings showed that EGFR is both highly expressed and functional in human ES cell lines, thereby providing a rationale for examining the effect of EGFR activation and blockade on the ES protumorigenic phenotype.

**EGFR activation induces the proliferation, cell-cycle progression, migration, and invasion of ES cells**

The functional effects of EGFR activation on ES cells were next examined. A significant ($P < 0.05$) increase in ES cell growth was observed after EGF stimulation as per MTS (100 ng/mL/24 h) and clonogenicity (100 ng/mL/14 d) assays (Fig. 2A). EGF induced G1 cell-cycle progression in serum-starved ES cells possibly, at least in part, through increase in the expression of the cell-cycle regulator cyclin D1 (Fig. 2B). Immunohistochemical analysis of the ES TMA also showed increased nuclear cyclin D1 expression in human tumors (Fig. 2B and Supplementary Table S2), possibly suggesting that a functional EGFR signaling network is operative in situ although additional mechanisms enhancing cyclin D1 expression might also be at play. Western blot analyses identified EGF to affect the expression of several additional G1 cell-cycle regulators; increased cyclin A1, cyclin B1, cyclin-dependent kinase (CDK) 2, and CDK4 and decreased p27 expression were noted, especially in the Epi544 cell line (Supplementary Fig. S1).

Modified Boyden chamber assays were conducted to evaluate effects of EGF on cell migration and invasion. To assure that the effects seen were not secondary to EGF impact on cell proliferation, experiments were limited to 6 hours. As depicted in Figure 2C, EGF enhanced the migration and invasion of both ES cell lines tested ($P < 0.05$). Metalloproteinases play an important role in cellular invasive capacity; consequently, we evaluated the effect of EGFR activation on both MMP2 and MMP9 expression. A significant increase in the mRNA levels of these 2 enzymes was noted after 24 hours of EGF stimulation (Fig. 2D). As in the case of cyclin D1, MMP2 and MMP9 were found to be commonly expressed in human ES specimens upon immunohistochemical staining of TMA (Fig. 2D). In summary, EGFR signaling was found to enhance ES proliferation, cell-cycle progression, migration, and invasion—all functions critical for tumor growth and progression.

**EGFR blockade inhibits ES cell growth in vitro and in vivo**

The orally available small molecule EGFR inhibitor erlotinib was used to evaluate the impact of EGFR blockade in our ES preclinical models. ES cells were treated with incremental drug doses for 4 hours; decreases in EGF-induced pEGFR were observed even at the lowest dose (1 μmol/L) tested (Fig. 3A). Furthermore, EGFR blockade resulted in a dose-dependent inhibition of pERK in both cell lines tested (Fig. 3A). Interestingly, while a marked effect on the phosphorylation of AKT and its downstream mTOR-regulated effectors P70S6 kinase and 4EBP1 was observed in Epi544 cells, only a limited effect on this signaling pathway was seen in VAESBJ cells (Fig. 3A).

Functionally, a dose-dependent decrease in ES cell growth in response to erlotinib (96 hours) was observed in both cell lines, although more pronouncedly in Epi544 (Fig. 3B). Similarly, EGFR blockade inhibited the colony formation capacity of ES cells (Fig. 3B). Next, the effects of EGFR inhibition on cell-cycle progression and apoptosis were evaluated. Erlotinib treatment (24 hours) resulted in a G1 cell-cycle arrest in both cell lines (Fig. 3B). This effect could, at least in part, be secondary to the observed decrease in cyclin D1 expression in treated cells (Fig. 3C). Furthermore, PI/Annexin V staining FACS analysis showed a significant ($P < 0.05$) increase in apoptosis induced by erlotinib in the ES cells after 96 hours (Fig. 3C). Concordantly, increased levels of PARP cleavage were noted in both cell lines in response to EGFR blockade (96 hours) further confirming the presence of treatment-induced apoptosis.

To evaluate the effects of erlotinib on ES cell migration and invasion while taking into account the effects of this inhibitor on ES cell growth and survival, assays were conducted with cells pretreated with erlotinib for 4 hours (only viable cells were used). A significant decrease in EGF-stimulated migration and invasion was identified ($P < 0.05$); qRT-PCR showed a marked decrease in MMP2 and MMP9 mRNA levels (Fig. 3D). Taken together, these results suggest that EGFR blockade results in decreased ES cell proliferation, G1 cell-cycle arrest, enhanced apoptosis, and abrogated migration and invasion capacities.

