Concomitant inhibition of epidermal growth factor and vascular endothelial growth factor receptor tyrosine kinases reduces growth and metastasis of human salivary adenoid cystic carcinoma in an orthotopic nude mouse model

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Abstract
We hypothesized that epidermal growth factor (EGF) receptor (EGFR) activation and vascular endothelial growth factor (VEGF)–induced angiogenic signals are important for the progression and metastasis of human salivary adenoid cystic carcinoma (ACC). To test this hypothesis, we evaluated the therapeutic effect of AEE788, a dual inhibitor of EGF and VEGF receptor (VEGFR) tyrosine kinases, on human salivary ACC. In clinical specimens of salivary ACC, EGFR and VEGF signaling proteins were expressed at markedly higher levels than in adjacent normal glandular tissues. We examined the effects of AEE788 on salivary ACC cell growth and apoptosis and on the phosphorylation of EGFR and VEGFR-2 in salivary ACC cells. Treatment of salivary ACC cells with AEE788, alone or in combination with chemotherapy, led to growth inhibition, induction of apoptosis, and dose-dependent inhibition of EGFR and VEGFR-2 phosphorylation. To determine the in vivo antitumor effects of AEE788, nude mice with orthotopic parotid tumors were randomized to receive oral AEE788 alone, paclitaxel alone, cisplatin alone, a combination of AEE788 plus paclitaxel, a combination of AEE788 plus cisplatin, or a placebo. AEE788 inhibited tumor growth and prevented lung metastasis in nude mice. To study the mechanism of interaction between AEE788 and chemotherapy, AEE788 was found to potentiate growth inhibition and apoptosis of ACC tumor cells mediated by chemotherapy. Tumors of mice treated with AEE788 and AEE788 plus chemotherapy exhibited down-regulation of activated EGFR and VEGFR-2, increased tumor and endothelial cell apoptosis, and decreased microvessel density, which correlated with a decrease in the level of matrix metalloproteinase-9 and matrix metalloproteinase-2 expression and a decrease in the incidence of vascular metastasis. These data show that EGFR and VEGFR can be molecular targets for therapy of salivary ACC.

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Angiogenesis is also crucial for the progression and metastasis of many types of human tumors. However, few studies have examined the implications of expression of angiogenesis-related factors in salivary cancer. A recent study reported that both vascular endothelial growth factor (VEGF) and basic fibroblast growth factor are major angiogenesis factors in salivary gland tumors (7). In another study, loss of heterozygosity on chromosome 6q in ACC correlated with decreased expression of thrombospondin-2, which is a potent inhibitor of tumor angiogenesis (8). Furthermore, microvessel density is considered to be a prognostic indicator for the incidence of distant metastasis in salivary ACC (9). These findings suggest that the angiogenic signaling pathways of salivary ACC are potential therapeutic targets.

Because of the rarity of ACC, very few clinical trials have been conducted to address the response of this tumor to conventional chemotherapy. Among the few tested agents, cisplatin was the most effective in a number of small trials (10, 11). In addition, paclitaxel has been found to potentiate the effect of new drugs, such as AEE788, against prostate and head and neck tumors in a number of preclinical trials (12, 13).

We hypothesized that EGFR activation and VEGF-induced angiogenic signals are important for the progression and metastasis of human salivary ACC, and therefore their inhibition could be integrated into treatment strategies for this tumor. In the present study, we evaluated the therapeutic effect of a dual-family EGFR/ErbB2 and VEGF receptor (VEGFR) tyrosine kinase inhibitor, AEE788, alone and in combination with paclitaxel and cisplatin, against established ACC growing in the parotid glands of nude mice.

Materials and Methods
Salivary Cancer Cells and Culture Conditions
A salivary cancer cell line, ACC3, was provided by Dr. Takashi Saku (Department of Pathology, Niigata University School of Dentistry, Niigata, Japan). The cells were maintained as monolayer cultures in RPMI 1640 containing 15% FCS, 1-glutamine, vitamins (Life Technologies, Inc., Grand Island, NY), and penicillin-streptomycin (Flow Laboratories, Rockville, MD). The cells were incubated in a mixture of 5% CO₂ and 95% air at 37°C. The cultures were free of Mycoplasma and pathogenic murine viruses.

