Antitumor Effects of Epidermal Growth Factor Receptor Antisense Oligonucleotides in Combination with Docetaxel in Squamous Cell Carcinoma of the Head and Neck

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ABSTRACT

Purpose: Antisense approaches targeting the epidermal growth factor receptor (EGFR) have been demonstrated to inhibit the growth of squamous cell carcinoma of the head and neck (SCCHN). Docetaxel is an effective chemotherapeutic agent in the treatment of SCCHN. The present study was undertaken to evaluate the antitumor mechanisms of EGFR antisense (AS) oligonucleotides administered in combination with docetaxel in preclinical models of SCCHN.

Experimental Design: SCCHN cells lines and xenografts were treated with an EGFR AS oligonucleotide targeting region 760-779 of EGFR mRNA (GenBank accession XM_004738) alone and in combination with docetaxel. Proliferation in vitro and tumor growth in vivo were examined in addition to determinations of EGFR expression and signaling pathways to evaluate antitumor mechanisms.

Results: A combination of docetaxel with EGFR AS resulted in increased cytotoxicity compared with treatment with docetaxel plus EGFR sense oligonucleotides or docetaxel alone after 24 h. Tumor volumes were significantly reduced in the mice treated with a combination of intratumoral EGFR AS and systemic docetaxel compared with mice receiving monotherapy. The combination of docetaxel plus EGFR AS resulted in decreased expression levels of EGFR, phosphotyrosine signal transducers and activators of transcription 3, vascular endothelial growth factor, and pAKT compared with expression levels after either treatment alone.

Conclusions: A combination of EGFR AS and docetaxel may be effective in the treatment of SCCHN with a reduced toxicity profile compared with standard chemotherapy regimens.

INTRODUCTION

Cancer therapeutics has evolved from nonspecific cytotoxic modalities toward selective molecular targeting of tumors based on aberrant expression of oncogenes and proto-oncogenes. The EGFR2 has been overexpressed in many solid tumors where EGFR expression levels have been correlated with decreased survival (1). The critical role of EGFR in carcinogenesis has led to the development of a number of therapeutic strategies targeting EGFR, with the aim of specifically inhibiting tumor growth.

Autocrine signaling involving EGFR and its ligands, generally TGF-α, has been implicated in the development and progression of a broad array of solid tumors [for review, see Ref. 2]. Ligand binding (TGF-α) induces dimerization of EGFR and autophosphorylation of tyrosine residues in the intracellular domain. Enhanced expression of TGF-α/EGFR has been detected in many human carcinomas where overexpression contributes to enhanced EGFR signaling. EGFR-targeting strategies are designed to decrease EGFR expression levels, abrogate EGFR activation (or phosphorylation), and/or inhibit EGFR downstream signaling. Several major classes of EGFR inhibitors are being tested in preclinical or clinical settings, including small molecule tyrosine kinase inhibitors, monoclonal antibodies, toxin conjugates, and AS nucleic acids. However, results of clinical studies, to date, suggest that EGFR targeting agents demonstrate only modest antitumor effects when administered as single agent therapy. More dramatic clinical responses have been observed when EGFR inhibitors are combined with standard cytotoxic regimens, including chemotherapy or radiation therapy (3, 4). We previously reported that AS oligonucleotides targeting key regulatory regions of the EGFR were more effective at inhibiting the proliferation of SCCHN cells in vitro when directly compared with tyrosine kinase-specific inhibitors or monoclonal antibodies (5).

Taxanes, including docetaxel, are widely used for the treatment of primary or recurrent SCCHN, demonstrating the highest single agent activity (6). When docetaxel is combined with other agents, response rates have been reported to be >50% in most cases (7, 8). Taxanes exert their cytotoxic effects by promoting microtubule assembly and stabilizing microtubule dynamics resulting in inhibition of cell proliferation and apoptosis. The clinical use of taxanes is often limited by potentially serious side effects such as myelosuppression, nausea, mucositis, diarrhea, and peripheral neuropathy. Few studies have examined the biological effects and mechanisms of docetaxel in SCCHN. In
this study, we tested the antiproliferative activity of EGFR AS in SCCHN cells expressing elevated levels of EGFR alone and in combination with docetaxel. Our results indicated that when coadministered with EGFR AS, the dose of docetaxel could be reduced without abrogating efficacy. Furthermore, EGFR AS and docetaxel appear to exert their antitumor effects through additive or synergistic modulation of downstream signaling pathways. These results provide a rationale for the translation of this combination strategy into the clinical setting.