On the basis of the above findings, we next sought to evaluate whether the impact of EGFR blockade can also be
Figure 2. EGFR activation enhances ES cell growth, cell-cycle progression, migration, and invasion. A, a significant increase in ES cell growth was observed after EGF stimulation as per MTS (top) and clonogenicity assays (bottom). B, EGF stimulation induced G1 cell-cycle progression in serum-starved ES cells (PI staining FACS analysis; top). Western blotting showed increase in cyclin D1 expression in response to EGF (middle). Representative photographs of ES TMA cyclin D1 immunostaining depicting low and high expressing tumors (bottom). C, enhanced ES cell migration and invasion in response to EGF stimulation was identified using modified Boyden chamber assays. D, EGF stimulation enhances MMP2 and MMP9 mRNA expression (qRT-PCR) in ES cells (top). Representative photographs of ES TMA MMP2 and MMP9 immunostaining depicting low and high expressing tumors (bottom). Graphs represent the average of at least 2 repeated experiments ± SD; *, statistically significant effects (P < 0.05).
Figure 3. EGFR inhibition exerts anti-ES effects in vitro. A, Western blot analysis identified EGFR phosphorylation inhibition in both cell lines response to erlotinib treatment. Furthermore, EGFR blockade resulted in a dose-dependent inhibition of pERK. Interestingly, whereas a marked effect on AKT phosphorylation and its downstream mTOR-regulated effectors p70S6 kinase (p70S6K) and 4EBP1 was observed in Epi544 cells, only a limited effect on this signaling pathway was seen in VAESBJ cells. B, erlotinib induced a dose-dependent decrease in ES cell growth (MTS; top) which was more pronounced in Epi544 cells. Similarly, EGFR blockade inhibited the colony formation capacity of ES cells (top middle). Furthermore, erlotinib treatment resulted in a G1 cell-cycle arrest in both cell lines (PI staining FACS analysis; bottom middle) and a dose-dependent decrease in cyclin D1 expression (Western blot; bottom). C, PI/Annexin V staining FACS analyses showed a significant increase in ES cell apoptosis induced by erlotinib (top). Concordantly, increased levels of PARP cleavage were noted in both cell lines (Western blot; bottom). D, a significant decrease in EGF-stimulated ES cell migration and invasion was observed in response to erlotinib (top). qRT-PCR showed a marked decrease in MMP2 and MMP9 mRNA levels (bottom). Graphs represent the average of at least 2 repeated experiments ± SD; *, statistically significant effects (P < 0.05).
observed in vivo. Using a recently published ES xenograft animal model resulting from the subcutaneous injection of VAESBJ cells (4), we compared the effect of erlotinib on tumor growth in severe combined immunodeficient mice with the impact of control vehicle administration. Therapy was initiated after tumor establishment (~5 mm in greatest dimension). Mice in both groups were followed for tumor size and toxicity; treatment was terminated when tumors in control group reached an average of 1.5 cm in largest dimension. Treatment with erlotinib induced significant \( (P = 0.002) \) tumor growth delay as compared with control vehicle–treated tumors (Fig. 4A). Average tumor weights recorded at termination of the study were control group: 1.19 ± 0.45 g versus erlotinib group: 0.63 ± 0.3 g \( (P < 0.05; \text{Fig. 4A}) \).

Next, formalin-fixed, paraffin-embedded tumor sections from mice of both study arms were immunohistochemically evaluated. Decrease in pEGFR expression in erlotinib-treated tumors confirmed EGFR activation blockade in vivo \( (P = 0.002) \) tumor growth delay as compared with control vehicle–treated tumors (Fig. 4A). Average tumor weights recorded at termination of the study were control group: 1.19 ± 0.45 g versus erlotinib group: 0.63 ± 0.3 g \( (P < 0.05; \text{Fig. 4A}) \).

Figure 4. EGFR blockade delays ES growth in vivo. A, VAESBJ xenografts (once average size tumor reached ~5 mm in largest dimension) were treated with erlotinib \( (n = 10) \) or vehicle control \( (n = 8) \). Erlotinib-treated mice exhibited slower growth (left graph; \( P = 0.002 \)) and a significantly decreased tumor weight at study termination \( (*, P < 0.05; \text{right graph}) \) as compared with control treated mice. B, immunohistochemical staining confirmed decreased pEGFR expression in erlotinib–treated tumors without significant change in total EGFR expression. Erlotinib–treated tumors exhibited decreased proliferation (measured by Ki67), increased apoptosis (measured via cleaved caspase 3 (CC3)), and decreased cyclin D1, MMP2, and MMP9 expression (original images were captured at \( \times 200 \) magnification).