Reagents
AEE788, which belongs to the 7H-pyrrrolo[2,3-d] class of pyrimidines, was provided for this study by Novartis Pharma AG (Basel, Switzerland). For in vivo administration, AEE788 was dissolved in 90% polyethylene glycol 300 plus 10% 1-methyl-2-pyrrolidinone to a concentration of 6.25 mg/mL. Paclitaxel was obtained from Sigma Chemical Co. (St. Louis, MO) and administered at 200 µg/wk, a dose regimen that was previously reported in the literature (15). Propidium iodide and tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were both purchased from Sigma Chemical.

The following antibodies for immunohistochemical analysis were purchased: rabbit polyclonal anti-EGF, anti-EGFR, anti-VEGF, and anti-VEGFR-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); mouse monoclonal anti-erbB2 and anti–mitogen-activated protein kinase (MAPK; p42), rabbit polyclonal anti-Akt, anti–phospho-Akt (pAkt), and anti–phospho-MAPK (pMAPK; Cell Signaling Technology, Beverly, MA); mouse anti–transforming growth factor-α (TGFA) and rabbit polyclonal anti–phospho-VEGFR-2 (pVEGFR-2; Oncogene, Cambridge, MA); rabbit polyclonal anti-MMP-9 and anti-MMP-2 (Chemicon, Temecula, CA); rabbit polyclonal anti–phospho-EGFR (pEGFR; Biosource International, Camarillo, CA); mouse anti–proliferating cell nuclear antigen (PCNA) clone PC-10 (DAKO A/S, Copenhagen, Denmark); rat monoclonal anti–CD31/platelet/endothelial cell adhesion molecule 1 (PharMingen, San Diego, CA); peroxidase-conjugated goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, West Grove, PA); peroxidase-conjugated rat anti-mouse immunoglobulin G2a (Seraotec; Harlan Bioproducts for Science, Inc., Indianapolis, IN); peroxidase-conjugated goat anti-rat immunoglobulin G1 (Jackson Research Laboratories); and Alexa Fluor 594–conjugated goat anti-rat immunoglobulin G and Alexa Fluor 488–conjugated anti-rabbit immunoglobulin G (Molecular Probes, Eugene, OR).

Immunohistochemical Analysis of EGF and VEGF Signaling Molecules in Human Salivary ACC
To determine the presence and the status of EGFR/ErbB2 and VEGF, 35 human salivary ACC samples were obtained from surgical specimens resected from patients who did not receive chemotherapy, under Health Insurance Portability and Accountability Act regulations at The University of Texas M.D. Anderson Cancer Center. Human salivary tumor specimens were evaluated for the presence of TGFA-α, EGF, EGFR/pEGFR, ErbB2, VEGF, and VEGFR-2/pVEGFR-2. Slides were prepared and viewed as previously described (12). Dilutions of primary antibodies were as follows: TGFA-α, 1:100; EGF, 1:100; EGFR, 1:200; pEGFR, 1:25; ErbB2, 1:50; VEGF, 1:500; VEGFR-2, 1:200; and pVEGFR-2, 1:400.

In vitro Cytotoxicity and Apoptosis Assays
For cytotoxicity assay, tumor cells were seeded into 38-mm² wells of 96-well plates and allowed to adhere for 24 hours. The cultures were then refed with medium with 2% serum. After 24 hours, cells were treated with different concentrations of paclitaxel with or without AEE788. Negative control cells were treated with medium alone. After 72-hour incubation, cell viability was assayed by measuring formazan produced by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. For apoptosis assay, tumor cells were plated at a density of 2 × 10⁴ per well in six-well plates (Costar, Cambridge, MA). After a 24-hour attachment period, the media were changed to 2% serum and cells were maintained for 24 hours.
24 hours and then treated with AEE788 and paclitaxel/cisplatin. After 72 hours, both adherent and detached cells were harvested, washed with PBS, and resuspended in propidium iodide 50 μg/mL in 0.1% sodium citrate for 20 minutes at 4°C. Flow cytometric analysis was done and the percentage of the sub-G0/G1 fraction was used as a measure of the percentage of apoptotic cells.

