COVER PAGE FOR PROTOCOL AND STATISTICAL ANALYSIS PLAN

Official Study Title: **Safety and Efficacy Evaluation of Radiation and Cetuximab plus Intratumoral EGFR Antisense DNA in Patients with Locally Advanced Head and Neck Squamous Cell Carcinoma (CTRC# 11- 47)**

NCT number: NCT 01592721

IRB Approval Date: 11/02/2016

Unique Protocol ID: CTRC 11-47

<u>Protocol Changes for the Revised Protocol dated October 25, 2016 for the study:</u> Safety and Efficacy Evaluation of Radiation and Cetuximab plus Intratumoral EGFR Antisense DNA in Patients with Locally Advanced Head and Neck Squamous Cell Carcinoma (CTRC# 11-47)

<u>PREVIOUSLY READ:</u> Cover page and Section 4 (page 11)

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<u>RATIONALE:</u> Principal Investigator changed from Dr. Woondong Jeong to Dr. Anand Karnad due to the departure of Dr. Jeong from CTRC.

CTRC# 11-47

Safety and Efficacy Evaluation of Radiation and Cetuximab plus Intratumoral EGFR Antisense DNA in Patients with Locally Advanced Head and Neck Squamous Cell Carcinoma

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IND 15043

RECEIVED
By Regulatory Affairs at 2:52 pm, Oct 25, 2016

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RESEARCH STUDY ABSTRACT

Background

The Epidermal Growth Factor Receptor (EGFR) is highly expressed in SCCHN and its overexpression is associated with poor patient outcome. EGFR is a promising target of anticancer therapy. We have developed EGFR antisense DNA as a safe and potentially efficacious treatment for SCCHN as shown in a previous phase I study conducted at the University of Pittsburgh. Cetuximab (Erbitux or C225) is a chimerized EGFR monoclonal antibody that has produced positive results in a phase III trial in SCCHN when added to radiation therapy and was approved by the FDA for the treatment of locally advanced SCCHN. Radiation plus cetuximab is considered a standard treatment, especially for patients who are not good candidates for chemotherapy. In the current study, we plan to evaluate the addition of intratumoral EGFR antisense DNA (EGFR AS) to standard radiation with concurrent cetuximab.

Objectives

1. To evaluate the safety of the combination of intratumoral EGFS AS DNA with standard cetuximab and radiation.
2. To evaluate the locoregional progression-free survival in selected patients with locally advanced SCCHN treated with intratumoral EGFR AS DNA combined with standard radiation plus cetuximab.
3. To evaluate other efficacy parameters, including the objective response rate, distant control and overall progression-free survival, and overall survival.
4. To determine the effect of EGFR antisense therapy on EGFR-related biomarkers. We will use reverse phase protein microarrays (RPPA) and immunohistochemical (IHC) analysis of tissue microarrays (TMA), on baseline and post-treatment tumor tissue, to determine the expression level and modulation of a panel of EGFR and EGFR-pathway related biomarkers, including (but not necessarily limited to) EGFR, pEGFR, Src, pMAPK, STAT3, pSTAT3, pSTAT5, pSTAT1, pAKT, p38, p21, p27, PARP, E-cadherin, p-ErbB3, and Ki67.
5. To examine the transfection of the EGFR antisense gene therapy *in vivo*.

Subject population

We will enroll patients with SCCHN who are suitable for intratumoral injections of EGFR antisense. Please see section 3 for detailed eligibility criteria.

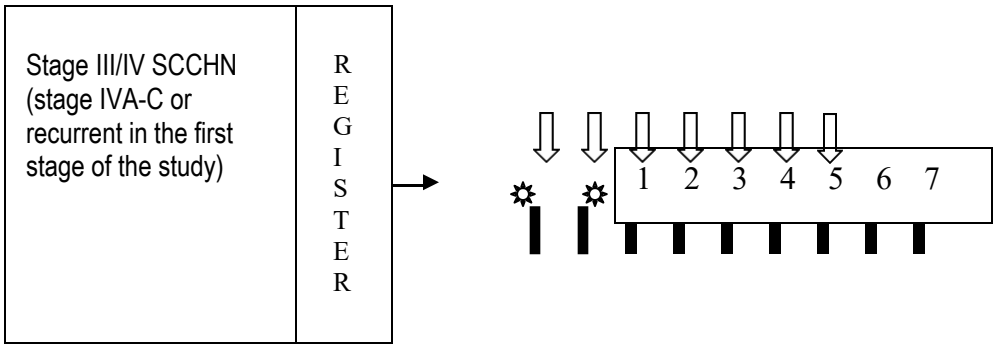
Treatment plan

EGFR AS will be administered by direct intratumoral injection using direct visualization, endoscopy, or imaging-guidance (ultrasound) as clinically determined (see sections 5 and 7 for detailed treatment plan and dose modifications). Patients will receive a total of up to 7 weekly intratumoral injections of EGFR antisense (or less if there is no identifiable tumor) starting 2 weeks prior to radiation (see schema on the next page). Patients will receive standard radiation 70 Gy/200 cGy/daily, 5 days/week, with concurrent cetuximab 250 mg/m^2, after a loading dose of 400 mg/m^2 2 weeks prior to starting radiation.

Statistical Design and Sample Size

The study will be conducted in two-stages. In the first stage, 11 patients with stage IVA-C or recurrent disease will be evaluated for safety. If the regimen is deemed safe, a total of 31 patients with stage III or IVA-B, previously untreated SCCHN will be enrolled in the second stage of the study (see section 11).

STUDY SCHEMA



Radiation once daily, 200 cGy/day, 5 days/week x 7 weeks, to 70 Gy

▌ Cetuximab 400 mg/m^2 IV day, loading dose 2 weeks prior to starting RT
Cetuximab 250 mg/m^2 IV weekly, 1 week prior to and during radiation (until radiation completion)

⇩ Intratumoral EGFR AS injections weekly until no identifiable tumor or to a maximum of 7 weeks, starting 2 weeks prior to radiation, after the initiation of cetuximab. The same lesion (primary tumor or lymph node), which will be the dominant and more accessible tumor, will be injected during treatment with EGFR AS.