Taken together, the impact of EGFR blockade on ES xenografts mirrors the effects noted in cell culture. However, taking into account the increasing body of clinical evidence, suggesting only minimal effects of compounds inducing EGFR blockade as single anticancer agents (20, 21), and our data showing tumor growth delay but not tumor abrogation in response to erlotinib suggested that it might be pertinent to identify EGFR blockade containing therapeutic combinations that possessed superior anti-ES effects.

mTOR pathway is commonly deregulated in ES

We sought to identify additional targets that could be blocked in combination with EGFR blockade as a treatment strategy for ES. Insights from experiments conducted above have suggested evaluating the potential deregulation of the AKT/mTOR in ES. As depicted in Figure 5A, both ES cell lines express pAKT and activated mTOR-regulated downstream targets. However, expression levels are markedly more pronounced in VAESBJ cells where pAKT expression is shown to be independent of serum conditions (Fig. 5A). In addition, 1 of the 2 ES cell strains showed pronounced AKT/mTOR activation (Supplementary Fig. S1). Phosphorylation of the AKT/mTOR pathway in Epi544 cells is decreased under serum-free culture conditions, suggesting that its activation might possibly occur via upstream signaling. VAESBJ-expressed pAKT/p4EBP1/p70S6K levels are similar to those observed in sarcoma cells exhibiting...
loss of PTEN (SW872 and MESA; ref. 22) and although not completely lost, PTEN expression in VAESBJ cells is much lower than in Epi544 cells (Fig. 5A).

To confirm that mTOR deregulation is a bona fide common event in human ES and that the results above are not just reflective of cell culture artifact, we determined the expression of p4EBP1 and pSRP (surrogate biomarkers for mTOR pathway activation) in our TMA (Fig. 5B and Supplementary Table S2). p4EBP1 and pSRP expression was 100% in both the samples (sample Figure 5. mTOR pathway is commonly deregulated in ES. A, Western blot analyses showed increased AKT/mTOR activation in ES cells which is more pronounced in VAESBJ cells as compared with Epi544 (top). VAESBJ pAKT expression level is independent of serum conditions (bottom) whereas in Epi544 cells, it is decreased under serum-free culture conditions (bottom). VAESBJ-expressed pAKT/p4EBP1/p70S6K levels are similar to those observed in sarcoma cells exhibiting loss of PTEN (SW872 and MESA) and while not completely lost, PTEN expression in VAESBJ cells is lower than in Epi544 cells (top). B, representative photographs of ES TMA pSRP and p4EBP1 immunostaining depicting low and high expressing tumors (top). PTEN immunohistochemistry (bottom) showed decreased to absent PTEN expression in the majority of ES samples as compared with levels observed in normal cells and other cancer tissues included on the TMA (PTEN expression level in a breast sample is depicted for comparison). C, rapamycin blocks mTOR signaling in ES cell lines (Western blot; left) and inhibits tumor cell growth (MTS; middle) and colony formation capacity (right).

Figure 6. Combined EGFR and mTOR targeting results in superior anti-ES effects as compared with monotherapy. A, combining erlotinib and rapamycin resulted in inhibition of both EGFR and mTOR signaling (Western blot); B, dual blockade resulted in a markedly superior ES cell growth inhibition as compared with each agent alone (MTS, P < 0.05; top). Isobologram analysis (middle) revealed that the growth inhibitory effect of this therapeutic combination is strongly synergistic in both cell lines, although more pronouncedly in VAESBJ cells, with CI < 0.5 for most combinations tested. Similarly, combination therapy induced a superior inhibitory effect on colony formation as compared with each agent alone (bottom). C, the most pronounced G1 cell-cycle arrest was found to occur in response to erlotinib and rapamycin combination (PI staining FACS analysis; top) as is also reflected in the most significant decrease in cyclin D1 expression observed in response to this regimen (Western blot; top middle). Similarly, erlotinib and rapamycin combination resulted in a more pronounced apoptotic rate as compared with monotherapy (PI/Annexin V staining FACS analysis; bottom middle) and PARP cleavage (bottom). D, the impact of combined therapy was assessed in vivo using VAESBJ xenografts growing in SCID mice. Both erlotinib and rapamycin as single agents inhibited tumor growth as compared with control (P < 0.05; left and middle top; v, vehicle; R, rapamycin; E, erlotinib; C, combination). Most importantly, combination therapy resulted in significant growth abrogation as compared with erlotinib, rapamycin, or control (P < 0.05). Combination-treated mice exhibited the most significant decrease in tumor weight as compared with all other therapeutic groups (right top). Immunohistochemical analyses showed a decrease in Ki67-positive staining cells and an increase in cleaved caspase 3 (CC3)-positive cells most pronounced in combination-treated tumors (bottom). *, statistically significant effects (P < 0.05).
Combining EGFR and mTOR blockade results in superior anti-ES effects

