Cetuximab and Irinotecan Interact Synergistically to Inhibit the Growth of Orthotopic Anaplastic Thyroid Carcinoma Xenografts in Nude Mice

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Abstract

Purpose: Anaplastic thyroid carcinoma (ATC) remains one of the most lethal known human cancers. Targeted molecular therapy with cetuximab, a monoclonal antibody against epidermal growth factor receptor, offers new treatment potentials for patient with ATC. Cetuximab has also been reported to have synergistic effects when combined with irinotecan, a topoisomerase inhibitor. Therefore, we hypothesized that cetuximab and irinotecan would be effective in inhibiting the growth and progression of ATC in a murine orthotopic model.

Experimental Design: The in vitro antiproliferative effects of cetuximab and irinotecan on ATC cell line ARO were examined. We also studied the in vivo effects of cetuximab and irinotecan on the growth, invasion, and metastasis of orthotopic ATC tumors in nude mice. The in vivo antitumor efficacy of cetuximab/irinotecan combination was also compared with that of doxorubicin.

Results: Cetuximab alone did not show any antiproliferative or proapoptotic effect on this cell line. However, when combined with irinotecan, cetuximab potentiated the in vitro antiproliferative and proapoptotic effect of irinotecan. Cetuximab, irinotecan, and cetuximab/irinotecan combination resulted in 77%, 79%, and 93% in vivo inhibition of tumor growth, respectively. Incidences of lymph node metastasis, laryngeal invasion, and tumor microvessel density were also significantly decreased in these treatment groups. Furthermore, the cetuximab/irinotecan combination was significantly more effective than doxorubicin in inhibiting the growth of orthotopic ATC xenografts.

Conclusions: Combination therapy with cetuximab/irinotecan inhibits the growth and progression of orthotopic ATC xenografts in nude mice. Given the lack of curative options for patients with ATC, combination therapy with cetuximab and irinotecan treatment warrants further study.

Carcinomas of the thyroid gland account for ~1% of all new malignant diseases in the United States (1). Relatively high cure rates can be achieved in well-differentiated thyroid carcinomas, such as papillary and follicular thyroid carcinomas. However, anaplastic thyroid carcinoma (ATC) is one of the most aggressive human malignancies known and carries a grave prognosis. Although ATC accounts for only 1.6% of all thyroid cancers, the median overall survival following diagnosis is only 6 months (2, 3).

The treatment of ATC is often multidisciplinary and frequently includes the use of doxorubicin for which single-agent response rates range from 5% to 20% (4, 5). Regardless, it is clear that no effective treatments exist for ATC. This may be due in part to the rarity of this disease but nevertheless reflects the inadequacy of the available treatment options and suggests an urgent need for development of novel treatment strategies.

It is well established that the epidermal growth factor receptor (EGFR) is a valid and promising therapeutic target in solid tumors that overexpress this receptor. In particular, multiple preclinical and clinical studies have shown the therapeutic efficacy of EGFR inhibition in lung, head and neck, and colorectal carcinoma (6, 7). Inhibition of the EGFR pathway as a therapeutic modality for ATC is a novel approach that has yet to be fully investigated. Ensinger et al. examined the expression of EGFR in the largest collection of ATC specimens to date (25 specimens) and found this receptor to be overexpressed in 40% of the specimens (8). ATC cell lines derived from human tumors have also been shown to express variable levels of EGFR (9). Inhibition of...
EGFR pathway using monoclonal antibodies and small-molecule inhibitors has shown antiproliferative effects on ATC cell lines in vitro (9, 10). Schiff et al. were the first to report the in vivo effects of EGFR inhibition on ATC xenografts in nude mice (11). In this study, the administration of gefitinib (Iressa), a small-molecule inhibitor of the EGFR tyrosine kinase, to nude mice bearing s.c. ATC xenografts resulted in significant inhibition of tumor growth. Kim et al. also showed that AEE788, a dual inhibitor of EGFR and vascular endothelial growth factor receptor tyrosine kinases, produced significant cytostatic and cytotoxic effects on ATC cell lines in vitro and also inhibited the growth of s.c. ATC xenografts in nude mice (12).