**Western Blotting**

Serum-starved ACC3 cells were treated for 1 hour with AEE788 (0.001–1 μmol/L) and with DMSO alone for controls, incubated with or without 40 ng/mL EGF for 15 minutes. Cell lysates were obtained and subjected to Western blot analysis as previously described (12), and the primary and secondary antibodies were bought from Cell Signaling (Danvers, MA) and Santa Cruz Biotechnology, respectively. Signals were visualized by the SuperSignal West Pico Chemiluminescent system from Pierce (Rockford, IL).

**Animals and Orthotopic Implantation of Tumor Cells**

Eight-week-old male nude mice were purchased from the Animal Production Area of the National Cancer Institute–Frederick Cancer Research and Development Center (Frederick, MD). The mice were maintained in a pathogen-free environment at facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U.S. Department of Agriculture, the U.S. Department of Health and Human Services, and the NIH. The mice were used in accordance with the Animal Care and Use Guidelines of our institute.

To produce tumors, ACC3 cells were harvested from subconfluent cultures. The cells were resuspended in Ca2+- and Mg2+-free HBSS. A total of 1 × 106 cells were resuspended in 30 μL of HBSS and injected into the parotid gland using a 30-gauge hypodermic needle and a tuberculin syringe. A well-localized bleb confirmed a successful injection without leakage of the tumor cells. The preauricular incision was approximated in one layer using a single metal clip.

**Treatment of Established Human Salivary Carcinomas Growing in the Parotid Glands of Nude Mice**

Eight days after injection of the tumor cells, tumor nodules were palpable. Mice with similarly sized tumors were randomized into one of four groups (n = 6-10): group 1, the control group, received an oral diluent for AEE788 (90% polyethylene glycol 300 plus 10% 1-methyl-2-pyrrolidinone) and i.p. HBSS; group 2, the paclitaxel group, received paclitaxel by i.p. injection once a week (200 μg/kg); group 3, the AEE788 group, received AEE788 (50 mg/kg) p.o. thrice a week; and group 4, the combination therapy group, received the i.p. paclitaxel regimen of group 2 and the oral AEE788 regimen of group 3 concomitantly. A similar treatment experiment was conducted with the substitution of paclitaxel for cisplatin (1.5 mg/kg) i.p. once a week. Treatments continued for 4 weeks. Tumor volume and body weight were measured about once a week. Tumor volume was measured using the formula (A × B × B) / 2, where A is the longest diameter and B is the shortest diameter of the tumor. For the incidence of lung metastasis, the AEE788-paclitaxel animal study design shown above was used; however, the duration of treatment was 7 weeks.

**Necropsy Procedures and Histologic Examinations**

The mice were euthanatized using carbon dioxide, and primary tumors in the parotid glands were excised and weighed. For immunohistochemical and routine H&E staining, one part of the tumor tissue was fixed in formalin and embedded in paraffin. Another part was embedded in optimum cutting temperature compound (Miles, Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at −70°C. Cervical lymph nodes and lungs were harvested and the presence of metastatic lesions was confirmed by histologic review.

**Immunohistochemical Determination of EGFR/pEGFR, Akt/pAkt, MAPK/pMAPK, MMP-9/MMP-2, PCNA, and CD31**

Paraffin-embedded tissues were prepared for detection of EGFR/pEGFR, MAPK/pMAPK, MMP-9/MMP-2, and PCNA. Frozen tissues were used for detection of Akt/pAkt and CD31. Slides were prepared as previously described (12). Dilutions of primary antibodies were as follows: EGFR, 1:200; pEGFR, 1:25; Akt, 1:100; pAkt, 1:100; MAPK, 1:100; pMAPK, 1:250; MMP-9, 1:50; MMP-2, 1:400; PCNA, 1:100; and CD31, 1:400. Immunohistochemical procedures were done as described above.

**Immunofluorescence Double Staining for CD31 and VEGFR-2, Phosphorylated VEGFR-2, or Terminal Deoxynucleobuclotidyl Transferase – Mediated dUTP Nick End Labeling**

For terminal deoxynucleobuclotidyl transferase–mediated dUTP nick end labeling (TUNEL) and double immunofluorescence assays, frozen tissues were used. After fixation with acetone, the samples were washed with PBS, incubated with protein-blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 minutes, and then incubated with a 1:400 dilution of anti-CD31 antibody overnight at 4°C. After washing with PBS, the slides were incubated with a 1:600 dilution of secondary antibody conjugated to Alexa Fluor 594 (red) for 1 hour in the dark. TUNEL assay was done using a commercial apoptosis detection kit (Promega Corp., Madison, WI) as previously described (12). Immunofluorescence double staining was done by staining frozen samples with CD31. The primary antibodies used were anti-VEGFR-2 (1:200) and anti–phospho-VEGFR-2 (1:400). Details of the Immunofluorescence double staining have previously been described (12).