We tested the effects of erlotinib and rapamycin combinations on ES cells. As anticipated and depicted in Figure 6A, combining these 2 compounds resulted in the inhibition of both EGFR and mTOR signaling. Of importance, this therapeutic combination abrogated rapamycin "feedback" induction of AKT phosphorylation. To determine effects on cell growth, ES cells were treated for 96 hours with increasing doses of erlotinib alone, rapamycin alone, or with different dose combinations of these 2 drugs. In both cell lines, combined therapy resulted in markedly superior effects as compared with each agent alone ($P < 0.05$; Fig. 6B). We further determined whether erlotinib and rapamycin interactions were additive or synergistic. Isobologram analysis revealed that the growth inhibitory effect of these drugs was strongly synergistic in both cell lines although more pronouncedly in VAESBJ cells, with CI $< 0.5$ for most combinations tested (Fig. 6B and Supplementary Table S3). Similarly, combination therapy induced a superior inhibitory effect on colony formation as compared with each agent alone (Fig. 6B). Although each compound individually leads to increased ES G1 cell-cycle arrest, the most pronounced effect was observed after combination therapy (24 hours; Fig. 6C). Similarly, the most significant decrease in cyclin D1 expression was noted in combination-treated cells (Fig. 6C). To determine the effect of the therapeutic combination on cell death PI/Annexin V staining FACS analyses were conducted after 96 hours of treatment: no apoptosis was found in response to rapamycin alone whereas similar to the findings described above, erlotinib did result in ES cell apoptosis. However, a significantly higher apoptotic rate was observed after combination therapy ($P < 0.05$; Fig. 6C). This observation was further strengthened by the marked increase in PARP cleavage also selectively observed after combination therapy (Fig. 6C).

Finally, a 4-armed therapeutic study was conducted to determine the comparative impact on tumor growth in vivo, assessing the effect of combination treatment to each drug alone or vehicle control. Both erlotinib and rapamycin as single agents inhibited VAESBJ xenograft growth as compared with control ($P < 0.05$; Fig. 6D). Most importantly, combination therapy resulted in significant growth abrogation as compared with erlotinib, rapamycin, or control alone ($P < 0.05$). Average tumor weights recorded at termination of the study were control group: $1.21 \pm 0.4$ g; erlotinib group: $0.53 \pm 0.3$ g; rapamycin group: $0.38 \pm 0.09$ g; and combination group: $0.15 \pm 0.11$ g (Fig. 6D). Immunohistochemical analysis showed a decrease in Ki67-positive staining cells and an increase in cleaved caspase 3-positive cells which was most pronounced in combination treatment tumors. These data suggest that EGFR blockade in combination with mTOR inhibition results in significant anti-ES effects in vitro and in vivo, a finding of potential clinical utility.

Discussion

The current study highlights several ES-associated molecular deregulations of potential translational and clinical importance. First, a recent observation (13) identifying EGFR overexpression as commonly occurring in ES was validated and was further expanded to show receptor activation in human ES samples. Moreover, a role for EGFR activation in supporting the malignant phenotype of ES was shown. Most importantly, EGFR blockade was found to inhibit the growth of ES cells in vitro and in vivo, inducing arrested cell-cycle progression, enhanced apoptosis, and abrogated tumor cell migration and invasion. While the limitations of only a small cohort of ES cell lines available for testing should be taken into consideration, these findings are encouraging and possibly support further investigation of EGFR blockade in the clinical ES context. ClinicalTrials.gov (http://clinicaltrials.gov/NCT00148109) reports a currently active phase II clinical trial using an EGFR monoclonal antibody for the treatment of metastatic or locally advanced bone and STSs. It is not certain whether any ES patients were included in this study; however, on the basis of observations made here, it might be useful to
establish the response rate of this subcohort, perhaps as a separate evaluation. Moreover, it is pertinent that a large body of evidence suggests that despite promising responses to monotherapy EGFR manipulation in multiple preclinical epithelial cancer models in which the receptor is overexpressed (23, 24), the clinical therapeutic benefit is minimal, even with high levels of EGFR expression within the tumor, and is mostly observed in tumors harboring a genetic deregulation of the receptor, that is, amplifications and activating mutations (11, 25). Furthermore, even tumors harboring such genetic modifications are bound to develop resistance to EGFR blockade (26). It is of note that no EGFR amplifications or gene mutations were previously identified in ES specimens (13). These findings suggest that enhanced EGFR expression and activation per se are not sufficient to predict response to EGFR blockade and that additional molecular deregulations may possibly decrease the efficacy of EGFR inhibitors as single agents. In the past few years, we have learned that rational combinations of targeted therapeutics may achieve more potent antitumor effects and help overcome initial therapeutic resistance (16); by implication, identifying other ES-associated molecular deregulations that can be targeted in combination with EGFR might be of great benefit.