☼ Tumor biopsies will be performed prior and after 2 weeks of cetuximab plus EGFR AS (prior to starting radiation).

1. BACKGROUND AND RATIONALE

Approximately 40,000 new cases of head and neck cancer are diagnosed annually in the United States.[1] Of these patients, two-thirds present with locoregionally advanced disease (AJCC stage III or IV) and one-third with early stage disease (AJCC stage I or II). At presentation 10% of patients may be found to have involvement of distant organs, most commonly the lung. In addition, 20% of patients will develop clinically detected distant metastases over the course of their disease. In autopsy series up to 50% of patients with head and neck cancer are found to have metastases.[2,3] Approximately 90% of all head and neck malignancies are squamous cell carcinomas.

1.1 Locoregionally Advanced Disease (AJCC Stages III and IV)

Systemic agents, such as traditional chemotherapy and more recently cetuximab, an EGFR inhibitor, have been incorporated into the standard therapy of locally advanced HNSCC. For certain primary sites, such as oropharynx, nasopharynx, and larynx, nonsurgical management with radiation plus systemic therapy is favored because it leads to good survival results and potentially better function outcomes and organ preservation. For patients with resectable locally advanced head and neck cancer, surgery and postoperative radiation plus cisplatin is now considered standard therapy.

A meta-analysis that included 63 randomized studies conducted between 1965 and 1993, demonstrated an absolute survival benefit of 8% at 5 years with concomitant chemotherapy and radiotherapy versus radiation therapy alone.[4] More recently, multiple randomized phase III trials have conclusively demonstrated that concurrent chemoradiotherapy is superior to radiation therapy alone in locally advanced head and neck cancer.[5-10] The above trials predominantly enrolled patients with unresectable head and neck cancer, whereas three of these trials were site-specific.[6,8,10] Three randomized trials used combined chemotherapy with platinum and 5-FU along with conventional or hyperfractionated radiotherapy.[5-7]and three others used cisplatin with conventional radiation.[8-10] Most of these studies favored concomitant chemoradiotherapy with an approximately 20% gain in survival at three to five years (approximately 30% versus 50%), whereas the study by Forastiere et al showed that concomitant chemoradiotherapy with cisplatin in resectable laryngeal cancer is superior in terms of laryngeal preservation.[10] Cetuximab has been added to radiation in a large randomized study.[11]

1.2 Comorbidities in Patients with Head and Neck Cancer

Due to their frequent history of exposure to tobacco and alcohol patients with head and neck cancer are in high risk for other co-morbidities associated with tobacco and alcohol, such as coronary artery disease, chronic obstructive pulmonary disease, and liver disease.[12,13] These comorbidities are significantly increased in the elderly.[14] In addition, patients with head and neck cancer are at high risk for the development of second primary tumors, synchronous or metachronous, that may involve the head and neck region as well as other organs, predominantly the lung.[15,16] A significant number of patients are ineligible for clinical trials due to poor performance status or comorbidities. The experience with chemotherapy and radiotherapy in these patients as well as the elderly is limited.

1.3 Epidermal Growth Factor Receptor (EGFR)

The epidermal growth factor receptor (EGFR) is a member of the erb B receptor tyrosine kinase family that includes erb B-2, erb B-3, and erb B-4.[17] It consists of an extracellular ligand-binding domain, a transmembrane region that anchors the receptor to the plasma membrane, and a cytoplasmic region containing a tyrosine kinase domain. Among the known natural ligands of EGFR include epidermal growth factor (EGF) and transforming growth factor ·(TGF-) which both activate the receptor by binding to the extracellular domain and inducing the formation of receptor homodimers or heterodimers, followed by internalization of the receptor/ligand complex and auto-phosphorylation. It is now accepted that the EGFR signal transduction network plays an important role in multiple tumorigenic processes, including cell cycle progression, angiogenesis, and metastasis, as well as protection from apoptosis.[17,18] In this signal network, erb B-2 is the major partner of EGFR because activated heterodimer complexes containing erb B-2 are more stable at the cell surface than complexes containing other EGFR family members.[19,20] In addition erb B-2 can decrease the rate of ligand dissociation from the cognate receptor EGFR.[21]

1.4 Cetuximab

Cetuximab binds specifically to the EGFR on both normal and tumor cells, and competitively inhibits the binding of epidermal growth factor and other ligands, such as transforming growth factor–alpha.[22] Binding of cetuximab to the EGFR blocks phosphorylation and activation of receptor-associated kinases, resulting in inhibition of cell growth, induction of apoptosis, and decreased matrix metalloproteinase and vascular endothelial growth factor production. The EGFR is constitutively expressed in many normal epithelial tissues, including the skin and hair follicle.

Human Pharmacokinetics

Cetuximab administered as monotherapy or in combination with concomitant chemotherapy or radiotherapy exhibits nonlinear pharmacokinetics.[22] The area under the concentration time curve (AUC) increased in a greater than dose proportional manner as the dose increased from 20 to 400 mg/m^2. Cetuximab clearance (CL) decreased from 0.08 to 0.02 L/h/m^2 as the dose increased from 20 to 200 mg/m^2, and at doses >200 mg/m^2, it appeared to plateau. The volume of distribution (Vd) for cetuximab appeared to be independent of dose and approximated the vascular space of 2-3 L/m^2.

Following a 2-hour infusion of 400 mg/m^2 of cetuximab, the maximum mean serum concentration (C_{max}) was 184 µg/mL (range: 92-327 µg/mL) and the mean elimination half-life was 97 hours (range 41-213 hours). A 1-hour infusion of 250 mg/m^2 produced a mean C_{max} of 140 µg/mL (range 120-170 µg/mL). Following the recommended dose regimen (400 mg/m^2 initial dose/250 mg/m^2 weekly dose), cetuximab concentrations reached steady-state levels by the third weekly infusion with mean peak and trough concentrations across studies ranging from 168 to 235 and 41 to 85 µg/mL, respectively. The mean half-life was 114 hours (range 75-188 hours).