Cetuximab (Erbitux, C225), a human-murine chimeric monoclonal antibody to EGFR, has been extensively studied in numerous preclinical and clinical studies (13, 14). Preclinical studies have shown that cetuximab is able to inhibit the growth of colon, head and neck, and pancreatic carcinoma xenografts in nude mice (15–17). More importantly, a randomized, phase III clinical trial showed that cetuximab prolongs the survival of patients with untreated head and neck cancers in combination with radiotherapy compared with treatment with radiotherapy alone (18).

Furthermore, multiple studies have shown synergism between the molecular inhibition of EGFR and DNA-damaging agents, such as radiation or the camptothecin class of chemotherapeutic agents, such as topotecan or irinotecan (19, 20). In particular, cetuximab has been approved by the Food and Drug Administration for use with irinotecan (Camptosar, CPT-11) in patients with irinotecan-refractory colorectal carcinoma (21). Irinotecan, an inhibitor of topoisomerase I, acts by preventing the relaxation of DNA supercoiling during DNA replication (22). This Food and Drug Administration approval of combination therapy with cetuximab and irinotecan was based on a randomized clinical trial, which showed that patients receiving the combination therapy showed higher response rate and significantly longer time to recurrence (21). Despite encouraging data, such as these, there are no studies in the literature that have examined the effects of cetuximab or irinotecan, as either single-agent therapy or combined therapy, against ATC. The aim of the present study was to investigate the therapeutic potentials of cetuximab and irinotecan against ATC using an orthotopic model of ATC in nude mice. We show that the coadministration of cetuximab and irinotecan significantly inhibited the growth, invasion, metastasis, and angiogenesis of orthotopic ATC xenografts in nude mice.

Materials and Methods

Reagents. For in vivo administration, irinotecan (Pharmacia and Upjohn Co., Kalamazoo, MI) was diluted in PBS to concentration of 5 mg/mL. Cetuximab (Imclone, New York, NY) was given undiluted at a concentration of 2 mg/mL. For in vitro experiments, both agents were diluted in tissue culture medium to the appropriate concentration. Propidium iodide (PI) and tetrazolium (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterazolium) were both purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Animals. Male athymic nude mice, ages 8 to 12 weeks, were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care 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 The University of Texas M.D. Anderson Cancer Center (Houston, TX) under a protocol approved by the Institutional Animal Care Use Committee.

Cell lines and culture conditions. ATC cell line ARO was used. This cell line was obtained from Sai-Ching Yeung, M.D., Ph.D. (Department of Endocrine Neoplasia and Hormonal Disorders, The University of Texas M. D. Anderson Cancer Center). The ARO cell line has been shown previously to express EGFR and to secrete EGF (11). The cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), penicillin, sodium pyruvate, and nonessential amino acids. Adherent monolayer cultures were maintained on plastic and incubated at 37°C in 5% CO2 and 95% air. The cultures were free of Mycoplasma species.

Measurement of cell proliferation. ARO cell line was plated at 2,000 cells per well in 96-well tissue culture plates. After 24 hours, the cells were treated with cetuximab (0.5 μg/mL) in RPMI 1640 supplemented with 2% FBS for 72 hours. To determine the effects of combined treatment with irinotecan and cetuximab on the proliferation of ATC cell lines, ARO cell line was plated as described above. After 24 hours, the cells were treated with various concentrations of irinotecan (0-6 μmol/L) with or without 0.5 μg/mL cetuximab for 72 hours. To measure the number of metabolically active cells after a 3-day incubation period, we used a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterazolium bromide assay as measured by a 96-well microplate reader (MR-5000; Dynatech Laboratories, Inc., Chantilly, VA) at an absorbance of 570 nm. These experiments were done at least in triplicates.