**Quantification of PCNA, Microvessel Density, Absorbance, and Apoptotic Tumor and Endothelial Cells**

For quantification analyses, five slides were prepared for each group and two areas were selected on each slide. The positively stained cells and total cells were counted in 10 random 0.159-mm2 fields of tumor area at ×100 magnification for PCNA expression and in 10 random 0.039-mm2 fields at ×200 magnification for TUNEL expression. The percentages of positively stained cells among the total number of cells were calculated and
compared. For the quantification of microvessel density, regions of high vascular density were identified by scanning the tumor sections at low microscopic power (×40). The vessels that were completely stained with anti-CD31 antibodies were counted in 10 random 0.159-mm² fields at ×100 magnification.

For quantification of staining intensity, the absorbance of 100 MMP-9- and MMP-2-positive cells in 10 random 0.039-mm² fields taken from treated tumor tissues was measured at ×200 magnification using the Optimas Image Analysis software. The samples were not counterstained so that the absorbance would be attributable solely to the product of the immunohistochemical reaction. MMP-9 and MMP-2 cytoplasmic immunoreactivity was evaluated using computer-assisted image analysis and expressed as mean density values. Quantification of apoptotic endothelial cells was expressed as the average of the ratios of apoptotic endothelial cells to the total number of endothelial cells in 10 random 0.039-mm² fields at ×200 magnification.

Statistical Analysis
To detect the difference in tumor volume over time, a repeated measures analysis was used with a LOWESS nonparametric smoother and the area-under-the-curve response feature between treatments. Tumor volume differences across groups and between groups were assessed using the Kruskal-Wallis test and the Wilcoxon rank-sum test, respectively. Associations between treatment groups and incidence of lung metastases were examined with Fisher’s exact test. The staining intensity of human ACC tumor tissue and adjacent normal salivary tissue was compared using the unpaired Student t test. Wilcoxon rank-sum test was used for the quantification of PCNA, TUNEL, microvessel density and CD31/TUNEL, and absorbance of MMP-9 and MMP-2 positive cells. A two-tailed P < 0.05 was considered significant. All statistical analyses were done using Stata 9.0 (StataCorp, College Station, TX).

Results
ACC Surgical Specimens Overexpress EGFR, ErbB2, and VEGFR-2
We first evaluated the expression of TGF-α, EGF, EGFR/pEGFR, ErbB2, VEGF, and VEGFR-2/pVEGFR-2 in 35 human salivary ACC samples. Adjacent normal salivary ducts, uninvolved by the tumor, served as controls. Most of the tumors, especially ACC with a tubular-cribriform pattern, were positive for all of these biomarkers (Fig. 1). The overall percentages of the 35 specimens expressing EGFR, ErbB2, and VEGFR-2 were 77%, 63%, and 77%, respectively. Activated forms of EGFR and VEGFR-2 were also expressed in the matching specimens. These were predominantly found in the tubular structures of tubular ACC and in the ductlike structures of the large cribriform pattern of ACC. In addition, strong TGF-α, EGF, and VEGF immunoreactivity was evident in the cytoplasm of tumor cells in solid, tubular, ductlike, and pseudocystic structures. Positive TGF-α, EGF, EGFR, ErbB2, and VEGFR-2 staining was also found in the ductal segments of normal salivary glands but at a significantly lower staining intensity than in ACC tumor cells (P < 0.01; data not shown).