MATERIALS AND METHODS

Cells and Reagents. Several well characterized SCCHN cell lines were used for these studies. The 1483 cell line was derived from a tumor of the retromolar trigone region of the oropharynx (9). The UM-22B cell line was derived from a hypopharyngeal tumor (10). The OSC-19 cell line was derived from a cervical metastatic lymph node from a patient with a cancer of the oral tongue (11). Cell lines were maintained in DMEM supplemented with 12% FCS and antibiotics (100 units/ml of penicillin and 100 units/ml of streptomycin). Doxorubicin (Taxotere RP56976) was obtained from Aventis Pharmaceuticals. The compound was suspended in ethanol at 10 mg/ml and stored at −20°C. Serial dilutions were prepared to make a final concentration of 100 μm. The final ethanol concentration was <0.05% by volume. All AS oligonucleotides targeted regions of human EGFR mRNA (GenBank accession XM_004738). The sequences of all AS and sense oligonucleotides used in these studies are listed in Table 1. Phosphorothioate oligonucleotides were synthesized on an Applied Biosystem 394 synthesizer by β-cyanoethylphosphoramide chemistry as described previously (5).

Table 1 Sequences and target sites of EGFR oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>EGFR mRNA location</th>
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<tbody>
<tr>
<td>Antisense 1</td>
<td>cgg agg gtc gca tgg cgg</td>
<td>173–190</td>
</tr>
<tr>
<td>Sense 1</td>
<td>cgg cga tgc gac cct cgg</td>
<td></td>
</tr>
<tr>
<td>Antisense 2</td>
<td>ccc cag cag ctc cca tgg gg</td>
<td>760–779</td>
</tr>
<tr>
<td>Sense 2</td>
<td>cca caa tgg gag ctt cgg ggg</td>
<td></td>
</tr>
<tr>
<td>Antisense 3</td>
<td>cct cgc tgg tca tgc tcc</td>
<td>3802–3819</td>
</tr>
<tr>
<td>Sense 3</td>
<td>gga gca tga cca cgg agg</td>
<td></td>
</tr>
</tbody>
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Cell Growth Assays. Cell growth was determined using the MTT (Sigma, St. Louis, MO) assay or cell count via vital dye exclusion. Representative SCCHN cell lines were plated at density 0.6 × 10^5/well in a 24-well polystyrene plate. The following day, the medium was removed, and fresh medium containing the investigational agents was added at various concentrations to each well in triplicate. For the MTT assay, MTT was diluted to 5 mg/ml in PBS. The diluted MTT was added to the wells as 500 μl/well. After the plates were incubated at 37°C in 5% CO2 atmosphere for 1 h, the media were carefully aspirated off leaving the dark blue formazan product in the bottom of the wells. The reduced MTT product was solubilized by adding 300 μl of DMSO, and 200 μl from each well were transferred to a 96-well microtiter plate. The absorbance of each well was measured using an automated plate reader. The growth inhibitory effects of each agent alone and their combination for a particular dosing schedule were analyzed by generating concentration effect curves as a plot of fraction of unaffected cells versus drug concentration. Dose response curves were plotted as percentages of the control cell absorbance. For each treatment, the percentage cytotoxicity was calculated by the following formula: [(P − A) / P] × 100%, where P equals mean of (absorbance − background absorbance) for controls and A equals background absorbance for treatment. The data were analyzed using a Prism (Graphpad Software, San Diego, CA) to determine the IC50.

Western Blot Analysis. Cells were treated with EGFR AS oligonucleotides (12.5 μM) or docetaxel (5 nM). After 4 days, cells were scraped into 1 ml of lysis buffer [1% NP40, 150 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate buffer (pH 7.2), 0.25 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin] in each well in a 24-well plate. For the in vivo studies, tumors were harvested and cell lysates were prepared and transferred to a clean Eppendorf tube and centrifuged for 30 min at 14,000 rpm. The supernatant was transferred to a clean tube, and protein concentrations were determined. Protein extracts (30 μg) were fractionated through 10% SDS-polyacrylamide gel and transferred to nitrocellulose filters (Protran Schleicher & Schuell, Inc.). The filters were blocked in 1× PBS with 0.2% Tween 20 and 5% nonfat milk overnight at 4°C. The filters were incubated with primary antibody and subsequently were washed with Blotto solution [50 mM Tris (pH 7.4), dry milk powder, 0.9% NaCl, 0.5% Tween 20] three times for 15 min. The filters were then incubated with secondary antibody for 1 h and washed with Blotto solution three times for 15 min. The filters were quickly rinsed with rinsing solution, and the blot was developed Luminol Regent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) by autoradiography. Antibodies used for blotting included an anti-EGF receptor monoclonal antibody (Transduction laboratories, Lexington, KY); actin (Ab-1) monoclonal mouse IgM (Oncogene Research Products, Boston, MA); phospho-p44/42 MAPK (Thr202/Tyr204; Cell Signaling Technology, Beverly, MA); p44/42 MAPK, (Cell Signaling Technology); phospho-AKT (Ser473; Cell Signaling Technology); and VEGF (A-20; Santa Cruz Biotechnology, Inc.).