Measurement of cell death. To measure cell death, ARO cells were plated at a density of 2 × 103 per well in 38-mm2 six-well plates (Costar, Cambridge, MA) and maintained for 24 hours before treatment with irinotecan. After 24 hours, the cells were treated with cetuximab (1 μg/mL) in RPMI 1640 supplemented with 2% FBS. After 48 hours of treatment with cetuximab, the extent of cell death was determined by PI staining of hypodiploid DNA. To determine the effects of combined treatment with irinotecan and cetuximab on the apoptosis of ATC cell lines, ARO cells were plated as described above. It has been shown previously that the cytotoxic effects of cetuximab and topotecan, another member of the camptothecin agents, can be maximized by the sequential treatment of the tumor cell lines first with topotecan followed by cetuximab (15). Therefore, 24 hours after plating, the cells were first treated with various concentrations of irinotecan (0-10 μmol/L) for 24 hours in RPMI 1640 with 2% FBS. After 24 hours, cetuximab was then added to the medium to a final concentration of 1 μg/mL. The cells were then incubated with both irinotecan and cetuximab for another 48 hours. After the 72-hour period, the apoptotic fraction was determined by PI staining of hypodiploid DNA. For PI staining, the treated cells were resuspended in a Nicoletti buffer (50 mg/mL PI, 0.1% sodium citrate, 0.1% Triton X-100) for 20 minutes at 4°C. Cells were then analyzed by flow cytometry, and the sub-G0-G1 fraction was measured. These experiments were done at least in triplicates.

Effects of cetuximab and irinotecan on the growth of orthotopic ATC xenografts in nude mice. Orthotopic xenografts in nude mice were established as described previously (23). Briefly, ARO cells were harvested from subconfluent cultures by trypsinization and washed. ARO cells (5 × 106) in a volume of 5 μL were injected into the right thyroid lobe of each mouse. The tumors were allowed to develop during the following 4 days. The mice were then randomized into four groups (14 mice in the control group and 10 mice in each of the treatment group), and the drugs were given as follows: (a) cetuximab 1 mg/injection i.p. twice weekly; (b) irinotecan 50 mg/kg i.p. once weekly; (c) both cetuximab 1 mg/injection i.p. twice weekly and irinotecan 50 mg/kg i.p. once weekly; or (d) 500 μL PBS given i.p. once weekly as placebo.
The mice were treated for 4 weeks and the weighed twice weekly. Our animal protocol required that the animals be killed if they lost >20% of body weight or if they became moribund. However, none met the criteria for sacrifice before the end of the treatment period. At the end of the 4-week treatment period, the mice were killed by CO2 asphyxiation, and necropsy was done. The cervical lymph nodes, lungs, and thyroid tumors were removed. At the time of the necropsy, the tumor sizes were measured in all three dimensions. The volumes of the tumors were determined using the formula: \( V = \frac{4}{3}\pi XYZ \), where \( X \), \( Y \), and \( Z \) represent the radius of the tumors in each dimension. Percentage of tumor inhibition was calculated according to the formula: 

\[
\left(1-\frac{T}{C}\right)\times 100, \text{ where } T \text{ and } C \text{ represent the mean tumor volumes of the treatment group and the control group, respectively.}
\]

**Effects of cetuximab and irinotecan on the survival of nude mice bearing orthotopic ATC xenografts.** Orthotopic ATC xenografts were established in nude mice as described above. Four days after the tumor cell injection, the mice were randomized into four groups (10 mice in each group) and treated with placebo, cetuximab, irinotecan, or both agents as per the schedules described in the previous section. The mice were weighed twice weekly and killed if the animals showed weight loss of >20% or appeared moribund. The mice were treated for 58 days.

**Comparison of the antitumor effects of cetuximab/irinotecan combination with doxorubicin.** Orthotopic ATC xenografts were established in nude mice as described above. Four days after the tumor cell injection, the mice were randomized into three groups (10 mice in each group): control, doxorubicin, and cetuximab/irinotecan combination groups. The control mice were given 500 \( \mu \)l PBS via i.p. injection once weekly. Doxorubicin was given by i.p. injection at a dose of 5 mg/kg once every 4 days. The cetuximab/irinotecan combination group received cetuximab and irinotecan as per the schedule described in the previous section. The mice were treated for 3 weeks and weighed twice weekly. At the end of the 3-week period, necropsy was done and the tumor sizes were measured in all three dimensions. The tumor volumes were then calculated as described above.