In vitro Cytotoxicity Mediated by AEE788 and Paclitaxel
To evaluate the effects of AEE788 on growth inhibition and paclitaxel-induced cytotoxicity in human salivary cancer cells, ACC3 cells were incubated for 72 hours in

Figure 1. Immunohistochemical analyses for the expression of EGF and VEGF signaling molecules in human salivary tumor tissues. Sixteen specimens were immunostained for expression of TGF-α, EGF, EGFR/pEGFR, ErbB2, VEGF, and VEGFR-2/pVEGFR-2. Adjacent normal salivary ducts served as controls. Representative results. Bar, 100 µm.
medium containing increasing concentrations (0–8 μmol/L) of AEE788. In addition, ACC3 cells were treated with increasing doses of paclitaxel in the absence or presence of AEE788. When grown in media with 2% serum, the growth of ACC3 cells was inhibited by AEE788 in a dose-dependent manner at an IC_{50} of 4.3 μmol/L (Table 1). Furthermore, the IC_{50} of 3.4 nmol/L for paclitaxel decreased to 2.6 and 0.9 nmol/L, respectively, when the cells were exposed to both paclitaxel and a noncytotoxic concentration of AEE788 (1.5 and 3 μmol/L). Therefore, the cytotoxicity of paclitaxel was intensified by AEE788, but AEE788 did not increase the level of growth inhibition achieved with higher concentrations of paclitaxel.

**In vitro Induction of Apoptosis by AEE788, Paclitaxel, and Cisplatin**

To assess the effects of AEE788 on the induction of apoptosis and on chemotherapy-induced apoptosis in human salivary cancer cells, ACC3 cells were incubated with paclitaxel or cisplatin in the absence or presence of AEE788 and then DNA fragmentation was quantified using flow cytometry with propidium iodide staining. Seventy-two hours after the start of treatment with AEE788, 50% of the ACC3 cells were apoptotic at 8.2 μmol/L (data not shown). At 12 nmol/L paclitaxel, 50% of the cells underwent apoptosis. By adding only 2 μmol/L AEE788, the concentration of paclitaxel needed to achieve 50% cell kill was reduced to 8 nmol/L. Similarly, at 14 μmol/L cisplatin, 50% of the cells were apoptotic. This concentration was reduced to 10 μmol/L on addition of 2 μmol/L AEE788 (Table 2).

**Inhibition of EGFR, VEGFR-2, Akt, and MAPK Phosphorylation in Salivary ACC Cells by AEE788**

To determine whether in vitro treatment of ACC3 cells with AEE788 can inhibit EGF-stimulated tyrosine phosphorylation of EGFR, VEGFR-2, Akt, and MAPK, ACC3 cells were incubated with AEE788 for 1 hour, followed by a 15-minute incubation with EGF. Untreated, EGF-stimulated cells exhibited high levels of phosphorylated EGFR and its downstream signaling kinases (pAkt and pMAPK) and also expressed high levels of phosphorylated VEGFR-2. Incubation of cells with AEE788 inhibited the phosphorylation of EGFR, VEGFR-2, Akt, and MAPK in a dose-dependent manner (Fig. 2). The phosphorylated forms of EGFR, VEGFR-2, Akt, and MAPK tyrosine kinases were inhibited at 1 μmol/L AEE788. However, the total levels of EGFR, VEGFR-2, Akt, and MAPK remained unchanged after AEE788 treatment.

**Table 1. Effects of AEE788 and paclitaxel on the induction of growth inhibition in ACC3 cells**

<table>
<thead>
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<th>Drug</th>
<th>IC_{50}</th>
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<tr>
<td>AEE788</td>
<td>4.3 μmol/L</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>5 nmol/L</td>
</tr>
<tr>
<td>Paclitaxel + AEE788(1.5 μmol/L)</td>
<td>3 nmol/L</td>
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**Inhibition of Tumor Growth and Vascular Metastasis**

To assess the effect of AEE788 on the in vivo growth of ACC3 cells, we used our murine orthotopic tumor model. Mice bearing orthotopic parotid tumors were treated for 4 weeks (Fig. 3). The Kruskal-Wallis test was used to detect at what day we start seeing difference between the treated groups. For the AEE788-paclitaxel and AEE788-cisplatin studies, a statistical difference between the treatment groups was evident at day 14 (P < 0.05) and day 21 (P < 0.05), respectively. For the AEE788-paclitaxel study, using the repeated measures analysis, the differences in the median tumor volume of the AEE788 + paclitaxel treatment group versus the paclitaxel treatment group (P < 0.01) and the AEE788 treatment group (P < 0.05) were statistically significant at the end of the study. As for the AEE788-cisplatin study and using the repeated measures analysis, the differences in the median tumor volume of the AEE788 + cisplatin treatment group versus the cisplatin treatment group (P < 0.05) was significant; however, difference in median tumor volume between the AEE788 + cisplatin group versus the AEE788 treatment group approached but did not reach statistical difference (P > 0.05) at the end of the study. At the end of the 4-week treatment, the median parotid tumor volumes (±SE) were 821.92 ± 187.92 mm³ in controls and 759.12 ± 99.24 mm³ in mice treated with paclitaxel. AEE788 treatment resulted in a significant reduction in tumor volume (454.11 ± 68.55 mm³; P < 0.05). Moreover, the combination of AEE788 and paclitaxel resulted in a highly significant reduction in tumor volume (311.73 ± 67.32 mm³; P < 0.01). Comparable results were obtained for the AEE788-cisplatin study, with the major difference being that the combination of cisplatin and AEE788 did not significantly add to the inhibitory effect of AEE788 (Fig. 1).