Clinical Studies of Cetuximab in Head and Neck Cancer

The addition of cetuximab to radiation produced promising results in a phase I trial in patients with locally advanced head and neck cancer.[23] Sixteen patients were treated with radiation therapy plus cetuximab. Cetuximab was delivered as a loading dose of 100 to 500 mg/m^2, followed by weekly infusions of 100 to 250 mg/m^2 for 7 to 8 weeks. There was one grade 4 allergic reaction. Most acute adverse effects were associated with radiation (xerostomia, mucositis, and local skin toxicity). All patients achieved an objective response (13 patients had a complete response). Based on these promising results, a phase III trial of radiation with or without cetuximab in stage III/IV head and neck cancer was conducted and recently reported results. The addition of cetuximab resulted in improved locoregional control and survival whereas the rate of mucositis was comparable between arms (see more details below).[24] Based on the results of this study, cetuximab was approved by the FDA for the treatment of locally advanced SCCHN in combination with radiation and as monotherapy for patients with SCCHN who failed cisplatin-based chemotherapy.

The efficacy and safety of cetuximab were studied in combination with radiation therapy in a randomized, controlled trial of 424 patients with locally or regionally advanced squamous cell carcinoma of the head and neck versus radiation alone. In a multicenter controlled clinical trial, 424 patients with Stage III/IV SCC of the oropharynx, hypopharynx, or larynx with no prior therapy were randomized 1:1 to receive cetuximab plus radiation therapy (211 patients) or radiation therapy alone (213 patients). Stratification factors were Karnofsky Performance Status (60-80 versus 90-100), nodal stage (N0 versus N+), tumor stage (T1-3 versus T4 using American Joint Committee on Cancer 1998 staging criteria), and radiation therapy fractionation (concomitant boost versus once-daily versus twice daily). Radiation therapy was administered for 6-7 weeks as once daily, twice daily, or concomitant boost. The planned radiation therapy regimen was chosen by the investigator prior to enrollment. For patients with ≥N1 neck disease, a post-radiation therapy neck dissection was recommended. Starting 1 week before radiation, cetuximab was administered as a 400 mg/m^2 initial dose, followed by 250 mg/m^2 weekly for the duration of radiation therapy (6-7 weeks). All cetuximab-treated patients received a 20 mg test dose on Day 1. Cetuximab was administered 1 hour prior to radiation therapy, beginning week **2.** Of the 424 randomized patients, 80% were male and 83% were Caucasian. The median age was 57 years (range 34-83). There were 258 patients enrolled in US sites (61%) and 166 patients (39%) in non-US sites. Ninety percent of patients had baseline Karnofsky Performance Status 80% or greater; 60% had oropharyngeal, 25% laryngeal, and 15% hypopharyngeal primary tumors; 28% had AJCC T4 tumor stage. The patient characteristics were similar across the study arms. Fifty-six percent of the patients received radiation therapy with concomitant boost, 26% received once-daily regimen, and 18% twice-daily regimen.

The primary endpoint of this phase III randomized trial was locoregional control and the sample size was adequate to show a 13% improvement in 1-year locoregional control (from 44% to 57%). Study results were remarkable and demonstrated an overwhelming superiority of the combined modality arm versus the radiation alone arm: locoregional control rate at 2 years was 50% versus 41% (HR 0.68, p=0.005); progression-free survival at 2 years was 46% vs. 37% (HR 0.70, p=0.006); overall survival at 3 years was 55% vs.

45% and the median survival 49 vs. 29 months (HR 0.74, p=0.03). The survival advantage achieved with the addition of cetuximab to radiation is comparable with the one achieved with the addition of chemotherapy to radiation. In a meta-analysis, the addition of concurrent chemotherapy to radiotherapy for the treatment of locally advanced SCCHN was shown to result in an 8% survival benefit at 5 years.[4] Similar to the study of Bonner et al. in most randomized studies that compared concurrent chemoradiotherapy to radiotherapy alone, the survival benefit seemed to have derived from an improvement in locoregional control.[25] Another important observation was that acute in-field toxicities, such as stomatitis and dermatitis, that are exacerbated when chemotherapy is added to radiation and are the most dreadful toxicities of combined modality therapy, were comparable between the two arms. Dermatologic toxicities, such as acneiform rash and pruritus, and infusional reactions were predominantly seen, as expected, in the cetuximab arm. Late toxicities were also comparable between the two arms.

Table 1 Clinical Efficacy of Radiation+Cetuximab in Locoregionally Advanced SCCHN

	Cetuximab + Radiation (n = 211)	Radiation Alone (n = 213)	Hazard Ratio (95% Confidence Interval)	Stratified Log-rank p-value
Locoregional Control				
Median Duration	24.4 mo	14.0 mo	0.68 (0.52-0.89)	0.005
1-year	63%	55%		
2-year	50%	41%		
3-year	47%	34%		
Overall Survival				
Median Duration	49.0 mo	29.3 mo	0.74 (0.57-0.97)	0.03
2-year	62%	55%		
3-year	55%	45%		

Single-Arm Trial in SCCHN

Cetuximab alone was studied in a single-arm, multicenter clinical trial in 103 patients with recurrent or metastatic SCCHN with documented progression within 30 days after 2-6 cycles of a platinum-based chemotherapy. Patients received a 20 mg test dose of cetuximab on Day 1, followed by a 400 mg/m^2 initial dose, and 250 mg/m^2 weekly until disease progression or unacceptable toxicity. Upon progression, patients were given the option of receiving cetuximab plus the platinum regimen that they failed prior to enrollment. Tumor response and progression were assessed by an Independent Radiographic Review Committee (IRC). The median age was 57 years (range 23-77), 82% were male, 100% Caucasian, and 62% had a Karnofsky Performance Status of 80% or higher. The objective response rate on the monotherapy phase was 13% (95% confidence interval (7%-21%). Median duration of response was 5.8 months (range 1.2-5.8 months). Since expression of EGFR has been detected in nearly all patients with head and neck cancer, patients enrolled in the head and neck cancer clinical studies were not required to have immunohistochemical evidence of EGFR expression prior to study entry.