**Immunohistochemical analysis.** For staining with rabbit anti-mouse CD31 (PharMingen, San Diego, CA), rat anti-mouse CD204 (Serotec, Raleigh, NC), and rat anti-mouse F4/80 antibodies (Serotec), frozen tumors were sectioned and mounted on positively charged Superfrost slides (Fisher Scientific, Houston, TX), air dried for 30 minutes, and fixed in cold acetone for 10 minutes. Endogenous blocking was done with 3% hydrogen peroxide followed by protein blocking using 5% horse serum with 1% goat serum (protein-blocking solution). The slides were incubated with primary antibody (1:800, 1:200, and 1:100 dilutions for anti-CD31, F4/80, and CD204 antibodies, respectively) for 18 hours at 4°C. The samples were then washed and blocked with protein-blocking solution for 10 minutes and incubated with appropriate secondary antibodies conjugated to horseradish peroxidase. Positive staining was visualized using 3,3'-diaminobenzidine chromogen and counterstained with Hoechst stain.

To image the 3,3'-diaminobenzidine-stained sections, we used a Microphot-FX microscope (Nikon, Melville, NY) equipped with a threechip charged coupled device color video camera (model DXC990; Sony Corp., Tokyo, Japan). To quantify microvessel density (MVD), the CD31-labeled endothelial cells were counted from four random 0.159-mm\(^2\) fields (\(\times 100\) magnification) per slide from total of five slides per study group. To quantify F4/80 and CD204 staining, computer-assisted image analysis was done using Image Pro Plus software (Media Cybernetics, Silver Spring, MD). The image analysis was done on three to four random 0.159-mm\(^2\) fields (\(\times 100\) magnification) per slide from total of five slides per group. The photomontages were prepared using Photoshop software (Adobe Systems, Inc., San Jose, CA).

**Statistical analysis.** To assess synergy between irinotecan and cetuximab, we first define synergy as follows: a combination treatment is said to be synergistic if the combined treatment results in more tumor inhibition than the tumor inhibition associated with the individual treatments alone. If we define \( \mu_c \) as the mean tumor inhibition for the cetuximab arm, \( \mu_i \) as the mean tumor inhibition for the irinotecan arm, and \( \mu_{\text{comb}} \) as the mean tumor inhibition in the combination arm, then we would declare the combination treatment to be synergistic if \( \min(\mu_c, \mu_i) > \mu_{\text{comb}} \). We used a Bayesian modeling approach and calculated the posterior probability \( Pr[\min(\mu_c, \mu_i) > \mu_{\text{comb}}|\text{data}] \) (i.e., the posterior probability that the minimum of the two posterior mean tumor sizes for irinotecan alone, \( \mu_i \), or cetuximab alone, \( \mu_c \), was greater than the mean posterior tumor size for the combination, \( \mu_{\text{comb}} \)). For these Bayesian analyses, we assumed that the data followed a normal distribution with each treatment group having its own mean and variance. Furthermore, we gave the mean variables noninformative, half-normal prior distributions (i.e., \( \mu \sim N(0,1E10)(0,\infty) \)). Bayesian methods, unlike classic methods, express uncertainty about treatment effects in terms of probability. Thus, in this report, we summarized our uncertainty about effects using posterior probabilities (i.e., the probability of synergy given the observed data). Posterior probabilities greater than 0.975 (analogous to a one-sided \( P = 0.025 \) within a classic framework) were considered statistically meaningful to show synergy.

The average tumor volumes of the control and treatment groups as well as tumor MVD, F4/80, and CD204 staining intensities were compared using the independent sample \( t \) test. The incidences of cervical metastases were compared using \( \chi^2 \) test. The survival data were analyzed by Kaplan-Meier methods and the survival periods were compared by the log-rank test.