We could not detect tumor cells in cervical lymph nodes or the lungs of mice in all groups at the end of the 4-week treatment studies. However, when the same AEE788-paclitaxel animal study was carried out for 7 weeks (versus 4 weeks), distant hematogenous lung metastasis was histologically detected in 5 of the 10 control mice, 2 of the 10 paclitaxel-treated mice, 0 of the 10 AEE788-treated mice, and 1 of the 10 mice in the combination therapy group. Thus, our data suggest that AEE788 was able to prevent hematogenous metastasis to the lung compared with controls (P < 0.05). AEE788 was well tolerated by the animals, without significant side effects.

**Table 2. Effects of AEE788 on paclitaxel- and cisplatin-induced apoptosis**

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC_{50}</th>
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<tbody>
<tr>
<td>Paclitaxel</td>
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<tr>
<td>Paclitaxel + AEE788(2 μmol/L)</td>
<td>8 nmol/L</td>
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<tr>
<td>Paclitaxel + AEE788(2 μmol/L)</td>
<td>3 nmol/L</td>
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<tr>
<td>Cisplatin</td>
<td>14 μmol/L</td>
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<tr>
<td>Cisplatin + AEE788(2 μmol/L)</td>
<td>10 μmol/L</td>
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effects, as determined by the maintenance of body weight (data not shown).

Effect of AEE788 on Salivary ACC in an Orthotopic Nude Mouse Model

To study the effect of treatment on the tumors grown in mice, we used immunohistochemical analysis on the tumors from the AEE788-paclitaxel study. Immunohistochemical analysis showed that tumors from mice in all four treatment groups expressed similar levels of EGFR and its major downstream effectors (Akt and MAPK; data not shown). However, tumors from mice treated with AEE788 or the combination of AEE788 plus paclitaxel had lower levels of activated EGFR, activated AKT, and activated MAPK compared with the control and paclitaxel-treated groups (data not shown). Furthermore, tumors from mice treated with AEE788 or combination therapy had lower PCNA levels, more TUNEL-positive cells, and lower expression of MMP-9 and MMP-2 compared with the controls (Table 3). Quantitatively, the mean microvessel density was highest in the control group (15.9 ± 8.9) and significantly lower in the paclitaxel (10.8 ± 3.2; P < 0.05), AEE788 (5.7 ± 2.6; P < 0.01), and AEE788 plus paclitaxel (5.5 ± 1.3; P < 0.01) treatment groups. To detect apoptotic endothelial cells, double staining for CD31 (red staining) and TUNEL (green staining) was done. The percentage of CD31/TUNEL* cells (yellow signal) was significantly higher in the tumors from mice treated with paclitaxel, AEE788, or AEE788 plus paclitaxel compared with controls (P < 0.01). However, there was no significant difference in microvessel density and apoptotic endothelial cells between the AEE788

Figure 2. Effect of AEE788 on phosphorylation levels of EGFR, VEGFR-2, Akt, and MAPK in ACC3 cells. Serum-starved ACC3 cells were treated with AEE788 (0–1 μmol/L) for 1 h and then incubated with or without 40 ng/mL EGF for 15 min. Western blotting was done on protein extracts that were probed with anti–phospho-EGFR (Tyr1068), anti–phospho-VEGFR-2 (Tyr1068), anti–phospho-Akt (Ser473), or anti-pMAPK (Thr202/Tyr204) antibody. Membranes were reprobed with anti-EGFR, anti-VEGFR-2, anti-Akt, anti-MAPK, or β-actin as protein and loading controls. The assay was done in triplicate independently with similar results.
plus paclitaxel-treated and AEE788-treated groups. Double staining for CD31/activated VEGFR-2, which was done with CD31 (red staining) and activated VEGFR-2 (green staining), revealed that only tumors from mice treated with AEE788 and AEE788 plus paclitaxel had decreased activation of the two biomarkers (Fig. 4).