Animal Studies. The 1483 cells were harvested by trypsination and resuspended in HBSS (Life Technologies, Inc., Rockville, MD) and centrifuged at 1300 rpm for 5 min. Cells were resuspended in Hank’s solution at a concentration of 1 × 10^6 cells/ml before s.c. implantation into mice. Female athymic nude mice nu/nu (4–6 weeks old; 20 ± 2 g; Harlan Sprague Dawley, Inc., Indianapolis, IN) were implanted with 1 × 10^6 cells into both the right and left flanks using a 27 gauge needle/0.5 ml tuberculin syringe ~5–7 days later when tumor nodules were palpable. Mice were randomly assigned to a treatment group: AS alone; docetaxel alone; AS plus docetaxel; sense plus docetaxel; or no treatment. There were 8–10 mice in each group, and the experiment was repeated three times. Intratumoral injection of oligonucleotide DNA (1 mg/kg in a volume of 50 μl) was administered three times/week. Docetaxel (20 or 5 mg/kg) was administered via i.p. injection once/week. Animals were treated until the tumors ulcerated or reached a maximum diameter of 2 cm. Tumor volume was calculated as...
length × width² / 2, and fractional tumor volume was calculated as the actual tumor volume relative to the tumor volume at the start of treatment. Animal care was in strict compliance with institutional guidelines established by the University of Pittsburgh, the Guide for the Care and Use of Laboratory Animals, and the Association for Assessment and Accreditation of Laboratory Animal Care and Accreditation of Laboratory Animal Care International.

RESULTS

EGFR AS Oligonucleotides Decrease EGFR Protein Expression and Inhibit SCCHN Growth. We have previously targeted several regions of the EGFR gene to achieve growth inhibition of SCCHN cells in vitro (5). AS oligodeoxynucleotides inhibit gene expression in a highly selective and target sequence-specific manner. Others have analyzed AS oligonucleotides targeting additional regions of the EGFR gene and found down-regulation of EGFR protein expression using an oligonucleotide-targeting coding region, position 157 (12), or an oligonucleotide targeting the opal translation termination codon at residues 3811–3825 (13). We compared the effects of AS oligonucleotides targeting the translation start site in addition to these other two regions of the EGFR gene and found that the sequence targeting 760–779 was most effective at inducing sustained down-modulation of EGFR protein expression in SCCHN cells (Table 1 and Fig. 1A). Additional investigation of the kinetics of EGFR down-regulation demonstrated that treatment of SCCHN cells with this AS sequence resulted in sustained down-modulation of EGFR protein expression levels up to 6 days, compared with treatment of cells with corresponding sense oligonucleotides (Fig. 1B; 67% decrease with EGFR AS versus 20% decrease with EGFR sense). In addition, this EGFR AS oligonucleotide was more effective at inhibiting SCCHN proliferation compared with other EGFR AS oligonucleotides sequences (Fig. 1C).
Docetaxel Inhibits SCCHN Cell Growth. Taxanes, including docetaxel, have demonstrated cytotoxicity against a variety of cancer cells in vitro (14). To determine the effects of docetaxel on SCCHN cells, the IC$_{50}$ dose was determined in three well-characterized cell lines derived from patients with head and neck cancer. The IC$_{50}$ was found to be $5\text{nM}$ in all three cell lines tested (Fig. 2).

**Antiproliferative Effects of EGFR AS plus Docetaxel.** We have previously reported inhibition of SCCHN proliferation by EGFR AS oligonucleotides (5). However, results of clinical trials, to date, suggest that EGFR-targeting strategies are more effective when combined with cytotoxic chemotherapy (3). To determine the cytotoxic effects of EGFR AS alone and in combination with docetaxel, cell growth assays were performed. Treatment of SCCHN cells with EGFR AS plus docetaxel resulted in increased cytotoxicity compared with treatment with EGFR AS alone, docetaxel alone, or with docetaxel plus control EGFR sense oligonucleotides at several time points (Fig. 3 and data not shown).

