Combining EGFR and mTOR blockade for the treatment of epithelioid sarcoma

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Statement of Translational Relevance

Epithelioid sarcomas (ESs), 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 EGFR activation in promoting the ES aggressive/metastatic phenotype and demonstrate 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.
Abstract

Purpose: Molecular deregulations underlying epithelioid sarcoma (ES) progression are poorly understood yet critically needed to develop new therapies. 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; IHC) and in human ES cell lines (WB and qRTPCR). Cell proliferation, survival, migration, and invasion effects of EGFR and mTOR activation and 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 SCID 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, MMP2, 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 TMA-evaluable ES tissues; 88% of samples had no or reduced PTEN expression. Similarly, both ES cell lines demonstrated 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.
Introduction

First described almost fifty 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) histological subtype is further classified into the distal and the more aggressive proximal subtypes, typically develops in young adults (third and forth 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 five year outcome rates (75-88%), local and systemic recurrences tend to occur later in the course of disease and underlie a dismal ten-year disease specific survival of <50% (1, 3-5). Unresectable locally advanced and metastatic ES are usually chemoresistant and consequentially 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.

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 anti-cancer 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 ‘drugable’, 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), a HER receptor family member (HER1/EGFR, HER2/neu, HER3 and HER4; (8)). Upon ligand binding, EGFR phosphorylation triggers the activation of downstream signaling pathways involved in critical cellular functions such as proliferation, survival, angiogenesis, etc. (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 tumorigenesis and disease progression (10). Similarly, increased EGFR expression has been described as occurring in several STS histological subtypes (11). Consequentially, significant efforts have been directed towards 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 demonstrated 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).

While 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 to single agent EGFR inhibition.
Materials and Methods

Cell-lines and reagents: The human ES cell lines VAESBJ (ATCC) 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 Supp information). Authentication of ES cell lines was conducted utilizing Short Tandem Repeat DNA fingerprinting (STR; Supp data). Recombinant human EGF was purchased from R&D (Minneapolis, MN), the EGFR inhibitor, erlotinib (Tarceva), from LC Laboratories (Woburn, MA), and the mTOR inhibitor, rapamycin, from the UTMDACC pharmacy. Commercially available antibodies were used for immunoblot or immunohistochemical detection of: pEGFR (Tyr1173), pERK (Thr202/Tyr404), ERK, pAKT (ser 473), AKT, p4EBP1 (Thr70), 4EBP1, p70S6K (Thr389), 70S6K, pSRP (Ser235/236), CDK2, CDK4, PARP, and PTEN (all from Cell Signaling, Danvers, MA); MMP2, MMP9 (R&D Systems, Minneapolis, MN); Ki67 (Thermo/Lab Vision, Fremont, CA); EGFR, Cyclin D1, Cyclin B1, p27, and beta-actin (Santa Cruz Biotechnology, Santa Cruz, CA); Cyclin A1, cleaved PARP (Abcam, Cambridge, MA); cleaved caspase 3 (Biocare Medical, Concord, CA); and, INI1 (BD Transduction Laboratories, Lexington, KY).

Immunohistochemistry: A recently established TMA containing tissues retrieved from human ES surgical specimens was used to immunohistochemically evaluate biomarker expression (4). Of note, eight 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 two independent observers (AJL and XX) 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 was the percentage of positive tumor nuclei were scored. For INI1, the tumors were scored as having nuclear expression intact or absent. Assessment of PTEN categorized tumors as: 0=no expression, 1=greatly reduced, 2=relatively normal or increased expression; intra-tumoral blood vessel endothelium was used as an internal positive control.

**Cellular assays:** A panel of *in vitro* cell culture-based assays was utilized. These included: MTS and clonogenicity assays to determine cell growth; PI staining and PI/Annexin V staining FACS analyses to evaluate cell cycle progression and rate of apoptosis, respectively; migration and invasion assays to assess these respective cellular phenotypes. Western blot analyses were used to evaluate levels of protein expression and phosphorylation, and qRT-PCR were used to determine MMP2 and MMP9 mRNA expression levels. All these experiments were conducted as we have previously described (15); further information is available as Supp Data.

**In vivo animal models:** All animal procedures/care was approved by UTMDACC 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 utilized as previously described (16). Information regarding the xenograft model and therapeutic schemas are provided in Supp 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, two-sample 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 (Fa) was calculated from cell viability assays, and CIs were generated using CalcuSyn software (Biosoft, Cambridge, UK). Differences in xenograft size, weight and lung weight in vivo were assessed using a two-tailed Student's t-test. Significance was set at P≤0.05.