Discussion
We report that blockade of EGF and VEGFR tyrosine kinases by AEE788 inhibits orthotopic tumor growth of ACC and prevents lung metastasis in nude mice through the induction of tumor and endothelial cell apoptosis. Furthermore, combination therapy of AEE788 plus paclitaxel and/or cisplatin produces a synergistic therapeutic response, resulting in a significant reduction in tumor growth and an increase in tumor cell apoptosis. Our data also provide experimental evidence for the relation of MMP-9 and MMP-2 in neoangiogenesis and vascular metastasis in human salivary ACC.

To initiate the current study, we determined that human salivary ACC expresses EGF and VEGF signaling proteins, which can provide survival and metastatic advantages to tumor cells through multiple mechanisms, including increased angiogenesis. We had found that tumor-associated endothelial cells and stromal cells, such as infiltrating inflammatory cells and tissue-specific fibroblasts, as well as tumor cells, were positive for expression of TGF-α, EGF, and VEGF. These findings suggest that EGF and VEGF may play an important biological role as mitogens in the autocrine system and that specific organ microenvironments play an important role in the progression of salivary ACC.

In this study, we developed an orthotopic model of parotid ACC by injecting ACC3 cells into the parotid glands of athymic mice. One of the striking features of human salivary ACC is that metastases are usually hematogenous and most frequently involve the lungs. Lymph node metastases are uncommon and, when present, are more often the result of direct extension of the tumor into the lymph node rather than embolic spread (16). Our orthotopic nude mouse model of parotid ACC exactly followed the metastatic pattern of human tumors. Using this parotid tumor model, we could evaluate the effect of chemotherapeutic agents on vascular metastasis to the lung.

In the present study, AEE788, which is a reversible inhibitor of EGFFR and VEGFR kinases, potently inhibited the receptor phosphorylation in ACC3 cells in vitro and in vivo. Furthermore, AEE788 inhibits phosphorylation of Akt and MAPK, which are downstream effectors of the EGF and VEGF signaling pathways that promote cellular proliferation and survival (17, 18). AEE788 at 1 μmol/L, which is below those doses reported to be achievable in vivo (19), can inhibit the phosphorylation of EGFFR, VEGFR-2, Akt, and MAPK in ACC3 cells. Treatment of salivary ACC cells with AEE788 led to dose-dependent inhibition of cellular proliferation and induction of apoptosis. Oral administration of AEE788 significantly reduced human salivary tumor growth in this orthotopic murine model.

Expression of MMPs has been associated with tumor cell invasion and metastasis. Moreover, MMPs can have an angiogenic role by releasing matrix-bound proangiogenic factors (20). A recent study reported that the switch from vascular quiescence to angiogenesis involves MMP-9 (21). Therefore, MMP activity can enhance tumor growth and survival, invasion, angiogenesis, and metastasis. In the present study, we showed inhibition of MMP-9 and MMP-2 expression after treatment with AEE788.
Under normal resting conditions, MMP-9 expression is low or absent, and a stimulus is required to increase its production. The EGF signaling pathway has been implicated in the up-regulation of MMP-9 in head and neck squamous carcinoma cells (22) and non–small-cell lung cancer (23). Several reports strongly suggest a role of MAPK in MMP-9 regulation through the regulation of activator protein-1, which is one of the major governing elements in the MMP-9 gene (24–26). These studies support our immunohistochemical results, which showed decreased protein expression of MMP-9 after treatment with the EGFR inhibitor AEE788. Furthermore, other research has shown that blocking of phosphatidylinositol 3-kinase by selective inhibitors effectively inhibited beta-cellulin-enhanced MMP-9 expression in head and neck squamous cell carcinoma cells, indicating that phosphatidylinositol 3-kinase/Akt is required in this process (27, 28). MMP-9 has a nuclear factor κB (NF-κB) binding site in its promoter region and, therefore, expression of MMP-9 is regulated by NF-κB transcriptional activity (28). NF-κB transcriptional activity, in turn, has been shown to be affected by Akt (29, 30). In addition, phosphatidylinositol 3-kinase/Akt signaling is also reported to be associated with MMP-2 activity in malignant gliomas (31). Induction of membrane type 1 MMP, which is a major activator of MMP-2, was blocked by a phosphatidylinositol 3-kinase inhibitor (32). Consistent with the above studies, our immunohistochemical analysis suggests that inhibition of EGFR by AEE788 decreased Akt activity and subsequently decreased MMP-9 and MMP-2 production. Furthermore, intratumoral microvessel density was decreased significantly and metastases were suppressed after treatment with AEE788.