**EGFR AS plus Docetaxel Modulates EGFR Expression and Signaling Pathways in Vitro.** We have previously reported that down-modulation of EGFR using AS approaches in SCCHN cells leads to decreased EGFR expression (5, 15). To begin to explore the antiproliferative mechanisms of the combination of EGFR AS plus docetaxel, SCCHN cells were treated with EGFR AS (or control sense oligonucleotides) with or without docetaxel, followed by EGFR immunoblotting. As shown in Fig. 4, EGFR AS resulted in down-modulation of EGFR protein expression, an effect that was augmented by docetaxel.

**Activation of MAPKs has been shown to be decreased after EGFR blockade in SCCHN cell lines (16). To determine the effects on MAPK activation by combination therapy, SCCHN cells were treated with EGFR oligonucleotides with or without docetaxel. As shown in Fig. 4, docetaxel induced MAPK activation as determined by phosphorylated p44/42 MAPK expression in SCCHN cells. This effect appeared to be independent of EGFR AS because MAPK activation was not modulated by treatment of SCCHN cells with oligonucleotides. We and others (17, 18) have reported that activation of STAT3 and AKT are downstream of EGFR in SCCHN cells. As shown in Fig. 4,
EGFR Blockade and Docetaxel in Head and Neck Cancer

Both docetaxel (P < 0.0017) reduced tumor growth compared with control and these effects were additive (i.e., they did not interact, P = 0.8506; data not shown).

EGFR AS plus Docetaxel Inhibits EGFR Signaling Pathways in Vivo. To determine whether the increased antitumor effects observed with combined EGFR AS plus docetaxel treatment were accompanied by additive or synergistic modulation of signaling pathways, we examined expression levels of EGFR and activated EGFR signaling proteins in the tumors. As shown in Fig. 6, although EGFR AS or docetaxel alone resulted in a slight decrease of EGFR, VEGF, phosphotyrosine STAT3, or phospho-AKT, the tumors in the combined treatment group demonstrated increased down-modulation of EGFR and downstream signaling pathways. Specifically, docetaxel plus EGFR AS oligonucleotide treatment resulted in a 95.3% decrease in pAKT, compared with 9.4% reduction after docetaxel alone or 66.8% decrease with EGFR AS alone. Similarly, expression of phosphotyrosine STAT3 was decreased by 97.4% with combined treatment, compared with 66.8% with docetaxel or 51.1% with EGFR AS.

DISCUSSION

Overexpression and activation of EGFR is frequently detected in SCCHN where EGFR levels in the tumor correlate with survival, independent of known prognostic parameters, including nodal staging (1). Activation of EGFR by autocrine ligands such as TGF-α induces receptor phosphorylation, dimerization, and signaling via several well-characterized downstream pathways. The integrated biological responses to EGFR signaling are pleiotropic, including mitogenesis or apoptosis, enhanced cell motility, protein secretion, differentiation, or angiogenesis. In addition to its role in organ morphogenesis, maintenance, and repair, up-regulated EGFR signaling has been implicated in tumor progression. EGFR-directed therapies offer the opportunity to selectively target cells that express elevated levels of this oncogenic growth factor receptor. However, results of clinical trials, to date, suggest that only limited antitumor effects are observed when targeting EGFR alone as cancer therapy. We previously reported increased SCCHN growth inhibition using AS oligonucleotides compared with tyrosine kinase inhibitors or monoclonal antibodies (5). Additional investigation demonstrated that EGFR AS strategies down-modulated EGFR expression, as well as abrogation of mitogenic and anti-apoptotic signaling pathways in SCCHN cells (1, 20, 21). The results of this study demonstrate that increased antitumor effects...
are observed when EGFR AS oligonucleotides are combined with docetaxel in vitro and in vivo. Mechanistic determinations demonstrated that the augmented cytotoxicity occurred through both distinct and overlapping mechanisms, thus setting the stage for the rational design of combination therapeutic regimens.

AS DNA or RNA sequences can be designed that are highly specific to EGFR and/or its ligand(s). Binding of AS moieties to EGFR mRNA theoretically prevents transcription and translation. Moreover, EGFR is down-regulated via the degradation of AS DNA:mRNA hybrid by RNase H. AS moieties can be delivered using either viral or nonviral vectors. In vitro and in vivo administration of EGFR AS nucleic acids (full-length EGFR DNA, oligonucleotides, or RNA) have resulted in inhibition of tumor growth in a number of tumor types overexpressing EGFR (15, 22–24). Suppression of EGFR protein using an EGFR AS plasmid in SCCHN xenografts increased the rate of apoptosis detected by in situ staining for DNA fragmentation (15). We previously reported antitumor effects in vitro and in vivo using EGFR AS oligonucleotides or an EGFR AS sequences expressed under the control of the U6 small nuclear RNA promoter and delivered intratumorally with cationic liposomes (5, 15). Although the use of cationic lipids enhanced the antitumor effects of EGFR AS in vitro, we found that the addition of lipids did not improve transfection in vivo. Elimination of the need for the lipid carrier molecule in the clinical setting should reduce the potential toxicity of oligonucleotide delivery. We elected to use intratumoral administration of EGFR AS to insure that the oligonucleotides were delivered to the tumor cells. Phosphorothioate modifications were added to protect the oligonucleotide from degradation by endogenous