Safety of Cetuximab in Clinical Trials in Patients with Head and Neck Cancer

Except where indicated, the data described below reflect exposure to cetuximab in 208 patients with locally or regionally advanced SCCHN who received cetuximab in combination with radiation. Cetuximab was administered as monotherapy in 103 patients with recurrent or metastatic SCCHN. Of the 103 patients receiving cetuximab monotherapy, 53 continued to a second phase with the combination of cetuximab plus chemotherapy. Patients receiving cetuximab plus radiation therapy received a median of 8 doses (range 1-11 infusions). The population had a median age of 56; 81% were male and 84% Caucasian. Patients receiving cetuximab monotherapy received a median of 11 doses (range 1-45 infusions). The population had a median age of 57; 82% were male and 100% Caucasian. The most **serious adverse reactions** associated with cetuximab in combination with radiation therapy in patients with head and neck cancer were:

- Infusion reaction (3%)
- Cardiopulmonary arrest (2%)
- Dermatologic toxicity (2.5%)
- Mucositis (6%)
- Radiation dermatitis (3%)
- Confusion (2%)
- Diarrhea (2%)

Fourteen (7%) patients receiving cetuximab plus radiation therapy and 5 (5%) patients receiving cetuximab monotherapy discontinued treatment primarily because of adverse events.

The most common adverse events seen in 208 patients receiving cetuximab in combination with radiation therapy were acneform rash (87%), mucositis (86%), radiation dermatitis (86%), weight loss (84%), xerostomia (72%), dysphagia (65%), asthenia (56%), nausea (49%), constipation (35%), and vomiting (29%).

The most common adverse events seen in 103 patients receiving cetuximab monotherapy were acneform rash (76%), asthenia (45%), pain (28%), fever (27%), and weight loss (27%).

The data in the table below are based on the experience of 208 patients with locoregionally advanced SCCHN treated with cetuximab plus radiation therapy compared to 212 patients treated with radiation therapy alone.[26]

Table 2 Incidence of Selected Adverse Events (≥10%) in Patients with Locoregionally Advanced SCCHN

Body System Preferred Term	Cetuximab plus Radiation (n=208)		Radiation Therapy Alone (n=212)	
	Grades 1 – 4	Grades 3 and 4	Grades 1 – 4	Grades 3 and 4
	% of Patients			
Body as a Whole				
Asthenia/Malaise	56	4	48	5
Fever[1]	29	1	13	<1
Headache	19	<1	8	<1
Infusion Reaction[2]	15	3	2	0
Infection	13	1	9	1
Chills[1]	16	0	5	0
Digestive				
Mucositis/Stomatitis	93	56	94	52
Xerostomia	72	5	71	3
Dysphagia	65	26	63	30
Nausea	49	2	37	2
Constipation	35	5	30	5
Vomiting	29	2	23	4
Anorexia	27	2	23	2
Diarrhea	19	2	13	1
Dyspepsia	14	0	9	1
Metabolic/Nutritional				
Weight Loss	84	11	72	7
Dehydration	25	6	19	8
Respiratory				
Pharyngitis	26	3	19	4
Cough Increased	20	<1	19	0
Skin/Appendages				
Acneform Rash[3]	87	17	10	1
Radiation Dermatitis	86	23	90	18
Application Site Reaction	18	0	12	1
Pruritus	16	0	4	0

[1] Includes cases also reported as infusion reactions.
[2] Infusion reaction is defined as any event described at any time during the clinical study as “allergic reaction” or “anaphylactoid reaction” or any event on the first day of dosing described as “allergic reaction”, “anaphylactoid reaction”, “fever”, “chills”, “chills and fever” or “dyspnia”.
[3] Acneform rash is defined as any event described as “acne”, “rash”, “maculopapular rash”, “pustular rash”, “dry skin” or “exfoliative dermatitis”.

Late Radiation Toxicity

The overall incidence of late radiation toxicities (any grade) was higher in cetuximab in combination with radiation therapy compared with radiation therapy alone. The following sites were affected: salivary glands (65% versus 56%), larynx (52% versus 36%), subcutaneous tissue (49% versus 45%), mucous membrane (48% versus 39%), esophagus (44% versus 35%), skin (42% versus 33%), brain (11% versus 9%), lung (11% versus 8%), spinal cord (4% versus 3%), and bone (4% versus 5%). The incidence of

Grade 3 or 4 late radiation toxicities were generally similar between the radiation therapy alone and the cetuximab plus radiation treatment groups.

1.5 EGFR Antisense DNA therapy

We have previously demonstrated up-regulation of EGFR (mRNA and protein) in tumors and normal mucosa several centimeters away from the tumor, compared with expression levels in normal mucosa from patients without cancer.[27,28] We also detected increased EGFR levels in premalignant lesions (that correlate with the degree of dysplasia) which progress to invasive cancer suggesting that up-regulation of EGFR occurs early in squamous epithelial carcinogenesis.[29] Based on these results, we designed experiments to down-modulate receptor expression and examined the effects of such modulation strategies on proliferation and target protein expression. EGFR was targeted using 3 different approaches including down-modulation of EGFR mRNA using antisense oligonucleotides, and blocking the function of the mature protein at two sites, the ligand-binding domain and the kinase domain. All 3 strategies resulted in growth inhibition of SCCHN cells but had no effect on the proliferation rate of corresponding normal cells *in vitro*.[30]