**Results.**

Cetuximab enhances the in vitro antiproliferative and proapoptotic effects of irinotecan on ATC cell line ARO. Treatment of ARO cells with 0.5 \( \mu \)g/mL cetuximab alone for 72 hours did not result in antiproliferative effects (Fig. 1A). However, when combined with irinotecan, cetuximab enhanced the antiproliferative effects of irinotecan (Fig. 1B). The \( IC_{50} \) for the antiproliferative effect were 5.5 and 4 \mu\text{mol/L} in the absence and presence of cetuximab, respectively. The magnitudes of growth inhibition at the \( IC_{50} \) were ~18% and 30%. Likewise, treatment of ARO cells with 1 \( \mu \)g/mL cetuximab did not result in the induction of apoptosis (Fig. 1C). The addition of cetuximab at this concentration to irinotecan, however, increased the proapoptotic effect of irinotecan and decreased the \( IC_{50} \) for apoptosis from 3 to 1.8 \mu\text{mol/L}. The overall apoptosis was increased by ~30% (Fig. 1D). The \( IC_{50} \) for antiproliferative and proapoptotic effects of irinotecan was within the plasma concentration achievable in human patients (3.5 \mu\text{mol/L}) when given i.v. at dose of 350 mg/mm\(^2\) (24, 25). Although we used 2% FBS in our medium to minimize the effect of exogenous EGF, it may be argued that there are still sufficient EGF or other growth factors in 2% FBS to overcome the effect of exogenous EGF, it may be argued that there are still sufficient EGF or other growth factors in 2% FBS to overcome the effect of exogenous EGF, it may be argued that there are still sufficient EGF or other growth factors in 2% FBS.

Cetuximab and irinotecan inhibit the growth of orthotopic ATC xenografts in nude mice. After having shown in vitro activity of cetuximab and irinotecan on ATC cell line ARO, we examined the in vivo activity of the two agents. Both cetuximab and irinotecan produced significant growth inhibition of orthotopic ATC xenografts when used as single-agent therapy. However, the greatest growth inhibition was achieved by the coadministration of cetuximab and irinotecan (Fig. 2A-E). At the end of the 4-week treatment period, cetuximab, irinotecan, and the combination showed ~77%, 79%, and 93% decreases, respectively, in mean tumor volume compared with tumors from the placebo-treated group. All treatment groups produced...
Cetuximab, Irinotecan, and Anaplastic Thyroid Carcinoma

Final concentration of 1:24 hours. Cetuximab was then added to irinotecan. The ARO cells were treated with enhance the proapoptotic effects of D, assay on PI-stained cells. measured using a flow cytometry based assay in vitro. Cetuximab does not induce the apoptosis of ATC cell line ARO. ARO cells were incubated with 1 μg/mL cetuximab for 48 hours. The degree of apoptosis was then measured using a flow cytometry based assay on PI-stained cells. D. Cetuximab enhances the proapoptotic effects of irinotecan. The ARO cells were treated with various concentrations of irinotecan for 24 hours. Cetuximab was then added to a final concentration of 1 μg/mL and the cells were incubated in the presence of both cetuximab and irinotecan for additional 48 hours.

Fig. 1. A. Cetuximab does not inhibit the proliferation of ATC cell line ARO. ARO cells were incubated with 0.5 μg/mL cetuximab for 72 hours. The degree of proliferation was then measured with a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. B. Cetuximab enhances the antiproliferative effects of irinotecan. ARO cells were incubated with various concentrations of irinotecan with and without 0.5 μg/mL cetuximab for 72 hours. The degree of proliferation was then measured with a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. C. Cetuximab does not induce the apoptosis of ATC cell line ARO. ARO cells were incubated with 1 μg/mL cetuximab for 48 hours. The degree of apoptosis was then measured using a flow cytometry based assay on PI-stained cells. D. Cetuximab enhances the proapoptotic effects of irinotecan. The ARO cells were treated with various concentrations of irinotecan for 24 hours. Cetuximab was then added to a final concentration of 1 μg/mL and the cells were incubated in the presence of both cetuximab and irinotecan for additional 48 hours.

statistically significant differences in mean tumor volume when compared with the control group ($P < 0.05$). The differences in mean tumor volume between each single-agent treatment group and the combination treatment group were also statistically significant ($P < 0.05$).

The degree of tumor growth inhibition by the single-agent treatment groups was compared with that of the combination therapy group to assess for possible synergism between cetuximab and irinotecan. For this purpose, we used a Bayesian analysis where synergism between cetuximab and irinotecan was established if the variable $Pr[\min(\mu_j - \mu_l) > \mu_C|data]$ (as defined in Materials and Methods) was $> 0.975$. Our analysis showed $Pr[\min(\mu_j - \mu_l) > \mu_C|data]$ to be 0.981 or that there were 98.1% chance that the minimum of the two posterior mean tumor sizes for the single-agent treatment groups (cetuximab and irinotecan) is greater than the posterior mean tumor size for the combination treatment group. Based on this analysis, we conclude that the coadministration of cetuximab and irinotecan produced synergy in the inhibition of orthotopic ATC tumor growth.