The pivotal mTOR pathway, a convergence downstream node of a large number of molecular processes including EGFR signaling, has been shown to play a major role in cancer progression and metastasis (27). mTOR is known to act as a master switch for cellular catabolism and anabolism, enhancing cancer cell growth and proliferation (27, 28). In addition, mTOR can induce cell-cycle progression, enhance cell survival, and block cell death as dictated by the cellular context and specific downstream targets (27, 28). Rapamycin and its derivatives that block the mTORC1 complex have been developed and are currently being evaluated in several clinical trials for a variety of hematologic and solid malignancies (29, 30). To the best of our knowledge, no previous studies have evaluated mTOR deregulation and function in ES. Our findings identify ubiquitous phosphoinositide-3-kinase (PI3K)/mTOR pathway activation in human ES specimens (to levels similar to those found in breast cancer samples) and cell lines possibly mediated, at least in part, by reduced or lost expression of the negative regulator PTEN, although additional molecular mechanisms are potentially relevant. Of potential importance, we found that the ES cell line exhibiting PTEN loss (VAESBJ) was most pronouncedly sensitive to rapamycin. To that end, it is possible that PTEN loss should be further evaluated as a molecular biomarker for appropriate patient selection for inclusion in mTOR blockade–based human ES clinical trials. Reduced PTEN expression in cancer can occur through genomic alterations or epigenetic modifications such as DNA methylation (31). Loss of INI1, the product of the hSNF5/SMARCBI/BAF47 gene, occurs in the vast majority of ES (2). INI1 is a component of the hSWI/SNF chromatin remodeling complex regulating gene transcription by modulating nucleosomal structures in an ATP-dependent manner (32). The possible role this epigenetic modifier plays in the activation of the PI3K/mTOR pathway and in decreased PTEN expression is currently unknown and should be further explored.

Interestingly, response to EGFR inhibitors has been shown to inversely correlate with PI3K/mTOR signaling overactivation (33–36). For example, breast cancer cells expressing enhanced mTOR activation secondary to PTEN loss were found to be EGFR blockade resistant; PTEN reintroduction into these cells results in increased response to EGFR inhibition (33). Similarly, loss of PTEN has been found to predict resistance to EGFR blockade in colorectal cancer, lung cancer, and glioblastoma (34–36); our studies expand these observations to now include ES—a tumor lacking effective systemic treatment. Although erlotinib inhibited the growth of both available ES cell lines, a more limited response was observed in VAESBJ cells. These cells exhibit uncoupling of EGFR from its downstream PI3K/mTOR signaling, a higher level of constitutive PI3K/mTOR activation, and consequently survive independent of EGFR. Multiple preclinical studies have shown that dual targeting of EGFR and mTOR results in enhanced anticancer effects in several different cancer types (37–39). Such an approach was shown to be effective in experimental models exhibiting relative sensitivity to EGFR blockade as a single agent and also for targeting resistant cells where mTOR blockade restores therapeutic sensitivity (37, 39). In light of these findings, several phase I/II trials combining EGFR and mTOR blockade for the treatment of a variety of epithelial cancers have recently been initiated (ClinicalTrials.gov). In the present study, we found EGFR inhibition in combination with mTOR blockade to exert a synergistic anti-ES effect in vitro and in vivo. This combination resulted in significant ES cell growth arrest, inhibition of cell-cycle progression, enhanced apoptosis, and blocked migration and invasion. Together, these data support the inclusion of ES patients in future EGFR/mTOR blockade clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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