A novel approach for EGFR inhibition is the use of antisense oligonucleotides that can be injected intratumorally and inhibit EGFR and its signaling pathway. In the past several years, we have investigated successfully the down-modulation of EGFR mRNA using antisense oligonucleotides.[30] To extend these initial observations to an *in vivo* model, we generated an EGFR antisense expression construct designed for intratumoral injection. The criteria for selecting a gene transfer vector were to: 1) transfect a high percentage of tumor cells *in vivo*; 2) generate high expression levels of EGFR antisense RNA in each cell; and 3) demonstrate an anti-tumor response following treatment. To accomplish this, we selected the U6 small nuclear RNA promoter, which has been engineered to express short antisense RNA sequences under the control of RNA polymerase III.[31] U6 is a small, stable RNA that exists as an abundant nuclear ribonucleoprotein (U6 snRNP) in all human cells where it plays central roles in both spliceosome assembly and catalysis in nuclear pre-mRNA splicing. Compared with other RNA polymerase III-transcribed genes, such as tRNA, the U6 gene has no control region located within the sequence encoding the structural component of the RNA. Thus, nearly all of the structural U6 core can be replaced with any other sequence without affecting transcript production.[32]

We cloned an oligonucleotide (39 bp) targeting the ATG start site of human EGFR in the antisense orientation under the control of the U6 promoter into the pNGVL1 plasmid backbone containing a kanamycin resistance marker. The pNGV1 plasmid had been previously approved for use in clinical trials. The pNGVL1 backbone was modified to eliminate the residual CMV promoter sequences to avoid potential interference with the U6 promoter.

Using a murine xenograft head and neck squamous cell carcinoma model system, repeated intratumoral administration of the antisense (but not the corresponding sense) EGFR gene therapy construct (25 µg/3x week for a total of approximately 8 injections), significantly inhibited the growth of established tumors (approximately 2 mm in diameter). The inhibition of tumor growth was sustained up to several weeks following cessation of therapy. Examination of the treated tumor nodules revealed diminished expression of the EGFR protein. Although the mechanism of

decreased tumor growth is not completely understood, increased apoptosis rates were detected in the EGFR antisense-treated tumors compared with tumors treated with the corresponding sense construct.[33] Although the preclinical studies were originally performed using cationic liposomes, recent studies have suggested that liposomes only modestly augment gene transfer *in vivo* and may contribute to toxicity. We subsequently repeated our experiments in tumor-bearing animals and found that anti-tumor effects were observed without the use of liposomes. Therefore, we used injection of DNA alone (naked DNA) in our phase I clinical study.

1.6 Phase I Trial of EGFR AS in Patients with Recurrent SCCHN

Previous studies have reported negligible toxicity following intratumoral administration of DNA.[26,34] Our preclinical studies suggest that 60 µg of antisense DNA decreases tumor progression.[33] Therefore, we implemented a rapid dose escalation (100% increment increase between tiers) in the phase I clinical study of EGFR AS. Eligible patients had recurrent SCCHN and have previously failed other treatments. Patients received weekly intratumoral injections for 4 weeks. The dose of EGFR AS was escalated in successive cohorts of patients (classic "3+3" design) as follows: level 1 - 60 µg/60 µL, level 2 - 120 µg/120 µL, level 3 - 240 µg/240 µL, level 4 - 480 µg/480 µL, level 5 - 960 µg/960 µL, and level 6 - 1.92 mg/1.92 mL. A total of 17 patients were enrolled. We reported results at the ASCO 2007 meeting.[35] No grade 3 or 4 or any dose limiting toxicities were observed at any dose level. A dose of up to 1.92 mg/1.92 mL of EGFR AS was well tolerated. There were four adverse events related to EGFR AS in this trial. All events were grade 1 and included two episodes of injection site pain/swelling, one episode of edema and one episode of lethargy. No dose limiting toxicities were observed at any dose level. One patient developed dehydration that was thought to be unrelated to the drug injection. Five of 17 patients (29%) achieved an objective response: 2 patients had a complete response and 3 patients had a partial response. In order to demonstrate transfection of the EGFR AS gene into the tumors, we performed polymerase chain reaction (PCR) analysis on DNA extracted from pre- and post-treatment patient tissue specimens. We were able to detect EGFR AS in the tissue specimens taken two weeks after the last EGFR AS injection in both non-responders and responders. Moreover, we observed that STAT3 and EGFR expression levels at baseline correlated with efficacy outcomes. The final manuscript is in preparation.

1.7 Study Rationale

The incorporation of novel targeted therapies to radiation therapy is of particular interest in head and neck cancer, and may improve efficacy without significantly increasing toxicity. Cetuximab is an EGFR inhibitor that is a potent radiosensitizer and has increased the survival of patients with stage III/IV head and neck cancer when added to radiation therapy. We would like to enhance the standard therapy of locally advanced SCCHN by incorporating intratumoral EGFR AS injections, a novel and promising locoregional treatment, to standard radiation and cetuximab. We hypothesize that the addition of a second EGFR-targeted agent that inhibits EGFR at the intracellular level will improve the antitumor effect of standard radiation and cetuximab. Preliminary preclinical data from our group support this hypothesis. Based on our data, intratumoral EGFR AS therapy has shown promise as single treatment modality. The addition of EGFR AS to standard radiation and cetuximab may further increase treatment efficacy and is of major interest. The goal of this study is to evaluate the safety, efficacy, and secondarily, the biologic effects in patients with locally advanced SCCHN of an antisense gene targeting the EGFR in combination with standard therapy

with radiation and cetuximab, a systemic EGFR inhibitor.

2. OBJECTIVES

- To evaluate the safety of the combination of intratumoral EGFR AS DNA with standard cetuximab and radiation.
- To evaluate the locoregional control (primary endpoint), in patients with locally advanced SCCHN treated with intratumoral EGFR antisense DNA combined with standard radiation plus cetuximab.
- To evaluate the toxicities associated with the above treatment.
- To evaluate other efficacy parameters, including the objective response rate, distant control and overall progression-free survival, and overall survival.
- To determine the effect of EGFR antisense therapy on EGFR and EGFR-related biomarkers. We will use reverse phase protein microarrays (RPPA) and immunohistochemical (IHC) analysis of tissue microarrays (TMA), on baseline and post-treatment tumor tissue, to determine the expression level and modulation of a panel of EGFR and EGFR-pathway related biomarkers, including (but not necessarily limited to) EGFR, pEGFR, Src, pMAPK, STAT3, pSTAT3, pSTAT5, pSTAT1, pAKT, p38, p21, p27, PARP, E-cadherin, p-ErbB3, and Ki67. We hypothesize that the baseline expression or modulation of one or more EGFR signaling proteins will correlate with objective response rate and locoregional control after combined modality treatment.
- To examine the transfection of the EGFR antisense gene therapy in vivo.