The incidence of laryngeal invasion was >90% in the control group compared with 30%, 50%, and 11% in the cetuximab, irinotecan, and combination treatment groups, respectively ($P < 0.05$; Table 1). The incidences of laryngeal invasion between the single agent and the combination treatment groups were not statistically significant ($P > 0.05$). The preferential route of laryngeal invasion was through the inferior constrictor musculature posterior to the thyroid cartilage (Fig. 2F and G). Cetuximab and irinotecan also significantly decreased the incidence of lymph node metastasis (Fig. 2H and I; Table 1). The incidences of lymph node metastasis in the cetuximab, irinotecan, and combination treatment groups were 10%, 20%, and 10%, respectively, compared with 64% in the control group ($P < 0.05$). The incidences of lymph node metastasis between the single agent and the combination treatment groups were not statistically significant ($P > 0.05$). None of the mice showed pulmonary metastasis on histologic examination of the lungs. Lastly, cetuximab and irinotecan were well tolerated by the animals without significant weight loss and none of the animals required sacrifice before the end of the study (data not shown).

Cetuximab decreases the tumor MVD and induces tumor infiltration by macrophages. Although cetuximab alone did not affect the proliferation or the apoptosis of ATC cell lines in vitro, the administration of cetuximab to tumor-bearing mice resulted in >70% tumor inhibition. It has been shown in several tumor models that this discrepancy between in vitro and in vivo effects of cetuximab is due to its ability to inhibit tumor angiogenesis (26). To determine the effects of cetuximab and irinotecan on tumor angiogenesis, CD31 staining was used to quantify the degree of tumor MVD. Treatment with cetuximab, irinotecan, and cetuximab/irinotecan combination produced 28%, 15%, and 39% decrease in the mean tumor MVD compared with the control group (Fig. 3A and B). The decreases in MVD of all three treatment groups were statistically significant compared with the control group.

It has also been postulated that therapeutic monoclonal antibodies directed against transmembrane receptors can elicit host immune responses, such as antibody-dependent cell cytotoxicity (ADCC; ref. 27). To assess for evidence of host response to the tumor xenografts, immunohistochemical staining was done against murine macrophage markers F4/80 and CD204. The mean staining intensities of both F4/80 and CD204 were increased by ~2- and 4-fold in the tumors from the cetuximab and combination treatment groups, respectively (Fig. 3A, C, and D). These increases in tumoral macrophage infiltration were statistically significant ($P < 0.05$) for both F4/80 and CD204.

Comparison of doxorubicin to cetuximab/irinotecan combination therapy in athymic nude mice bearing orthotopic ATC xenografts. Although no single chemotherapeutic agent has
shown significant activity against ATC, the agent used most often in the setting of multimodality therapy include the taxanes and doxorubicin (28, 29). To compare the tumor inhibitory effects of doxorubicin with that of the combined therapy with cetuximab and irinotecan, nude mice with orthotopically established ATC xenografts were treated with placebo, doxorubicin, or combination of cetuximab and irinotecan. At the end of the 3-week treatment period, the mean tumor volume in the doxorubicin group was decreased by only 7.5% compared with the control treatment period, the mean tumor volume in the doxorubicin group and irinotecan group was decreased by 97% (P = 0.000).

Despite its relative ineffectiveness in the inhibition of tumor growth, doxorubicin showed significant toxicity in the test animals as manifested by progressive loss of weight (data not shown). The weight loss exhibited by the doxorubicin group is unlikely to be due to the growth of thyroid tumors, as this effect typically occurs after 3 to 4 weeks into the treatment period.

**Cetuximab and irinotecan improve the survival of athymic nude mice bearing orthotopic ATC xenografts.** In the absence of any treatment, all of the control mice in the survival study succumbed to the thyroid tumors by day 50 due to the obstruction of the upper aerodigestive tract. In contrast, all of the mice in the combination treatment group were alive at the end of the survival study (Fig. 5). The median survival period for control, cetuximab, irinotecan, and combination groups were 38, 51, 51, and 58 days, respectively. The differences in survival between the treatment groups compared with the control group were statistically significant when compared by log-rank test (P < 0.001). Furthermore, each single-agent treatment groups also showed statistically significant difference in survival compared with the combination treatment group (P < 0.01).