Results

EGFR is highly expressed and activated in human epithelioid sarcoma (ES)

Prior to evaluating the potential utility of EGFR as an ES therapeutic target we sought to expand previous observations of enhanced EGFR expression in this STS histological subtype (13). A pre-constructed TMA containing human ES specimens retrieved from 27 patients was used for immunohistochemical analysis (Fig 1A and Table S2). Twenty of the evaluable specimens (77%) expressed EGFR (11 moderate to high expression and 9 low); only six did not express EGFR, corroborating the above described previously published observation. Of potential importance, only two 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 one 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 two human ES cell lines available to us. Immunoblotting demonstrated loss of INI1 expression in both of these cell lines as compared to 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 epithelioid sarcoma origin (Fig
Increased EGFR expression was noted in ES cells as compared to normal controls at levels comparable to those observed in several other cancer cell lines (Fig 1B). Increased EGFR expression was similarly noted in two human ES primary cultures (early passages; 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 to Epi544. We evaluated whether ES cell-expressed EGFR can be further activated with the addition of an appropriate ligand. Towards 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 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 demonstrated 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 (100ng/ml/24h) and clonogenicity (100ng/ml/14d) 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 demonstrated increased nuclear cyclin D1 expression in
human tumors (Fig 2B and 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. WB analyses identified EGF to affect the expression of several additional G1 cell cycle regulators; increased cyclin A1, cyclin B1, CDK2, and CDK4 and decreased p27 expression were noted, especially in the Epi544 cell line (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 six 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 two enzymes was noted after 24h 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 IHC 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 utilized to evaluate the impact of EGFR blockade in our ES pre-clinical models. ES cells were treated with incremental drug doses for 4h; decreases in EGF-induced pEGFR was observed even at the lowest dose (1μM) 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 (96h) 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 (24h) 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 demonstrated a significant (p<0.05) increase in apoptosis induced by erlotinib in the ES cells after 96h (Fig 3C). Concordantly, increased levels of PARP cleavage were noted in both cell lines in response to EGFR blockade (96h) 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 four h (only viable cells were utilized). A significant decrease in EGF stimulated migration and invasion was identified (p<0.05); qRTPCR demonstrated 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.

Based on the above findings, we next sought to evaluate whether the impact of EGFR blockade can also be observed in vivo. Utilizing 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 SCID mice compared to the impact of control vehicle administration. Therapy was initiated after tumor establishment (~5mm 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 1.5cm in largest dimension. Treatment with erlotinib induced significant (p=0.002) tumor growth delay as compared to control vehicle-treated tumors (Fig 4A). Average tumor weights recorded at termination of the study were control group: 1.19g±0.45 vs. erlotinib group: 0.63g±0.3 (p<0.05; Fig 4A).

Next, formalin-fixed paraffin-embedded tumor sections from mice of both study arms were immunohistochemicaly evaluated. Decrease in pEGFR expression in erlotinib-treated tumors confirmed EGFR activation blockade in vivo (Fig 4B); as expected no decrease in the total EGFR expression level was observed. A decrease in the number of Ki67 (a nuclear marker for proliferation) expressing tumor cells and an increase in the number of cleaved caspase 3 (marker for apoptosis) positive cells was observed in erlotinib-treated samples (Fig 4B). Furthermore, as was found in vitro, erlotinib induced a decrease in cyclin D1, MMP2, and MMP9 expression in vivo (Fig 4B).

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 anti-cancer agents (20, 21) and our data demonstrating 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, one of the two ES cell strains demonstrated pronounced AKT/mTOR activation (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/pP70S6K levels are similar to those observed in sarcoma cells exhibiting loss of PTEN (SW872 and MESA; (22)) and while 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 Table S2). p4EBP1 and pSRP expression was found in 100% (moderate-high = 78%, low = 22%) and 100% (moderate-high = 87%, low = 13%) of samples, respectively. Furthermore, complete loss of PTEN expression in tumor cells was found in 40% of ES specimens. Interestingly, of those samples expressing PTEN (n=15), 80% exhibited a relative low staining intensity whereas in only 20% was expression noted that was equivalent to the level observed in intra-tumoral and surrounding normal cells (lymphocytes and endothelial cells) used as controls (Fig 5B and Table S1). These findings suggest that PTEN deregulation is a common molecular
aberration in human ES. Similar to ES expression pattern, pSRP and p4EBP1 were commonly expressed in the breast cancer samples evaluated: all 8 specimens were found to express moderate-high levels of pSRP; low p4EBP1 expression was noted in one breast cancer sample while moderate-high expression was found in 7. None of these samples exhibited loss of PTEN expression (reduced expression as compared to normal stroma was noted in three cases), suggesting that other molecular mechanisms possibly drive AKT/mTOR activation in these tumors.