3. SELECTION OF PATIENTS

3.1 Eligibility Criteria

- First stage
  Patients with AJCC 7th edition stage IVA-IVC or recurrent or metastatic head and neck cancer will be eligible. Patients with M1 disease must have asymptomatic or low volume distant metastasis and require palliation for local and regional disease.
- Second stage (phase II part)
  Patients with AJCC 7th edition stage III-IVB (T_1-T_4, $N_{1-3}M_0$) head and neck cancer, except WHO type II and III nasopharyngeal cancer, including unknown primary tumors.
- Histologically or cytologically confirmed diagnosis of squamous cell carcinoma or variants or poorly differentiated carcinoma.
- Unidimensionally measurable disease (RECIST criteria).
- ECOG Performance Status of 0-2

- In the second stage of the study, therapy will be administered with a curative intent and patients should not have recurrent disease or distant metastasis.
- Primary tumor and/or lymphadenopathy should be technically suitable for intratumoral injections. The Otolaryngologist specialist on the head and neck team will determine this feasibility.

- Participating patients should agree to undergo a tumor biopsy at baseline as well as approximately 2 weeks later as specified in study schema.
- Prior treatment
    - First stage: any prior treatment, except prior therapy, which specifically and directly targets the EGFR pathway, administered within the last 6 months. No prior radiation therapy to the head and neck.
    - Second stage: no prior chemotherapy, biologic/molecular targeted therapy (including any prior therapy which specifically and directly targets the EGFR pathway), or radiotherapy for head and neck cancer.
- Prior surgical therapy will consist only of incisional or excisional biopsy, including tonsillectomy, and organ sparing procedures, including neck dissection. Any non-biopsy surgical procedure for head and neck cancer must have taken place at least one month before initiating protocol treatment, at the treating physician's discretion.
- Patients must have organ and marrow function as defined below:

Absolute neutrophil count	1,000/µL
Platelets	75,000/µL
Hemoglobin	·10 g/dL
Total bilirubin	<2 x upper normal institutional limits
Creatinine clearance	> 20 mL/min

- Age of 18 years.
- Because radiation therapy is known to be teratogenic and EFGR inhibitors may have teratogenic potential, women of childbearing potential and men must agree to use adequate contraception (hormonal or barrier method of birth control) prior to study entry and for the duration of study participation, and for 3 months after completing study treatment. Should a woman become pregnant or suspect she is pregnant while participating in this study, she should inform her treating physician immediately.
- Informed consent must be obtained from all patients prior to beginning therapy. Patients should have the ability to understand and the willingness to sign a written informed consent document.

3.2 Exclusion Criteria

- Severe renal insufficiency (creatinine clearance < 20 mL/min)
- Treatment with anticoagulants, except when used to maintain the patency of a central venous line, or INR >1.5, or PTT ratio >1.5.
- Uncontrolled intercurrent illness including, but not limited to, ongoing or active infection or psychiatric illness/social situations that would limit compliance with study requirements. Significant history of uncontrolled cardiac disease; i.e., uncontrolled hypertension, unstable angina, uncontrolled congestive heart failure.
- Patients may not be receiving any other investigational agents.
- No history of prior malignancy, with the exception of curatively treated squamous cell or basal carcinoma of the skin or in situ cervical cancer, DCIS or LCIS of the breast, localized early stage

prostate cancer, or malignancy that has been treated with a curative intent with a 3-year disease-free survival.

- Pregnant women are excluded from this study because cetuximab, EGFR AS, and radiation have the potential for teratogenic or abortifacient effects. Because there is an unknown but potential risk for adverse events in nursing infants secondary to treatment of the mother with cetuximab and EGFR AS, breastfeeding should be discontinued if the mother is treated with cetuximab. The effects of cetuximab and EGFR AS on the developing human fetus at the recommended therapeutic dose are unknown. For this reason women of child-bearing potential and men must agree to use adequate contraception (hormonal or barrier method of birth control) prior to study entry and for the duration of study participation. Should a woman become pregnant or suspect she is pregnant while in this study, she should inform her treating physician immediately.
- HIV-positive patients receiving combination anti-retroviral therapy are excluded from the study because of possible drug interactions with cetuximab. Appropriate studies will be undertaken in patients receiving combination anti-retroviral therapy when indicated. HIV status of the patient will be obtained from the patient's history via discussion with the investigator. HIV testing is not required.
- Prior severe infusion reaction to a monoclonal antibody.
- Patients who are not informed of and are not willing to comply with the investigational nature of the study and have not signed a written informed consent in accordance with institutional and good clinical practice guidelines.
- Phase 2 **ONLY** (second stage) – Subjects with M1 disease will be excluded.

4. PATIENT REGISTRATION

Patients must not start protocol treatment prior to registration. Treatment should start within five working days after registration. Subjects can be enrolled after eligibility criteria are met by calling the site research nurse or study liaison:

Ofelia Romero, RN
Clinical Research Nurse III
Department of Clinical Investigations
Institute for Drug Development
Cancer Therapy and Research Center at UTHSCSA
Mail Code 8229
7979 Wurzbach Road, Suite Z413
San Antonio, Texas 78229
Office: (210) 450-1810
Fax: (210) 614-4418
Email: romeroo@uthscsa.edu

For questions regarding the eligibility of subjects, Anand Karnad, MD, should be contacted at (210) 450-1267.