**Discussion**

In the present study, the concurrent use of irinotecan and the molecular blockade of the EGFR signaling pathway with cetuximab resulted in the inhibition of the growth, invasion, metastasis, and angiogenesis of orthotopic ATC xenografts in nude mice. Statistical analysis also showed that cetuximab and irinotecan interacted synergistically to produce their antitumor effects. Furthermore, combination therapy with cetuximab and irinotecan was shown to be more effective and yet significantly less toxic in this model than doxorubicin, which is used frequently for the treatment of ATC.

Although cetuximab was able to moderately enhance the in vitro cytostatic and cytotoxic effects of irinotecan, cetuximab alone did not exhibit any effects on the proliferation or the apoptosis of ATC cell line ARO. However, when given in vivo as single-agent therapy, cetuximab was able to produce significant inhibition of ATC tumor growth (~70%). This discordance between in vitro and in vivo activities of cetuximab has also been reported in preclinical study of other tumor types, such as renal cell carcinoma (30).

A possible explanation for this observation may be the potential for cetuximab to induce ADCC (27). Consistent with this possibility, our study showed that orthotopic ATC xenografts treated with cetuximab undergo a significant increase in tumor infiltration by macrophages. The human portion of cetuximab is of IgG1 subclass and consequently is able to interact with Fc receptors of effector cells, such as natural killer cells. Although the ability of C225 to induce ADCC has not yet been shown directly, Bleeker et al. showed that a human IgG1 anti-EGFR antibody was able to induce efficient ADCC in vitro against A431 cells that overexpress EGFR (31). Another example of the mobilization of ADCC by antibodies engineered to target membrane receptors is rituximab. Rituximab is a chimeric
(human-murine) monoclonal antibody to CD20 that has emerged as an effective therapy for B-cell malignancies and non-Hodgkin’s lymphoma (32). In the case of rituximab, the induction of ADCC by the Fc portion of rituximab is critical in its cytotoxic effects (33).

Another potential explanation for the enhanced in vivo activity of cetuximab may be its antiangiogenic property. EGFR blockade with cetuximab has been shown to down-regulate the production of vascular endothelial growth factor by tumor cells and decreased the MVD in nude mice xenografts of transitional cell carcinoma, renal cell carcinoma, and colorectal carcinoma (26, 30, 34). This study showed that cetuximab is also able to inhibit the angiogenesis of orthotopic ATC xenografts showing that the endothelium of thyroid gland is sensitive to the antiangiogenic effects of cetuximab. Therefore, it is likely that the antineoplastic effect of cetuximab is multifaceted and is the result of combination of effects, including the blockade of tumor-expressed EGFR, recruitment of immune responses, and antiangiogenesis.

Although irinotecan has not yet been evaluated for the treatment of ATC, its role in the treatment of colorectal, ovarian, cervical, small cell lung, and non–small cell lung carcinoma has been evaluated in several studies (22, 35). In particular, two phase III clinical trials have shown that combining irinotecan with 5-fluorouracil in the treatment of colorectal carcinoma had survival benefit in patients with metastatic colorectal carcinoma over treatment with either agent alone (36, 37). Given the activity of irinotecan against

Table 1. Effect of cetuximab and irinotecan on the incidence of lymph node metastasis and laryngeal invasion in nude mice bearing orthotopic ATC xenografts

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Incidence of cervical lymph node metastasis (%)</th>
<th>P*</th>
<th>Incidence of laryngeal invasion (%)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9/14 (64)</td>
<td></td>
<td>12/13 (92)</td>
<td></td>
</tr>
<tr>
<td>Cetuximab</td>
<td>1/10 (10)</td>
<td>0.011</td>
<td>3/10 (30)</td>
<td>0.003</td>
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<tr>
<td>Irinotecan</td>
<td>2/10 (20)</td>
<td>0.040</td>
<td>5/10 (50)</td>
<td>0.035</td>
</tr>
<tr>
<td>Cetuximab + irinotecan</td>
<td>1/10 (10)</td>
<td>0.011</td>
<td>1/9 (11)</td>
<td>0.000</td>
</tr>
</tbody>
</table>

*Independent-sample t test.
†One tissue specimen each from the control group and cetuximab plus irinotecan group could not be accurately evaluated for laryngeal invasion due to partial loss of tissue during processing.