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**Fig. 6** EGFR AS plus docetaxel treatment additionally down-modulates expression of EGFR and downstream signaling pathways. Xenograft tumor tissues were harvested from athymic nude mice treated with docetaxel, EGFR AS, or both. Western blotting was performed with 30 μg of protein extracts. A, cumulative results showing relative protein expression levels as calculated by determining expression relative to β-actin using densitometry. B, representative blot showing down-regulation of EGFR, phosphotyrosine STAT3, VEGF, and pAKT by combined treatment (tumors from three representative animals were analyzed in each treatment group). C, representative immunoblotting from a mouse treated with docetaxel plus intratumoral injection of EGFR sense oligonucleotides (right flank) or intratumoral administration of EGFR AS oligonucleotides (left flank). D, cumulative results from 9 animals with bilateral tumors treated with EGFR sense or EGFR AS oligonucleotides plus docetaxel.
nucleases. Cancer patients have been treated with systemic administration of AS oligonucleotides targeting a number of molecules thought to contribute to cancer progression. Recent studies demonstrate the feasibility and an acceptable toxicity profile associated with systemic administration of phosphorothioated AS oligonucleotides in cancer patients (25). In addition, down-modulation of target gene expression has been reported in conjunction with the clinical administration of AS oligonucleotides (26).

Docetaxel has been widely used in the treatment of patients with advanced head and neck cancer, as well as in the neoadjuvant setting (7, 8, 27–29). In preclinical models, docetaxel has been reported to enhance response to radiation therapy (30). In a nude mouse SCCHN model, docetaxel treatment resulted in tumor growth inhibition without complete tumor regression (31, 32). As with other taxanes, docetaxel is an antimicrotubulin agent that promotes tubulin assembly in microtubules, inhibits depolymerization, acts as a mitotic spindle poison, and induces a mitotic block in proliferating cells (14). The molecular mechanism of the growth inhibitory effects of docetaxel in SCCHN models has not been extensively explored.

Accumulating preclinical and clinical evidence suggests that a combination of EGFR inhibitors with traditional cytotoxic therapy such as chemotherapy or radiation therapy may result in additive or synergistic therapeutic effects. The rationale behind the use of combination therapy is that when therapeutic agents with different mechanisms of actions are used, toxicities as well as resistance to single agents will be minimized and possible synergistic effects can be achieved. A combination of the small molecule EGFR tyrosine kinase inhibitor, ZD1839, with cytotoxic drugs produced a dose-dependent, supra-additive growth inhibition for cancer patients. Although paclitaxel has been shown to induce MAPK activation, the effects of docetaxel have not been previously reported. We found that docetaxel induced activation of MAPK in all SCCHN cell lines, independent of the effects of EGFR AS treatment. Our in vivo results support additive or synergistic effects of docetaxel plus EGFR AS in down-modulating expression of VEGF and activated STAT3 and AKT. We have previously demonstrated that blockade of EGFR results in decreased STAT3 activation and VEGF production (17, 38). Others have reported activation of phosphatidylinositol 3’-kinase and AKT downstream of EGFR in SCCHN cells (18).

Cumulative evidence suggests that the cellular damage induced by chemotherapy can convey EGFR ligands from growth factors to survival factors for cells that express functional EGFR (39). By blocking EGFR in conjunction with cytotoxic chemotherapy, cancer cells undergo apoptosis. Results from preclinical studies and preliminary clinical data strongly suggest that a combination of EGFR inhibitors with conventional cytotoxic therapy can greatly improve clinical efficacy for cancer treatment. The results of the present study demonstrate that targeting EGFR using AS oligonucleotides can enhance the antitumor activity of the chemotherapeutic agent, docetaxel. Mechanistic studies suggest that the augmented antitumor effects observed with combined therapy arise through both distinct and overlapping pathways. These results support the design of treatment strategies that combine docetaxel with EGFR targeting for cancer patients.

REFERENCES


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