To further confirm that mTOR activation has a functional role in ES, we sought to test the effects of the mTOR inhibitor rapamycin on tumor cell growth. As shown in Figure 5C, rapamycin blocked mTOR signaling in ES cell lines. A rapamycin dose dependent decrease (one that plateaus after 10nM) in tumor cell growth and colony formation capacity was observed in both cell lines. Of note, VAESBJ cells exhibited a more pronounced sensitivity to the drug possibly secondary to PTEN loss (Fig 5C). In sum, our data suggest that mTOR pathway activation is a common molecular deregulation in human ES contributing to tumor cell growth, thereby providing the rationale for testing therapeutic combinations that target this pathway in conjunction with EGFR blockade.

**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 two compounds resulted in the inhibition of both EGFR and mTOR signaling. Of importance, this therapeutic combination abrogated rapamycin “feed-back” induction of AKT phosphorylation. To determine effects on cell growth, ES cells were treated for 96h with increasing doses of erlotinib alone, rapamycin alone, or with different dose combinations of these two drugs. In both cell lines combined therapy resulted in markedly superior effects as compared to
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 Table S3). Similarly, combination therapy induced a superior inhibitory effect on colony formation as compared to each agent alone (Fig 6B). While each compound individually leads to increased ES G1 cell cycle arrest, the most pronounced effect was observed after combination therapy (24h; 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 96h 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).

Lastly, a four-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 to control (p<0.05; Fig 6D). Most importantly, combination therapy resulted in significant growth abrogation as compared to erlotinib, rapamycin, or control alone (p<0.05). Average tumor weights recorded at termination of the study were control group: 1.21g±0.4; erlotinib group: 0.53g±0.3; rapamycin group: 0.38g±0.09; and combination group: 0.15g±0.11 (Fig 6D). IHC analysis demonstrated 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 over-expression as commonly occurring in ES was validated, and was further expanded to demonstrate receptor activation in human ES samples. Moreover, a role for EGFR activation in supporting the malignant phenotype of ES was demonstrated. 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 utilizing an EGFR monoclonal antibody for the treatment of metastatic or locally advanced bone and soft tissue sarcomas. It is not certain if any ES patients were included in this study; however, based on observations made here, it might be useful to establish the response rate of this sub-cohort, 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 over-expressed (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, i.e., 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 is 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 rapamycin derivatives that block the mTORC1 complex have been developed and are currently being evaluated in several clinical trials for a variety of hematological 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 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/SMARCB1/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 over-activation (33-36). For example, breast cancer cells exhibiting enhanced mTOR activation secondary to PTEN loss were found to be EGFR-blockade resistant; PTEN re-introduction 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. While 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 demonstrated 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 \textit{in vitro} and \textit{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.
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Figure Legends

Figure 1: EGFR is highly expressed and activated in human ES. A. Representative photographs of ES tissue microarray EGFR and pEGFR (Tyr1173) immunostaining (original images were captured at x200 magnification) depicting low and high expressing tumors; B. Western blot (WB) analyses demonstrating INI1 loss and pEGFR and EGFR expression in protein extracts of human ES cell lines (VAESBJ and Epi544) grown under regular serum containing conditions and compared to expression in normal cell controls (NHDF and HC-SMC) and other cancer cell lines (SW872 [liposarcoma], SKLMS1 [leiomyosarcoma], MESA [uterine sarcoma], and A549 [lung carcinoma]); C. WB depicting enhanced EGFR phosphorylation and downstream signaling in response to EGF. Cells were grown in serum free media overnight prior to EGF stimulation. Interestingly, a high basal level of pAKT was noted in VAESBJ cells even under serum free conditions. EGF stimulation induced only a minimal increase in pAKT expression in VAESBJ cells in contrast to the marked pAKT induction observed in Epi544 cells.

Figure 2: EGFR activation enhances ES cell growth, cell cycle progression, migration invasion. A. A significant increase in ES cell growth was observed after EGF stimulation as per MTS (upper panel) and clonogenicity assays (lower panel); B. EGF stimulation induced G1 cell cycle progression in serum starved ES cells (PI staining FACS analysis; upper panel). WB demonstrated increase in cyclin D1 expression in response to EGF (middle panel). Representative photographs of ES tissue microarray cyclin D1 immunostaining depicting low and high expressing tumors (lower panel); 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 (upper panel). Representative photographs of ES tissue microarray MMP2 and MMP9 immunostaining depicting low
and high expressing tumors (lower panel). [Graphs represent the average of at least two repeated experiments ±SD; * denotes statistically significant effects (p<0.05)]