Paclitaxel inhibits cell replication and subsequently activates cellular apoptosis (33). The antitumor activity of paclitaxel has been shown to be potentiated by combination with EGFR tyrosine kinase inhibitors such as ZD1839 or PKI166 (34, 35). A recent study reported that ZD1839 promoted paclitaxel-induced apoptosis of tumor cells by blocking paclitaxel-induced activation of the EGFR/extra-cellular signal-regulated kinase antiapoptotic pathway (36). Although anticyclic agents are not as effective in salivary tumors because the growth rate of these tumors is so slow, paclitaxel is currently being evaluated in salivary malignancies. Morelli et al. (37) showed that a synergistic antiproliferative activity was obtained when chemotherapy (platinum or taxane-based) was followed by treatment with EGFR antagonists (gefitinib, ZD6474, cetuximab) in esophageal cancer cells. Similarly, in our study, there was an interaction between AEE788 and paclitaxel/cisplatin treatment. In a multicenter phase II study undertaken to study the efficacy and safety of cetuximab, an anti-EGFR

![Figure 4](image_url)
monoclonal antibody, in conjunction with cisplatin to patients with refractory metastatic or recurrent squamous cell carcinoma of the head and neck, cetuximab and cisplatin were found to be an active regimen (38). In another study, the addition of bevacizumab, an anti-VEGF antibody, to paclitaxel-carboplatin chemotherapy extends survival in a select group of patients with nonsquamous cell lung cancer (39). In the present study, AEE788 inhibited the in vivo growth of ACC xenografts in nude mice. However, the highest growth inhibition was achieved by concomitant administration of AEE788 and paclitaxel. In addition, our quantitative immunohistochemical analysis suggests that paclitaxel plus AEE788 has a synergistic effect on the inhibition of tumor cell proliferation (PCNA+) and the induction of tumor cell apoptosis (TUNEL+) compared with each single treatment group (P < 0.05). There is another possible scenario that may explain the interaction between AEE788 and chemotherapy in our study and that needs to be verified in future studies: The VEGF ligand is possibly inducing a multidrug resistance phenotype in endothelial cells to cisplatin and taxol. Actually, Zhang et al. (40) have reported that in human-derived dermal endothelial cells, VEGF provides this protective effect partly by inducing the up-regulation of lung drug resistance protein and multidrug resistance–associated protein, as well as the down-regulation of Bax protein. Thus, by inhibiting the activation of VEGFR-2 on the surface of tumor-associated endothelial cells, AEE788 is possibly removing that protective shield that is allowing the endothelial cells to resist chemotherapy-induced apoptosis.

In summary, we have shown that EGF and VEGFR and their ligands are present in most human salivary ACC, and the blockade of EGF and VEGFR by AEE788 has significant antitumor effects on human salivary ACC cell xenografts in nude mice. The inhibition of in vitro and in vivo growth of tumor cells is mediated by both antitumor effects and antiangiogenic effects. The antitumor effect of AEE788 was enhanced when it was combined with paclitaxel and/or cisplatin. The decreased expression levels of MMP-9 and MMP-2 after treatment with AEE788 likely contributed to decreased microvessel density, which in turn was correlated with primary tumor growth and vascular metastasis to the lung. Therefore, AEE788 is a promising candidate for study in clinical trials in salivary ACC, which has a propensity for delayed lung metastasis.

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References


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Concomitant inhibition of epidermal growth factor and vascular endothelial growth factor receptor tyrosine kinases reduces growth and metastasis of human salivary adenoid cystic carcinoma in an orthotopic nude mouse model

Maher N. Younes, Young Wook Park, Yasemin Dakak Yazici, et al.

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