![Fig. 3. Immunohistochemical analysis.](image)

After 4-week treatment with cetuximab, irinotecan, or both agents, the ARO tumor sections were immunostained for CD31 (to quantify the MVD) as well as F4/80 and CD204 (to assess tumoral macrophage infiltration). A, representative staining from each group. Original magnification, ×100. Quantification of (B) CD31, (C) F4/80, and (D) CD204 staining intensity. The unit for staining intensity is absorbance units (ODU). Columns, mean staining intensity; bars, SD.
other adenocarcinomas and the data presented in this study, the role of irinotecan in the treatment of ATC seems promising.

The synergy between EGFR inhibition and DNA-damaging agents, such as radiation or irinotecan, has been reported in multiple studies (16, 17). However, the mechanism responsible for this interaction remains to be fully elucidated. It is clear, however, that the molecular blockade of EGFR by cetuximab results in changes in the expression or the activity of several cellular factors, including CDK2, cyclin A, cyclin E, and p27KIP1, and that these changes may modify the response of a tumor cell to external stress (38). Huang et al. showed that the treatment of cultured SCC cells with cetuximab resulted in the suppression of postradiation DNA damage repair (39). Another interesting observation is that cellular stress, such as irinotecan and UV radiation, increases the phosphorylation of EGFR, lending further support to the hypothesis that the cellular survival responses depend on the integrity and possibly the upregulation of the EGFR activity (40). Therefore, it is likely that the cellular changes induced by the inhibition of EGFR pathway results in the impairment of effective DNA repair and recovery and thereby amplify the apoptotic and antiproliferative effects of agents, such as irinotecan.

Toxicity is a major concern in the treatment planning of any elderly patient or those with compromised medical status. ATC occurs mainly in the elderly with the mean age of 70 years (24, 25). Furthermore, these patients often have compromised medical and nutritional status due to the obstruction of the upper aerodigestive tract. A major advantage of cetuximab over other EGFR inhibitors is its safety profile that has been established in several clinical trials. Cetuximab, when given as monotherapy, results frequently in mild toxic effects most commonly manifesting as a macular-papular rash (19, 41). Irinotecan, on the other hand, has been associated with diarrhea and, less frequently, myelosuppression (21). However, it is possible that the coadministration of cetuximab may allow dose reduction of irinotecan and thereby alleviate its toxic effects in patients with impaired functional status. In our athymic, nude mice model, the prolonged administration of cetuximab/irinotecan combination did not produce weight loss and were better tolerated than doxorubicin.

Finally, the limitations of our study should be noted. First, the ARO cell line used in this study has been shown previously to express EGFR and to secrete EGF (11). Although the majority of ATC tumors express EGFR, the level of expression is more variable (8, 11). Before cetuximab could be considered for clinical study, its effectiveness against other frequently studied ATC cell lines, including those cell lines that express low EGFR level, needs to be evaluated. Second, because no single chemotherapeutic agent is active against ATC, several agents have been used in the treatment of this disease. Although we have compared the effectiveness of combined treatment with cetuximab and irinotecan with that of doxorubicin, we have not compared with combined treatment with other commonly used agents, such as paclitaxel. Thirdly, the increase in tumor-infiltrating macrophages in cetuximab-treated tumors by itself is only suggestive of ADCC. Further studies are necessary to validate this hypothesis.

In conclusion, we have provided data that support the study of cetuximab and irinotecan in the treatment of ATC. Both agents showed significant activity as single agent therapy as well as synergistic activity when coadministered in inhibiting the growth and progression of orthotopic ATC xenografts in nude mice. The regimen of cetuximab and irinotecan was significantly more effective than doxorubicin, an agent commonly used for this disease. Although our preclinical data require further validation, cetuximab and irinotecan may offer a novel therapeutic approach for this uniformly fatal disease.
References

Cetuximab and Irinotecan Interact Synergistically to Inhibit the Growth of Orthotopic Anaplastic Thyroid Carcinoma Xenografts in Nude Mice

Seungwon Kim, Christopher N. Prichard, Maher N. Younes, et al.