**Figure 3: EGFR inhibition exerts anti-ES effects in vitro.** A. WB 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, while a marked effect on AKT phosphorylation 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; B. erlotinib induced a dose dependent decrease in ES cell growth (MTS; upper pane) which was more pronounced in Epi544 cells. Similarly, EGFR blockade inhibited the colony formation capacity of ES cells (upper middle panel). Furthermore, erlotinib treatment resulted in a G1 cell cycle arrest in both cell lines (PI staining FACS analysis; lower middle panel) and a dose dependent decrease in cyclin D1 expression (WB; lower panel); C. PI/Annexin-V staining FACS analyses demonstrated a significant increase in ES cell apoptosis induced by erlotinib (upper panel). Concordantly, increased levels of PARP cleavage were noted in both cell lines (WB; lower panel); D. A significant decrease in EGF stimulated ES cell migration and invasion was observed in response to erlotinib (upper panels). qRTPCR demonstrated a marked decrease in MMP2 and MMP9 mRNA levels (lower panel). [Graphs represent the average of at least two repeated experiments ±SD; * denotes statistically significant effects (p<0.05)]

**Figure 4: EGFR blockade delays ES growth in vivo.** A. VAESBJ xenografts (once average size tumor reached ~5mm 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; right graph) as compared to control treated mice; B. Immunohistochemical (IHC) staining confirmed decreased pEGFR expression in erlotinib treated tumors without significant change in total EGFR expression. Erlotinib treated tumors exhibited decreased proliferation (measured by Ki-67), increased apoptosis (measured via cleaved caspase 3 [CC3]), and decreased cyclin D1, MMP2, and MMP9 expression (original images were captured at x200 magnification).

**Figure 5: mTOR pathway is commonly deregulated in ES.** A. WB analyses demonstrated increased AKT/mTOR activation in ES cells which is more pronounced in VAESBJ cells as compared to Epi544 (upper panel). VAESBJ pAKT expression level is independent of serum conditions (lower panel) while in Epi544 cells it is decreased under serum free culture conditions (lower panel). VAESBJ-expressed pAKT/p4EBP1/pP70S6K 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 (upper panel); B. Representative photographs of ES tissue microarray pSRP and p4EBP1 immunostaining depicting low and high expressing tumors (upper panel). PTEN immunohistochemistry (lower panel) demonstrated decreased to absent PTEN expression in the majority of ES samples as compared to 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 (WB; left panel) and inhibits tumor cell growth (MTS; middle panel) and colony formation capacity (right panel).

**Figure 6: Combined EGFR and mTOR targeting results in superior anti-ES effects as compared to monotherapy.** A. Combining erlotinib and rapamycin resulted in inhibition of both EGFR and mTOR signaling (WB); B. Dual blockade resulted in a
markedly superior ES cell growth inhibition as compared to each agent alone (MTS, p<0.05; upper panel). Isobologram analysis (middle panel) 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 to each agent alone (lower panel); C. The most pronounced G1 cell cycle arrest was found to occur in response to erlotinib and rapamycin combination (PI staining FACS analysis; upper panel) as is also reflected in the most significant decrease in cyclin D1 expression observed in response to this regimen (WB; upper middle panel). Similarly, erlotinib and rapamycin combination resulted in a more pronounced apoptotic rate as compared to monotherapy (Annexin V/PI staining FACS analysis; lower middle panel) and PARP cleavage (lower panel); 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 to control (p<0.05; left and middle upper panels [v=vehicle, R=Rapamycin, E=Erlotinib, C=combination]). Most importantly, combination therapy resulted in significant growth abrogation as compared to erlotinib, rapamycin, or control (p<0.05). Combination treated mice exhibited the most significant decrease in tumor weight as compared to all other therapeutic groups (right upper panel). IHC analyses demonstrated a decrease in Ki67 positive staining cells and an increase in cleaved caspase 3 positive cells most pronounced in combination treated tumors (lower panels). [* denotes statistically significant effects (p<0.05)].
Figure 1

A

Low

High

EGFR

pEGFR

B

INI1

pEGFR

EGFR

β-actin

C

EGF (100ng/ml/15min) – +

pEGFR

EGFR

pERK

ERK

pAKT

AKT

Epi544

VAESBJ

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Figure 2

A

EGF (100ng/ml/24h)  

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Migration  

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MMP2

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Cyclin D1

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β-actin

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Figure 3

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B

- **Erlotinib (5µM/14d)**
  - Epi544
  - VAESBJ

C

- **Erlotinib (5µM/96h)**
  - Epi544
  - VAESBJ

D

- **Migration**
  - Epi544
  - VAESBJ

- **Invasion**
  - Epi544
  - VAESBJ

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**MMP2**

- Epi544
- VAESBJ

**MMP9**

- Epi544
- VAESBJ

* Indicates significant difference.
Figure 5

A

B

C

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Combining EGFR and mTOR blockade for the treatment of epithelioid sarcoma

Xian-Biao Xie, Markus Ghadimi, Eric Young, et al.

*Clin Cancer Res* Published OnlineFirst August 5, 2011.

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