Registration will require the following information:

1) your name, telephone and pager number;
2) protocol name and number;
3) date treatment begins;
4) subject name;
5) date of birth;
6) subject hospital medical record number;
7) primary study physician;
8) primary treatment institution;
9) confirmation of eligibility;
10) copies of the informed consent signature page; and
11) verification that the informed consent was signed.

5. TREATMENT PLAN

- Loading dose of cetuximab 400 mg/m^2 IV over 120 minutes 2 weeks prior to starting radiation.
- Cetuximab 250 mg/m^2 IV over 60 minutes weekly, the week prior and during days of radiation, until radiation is completed (diphenhydramine hydrochloride [Benadryl] premedication 30-60 minutes prior to cetuximab infusion as required, see 5.1).
- Intratumoral EGFR AS injections weekly x 7 weeks (or less if there is no identifiable tumor), starting 2 weeks prior to radiation and after cetuximab is administered. Subsequent EGFR AS injections can be given before or after cetuximab is administered. Injections will be in the primary and/or lymph nodes. One site will be injected per weekly session. The intratumoral EGFR AS injections should occur during the same calendar day due to CTRC scheduling. If it is necessary the antisense injection can be scheduled within 1 business day of the original schedule date and then resume the original schedule.
- EGFR AS will be administered by direct intratumoral injection using direct visualization, endoscopy, or imaging-guidance (ultrasound) as clinically determined. The same lesion (primary tumor or lymph node) will be injected during treatment.
- Radiation therapy 70 Gy, 200 cGy/daily, 5 days/week (see 5.3). Radiation will start on a Monday, Tuesday, or Wednesday. Cetuximab will be given prior to radiation.
- A tumor biopsy will be performed prior to starting cetuximab and 2 weeks later but prior to starting radiation.
- In the first stage, after three (3) subjects are enrolled and have received all EGFR AS injections, there will be a two-week waiting period to assess adverse events before enrolling additional subjects. After the two-week period, additional subjects may be enrolled.

5.1 Cetuximab Administration

In an effort to prevent an infusion reaction, all patients should be premedicated with diphenhydramine hydrochloride [Benadryl] 50 mg (or an equivalent antihistamine) by IV given 30-60 minutes prior to the first dose of cetuximab. Premedication may be administered prior to subsequent doses, but at the Investigator's discretion, the dose of diphenhydramine (or a similar agent) may be reduced.

The initial dose of cetuximab is 400 mg/m^2 intravenously administered over 120 minutes, followed by weekly infusions at 250 mg/m^2 IV over 60 minutes. The infusion rate of cetuximab must never exceed 5 mL/min. Patients must be continuously observed during the infusion for signs of anaphylaxis.

Patients will be closely monitored for treatment-related adverse events, especially infusion reactions (see management of cetuximab adverse events) during the infusion and the post-infusion observation hour, if necessary. For the initial cetuximab infusion, vital signs should be monitored pre-infusion, ½ hour into the infusion, and at the end of the infusion, and, if needed, 1 hour post-infusion. For subsequent infusions, vital signs should be taken pre- and post-infusion; however, it is recommended that the patient be observed for 1 hour post infusion. Patients who have developed an infusion reaction in the past will be observed for >1 hour, if necessary.

For the duration that patients are on study therapy, adverse event monitoring will be done continuously. Patients will be evaluated for adverse events at each visit and are to be instructed to call their physician to report any adverse events between visits.

5.2 Intratumoral EGFR AS

Injections

The treatment will be administered in the outpatient setting. Pretreatment tumor biopsies will be performed in the operating room at the time of diagnosis or in the outpatient setting under local anesthesia. Intralesional (primary tumor or cervical metastases) EGFR AS will be administered weekly until there is no identifiable tumor or a maximum of 7 weeks.

- EGFR AS will be administered by direct intratumoral injection using direct visualization, endoscopy, or imaging-guidance (ultrasound) as clinically determined. Injection sites may be treated before injection with EMLA cream (or equivalent) to minimize discomfort. The primary tumor or cervical metastases will be injected with a single dose of DNA and monitored. The tumor will be inoculated with a 25-gauge needle in an attempt to maximize the distribution of the gene transfer formulation in the injected lesion. Specifically, the needle will be introduced into the center of the tumor and the injection will be performed slowly as the needle is withdrawn from the tumor. Each dose will be given 7 days (+/- 2 days) after the previous dose. The same lesion (primary tumor or lymph node), which will be the dominant and most accessible, will be injected during treatment in each patient. In the first stage, after three (3) subjects are enrolled and have received all EGFR AS injections, there will be a two-week waiting period to assess adverse events. After the two-week period, additional subjects may be enrolled.

To determine the *in vivo* efficacy of the antisense gene and the consequences of EGFR antisense gene therapy, we will obtain tumor biopsies under local anesthesia in the

outpatient setting, at the time or after the second dose of EGFR AS and after the second cetuximab dose, in addition to the pretreatment biopsy. Therefore, the tumor will be biopsied 2 times. When possible, we will biopsy adjacent areas of the tumor at each time point to determine heterogeneity of transfection and EGFR expression. When possible, additional biopsies will be obtained from non-injected areas that are more than 2 cm apart from an injection site. A comparison of biopsies from injected and non-injected lesions will provide the opportunity to assess the added effect of EGFR AS. Prior injection sites will be marked and attempts will be made to perform biopsies in the area of prior inoculation(s). Biopsies will be delivered to Drs. Carew and Nawrocki's laboratory at CTRC and to Dr. Grandis' laboratory at UPCI for molecular studies. The presence of EGFR antisense DNA in the tumors will be determined using PCR. EGFR and EGFR pathway will be evaluated using RPPAs and IHC (see correlative studies section).

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EGFR AS Dose

Patients will receive a total EGFR AS dose of 1.92mg/1.78mL on each weekly treatment. This dose may be delivered equally in the same tumor site per weekly session, the primary tumor or cervical lymph nodes.

Dose/Schedule Modifications

There will be no dose reductions. However, weekly doses will be omitted in the setting of locoregional toxicities (even if those are thought to be related to radiation).

Based on prior experience, the expected toxicities of EGFR AS therapy may include local discomfort at the injection site, edema, bleeding and cutaneous erythema when the treatment is administered.

Treatment with EGFR AS will be held in the setting of grade 3 or 4 mucositis/dermatitis.

Treatment with EGFR AS will be discontinued if there is cellulitis at the injection site requiring antibiotic therapy or grade 4 locoregional toxicities within the injected site develop (such as bleeding or tissue necrosis).

Duration of Therapy

Each patient will receive up to 7 weekly treatments. Treatment may be discontinued earlier if there is no identifiable tumor, a patient withdraws from study, develops intolerable toxicities, or their disease progresses (see section 7.8).

Gene Transfer Efficiency and Biologic Activity

These studies require a tissue biopsy and will be performed prior to initiation of therapy and on the second week of therapy. Tumor tissue will be biopsied for molecular studies as defined elsewhere. In order to demonstrate transfection of the EGFR AS gene into the tumors, we will perform polymerase chain reaction (PCR) analysis on DNA extracted from pre- and post-treatment patient tissue specimens. Samples will be taken directly to Dr. Grandis' laboratory and the assays will be performed under her direction at the time points indicated above. Samples from San Antonio will be sent to Dr. Grandis via Drs. Carew and Nawrocki's laboratory.

5.3 Radiation therapy

Radiation therapy will be standard of care for participating institutions.

5.3.1 Physical Factors

Equipment: Linear accelerator with appropriate photon and electron energies for supplemental boosting to the nodes.

Photon energies of 4 to 6 MV and/or appropriate electron energies for boosting the nodes are allowed. Photon energies > 6 MV may be utilized when appropriate to boost target localized centrally.

Minimum treatment distance must be ≥ 80 cm SSD (or SAD for isocentric techniques).

5.3.2 Localization Requirements

5.3.2.1 Simulation

Simulation of all fields is mandatory. Patients must be reproducibly

immobilized. Radio-opaque markers should be used to delineate the extent of nodal disease and whenever possible, the primary tumor, if conventional treatment planning is used. The use of customized Cerrobend blocks or multileaf collimator is recommended to shape the field and limit dose to normal structures. For CT simulation, IV contrast is recommended and the data should be acquired using 3 mm slice width and spacing using the spiral mode of scanner.

5.3.2.2 Verification

Checking the films should be done weekly. Beam portal films must be obtained to each field.

5.3.2.3 Electron fields utilized for supplemental nodal boosting must be verified by portal verification, simulation, or diagram.

5.3.3 Radiation Dose

Treatment to the primary tumor and upper neck will be given at 2 Gy/fraction once each day, five days a week. A total dose of 70 Gy/35 fractions/7 weeks will be delivered. Fields must be reduced to exclude the spinal cord at 36 Gy with dose calculated in the central plane. The entire neck must be irradiated to a total dose of at least 46- 50 Gy (even in N0 stage) at anatomic levels of lymph nodes usually 2-4 cm beneath the skin surface. Clinically positive neck nodes should also receive a dose of 70 Gy/35 fractions given continuously for 7-8 weeks. An electron beam should be used to supplement the dose to the posterior cervical chain nodes after the spinal cord has been shielded. Hence, the total dose to the primary tumor and clinically positive nodes will be 70 Gy. The anterior lower neck will be treated at 2 Gy/fraction once a day (3 cm depth or at depth determined by 3D imaging) from the anterior to a total dose of 46-50 Gy/25 continuous fractions (if clinically negative) in 5 weeks. The maximum dose permitted to the spinal cord will not exceed 45 Gy as determined by a separate off-axis point dose calculation.

5.3.4 Target Volume Irradiation and Radiation Doses

5.3.4.1 A combination of lateral opposing fields are recommended for the treatment of the primary tumor site and upper neck. A single anterior AP field will be used to treat the lower neck below the fields of the primary tumor/upper neck. When there is clinically involved lymph nodes in the lower neck, an additional posterior (PA) field may be necessary to deliver a supplemental dose to the positive nodes. All fields must be treated at each treatment session. The upper and lower fields should be matched using a “three dimensional” technique (i.e., appropriate rotation of the treatment table and appropriate angulation of the collimator or beam split technique). However, if such a three-dimensional match is not possible, the upper neck and supraclavicular fields may be abutted at the skin. The latter case, a small block should be placed either in the lower lateral position in the upper neck fields, or in the midline of the anterior

supraclavicular field (whichever is appropriate and does not block tumor) to the shield areas of potential overlap of diverging beams over the spinal cord.

5.3.4.2 Upper Neck Primary Volume

The primary treatment fields should encompass the primary tumor and/or suspected lymph node disease in the upper neck using the shrinking field technique as follows:

The initial target volume should include the primary tumor, positive nodes with an approximately 3.0 cm margin and the next echelon of uninvolved lymphatic nodal drainage sites to a dose of 50 Gy. Included in the target volume is the first field reduction occurring off the spinal cord at 36 Gy.

The second field reduction will occur at 50 Gy and continue to 60 Gy with the new target volume encompassing the primary tumor and known areas of nodal disease as well as areas at high risk for lymph node involvement with approximately 2.0 cm margin.

The third and final field reduction will occur at 60 Gy and continue to 70 Gy with the new target volume encompassing the primary tumor and known areas of nodal disease with at approximately 1.0 cm margin.

As a general rule, both ipsilateral and contralateral posterior cervical nodal chain and the adjacent echelon of uninvolved lymphatic nodal drainage sites will be treated to a dose of 46-50 Gy/5 weeks.

5.3.5 Lower Neck Volume

A single lower neck field will be used to treat the lower neck and the supraclavicular fossae. When there is (are) positive node(s) in the lower neck, an additional posterior field may be necessary to deliver a supplemental dose to the positive node(s). The boost dose should not exceed 4 Gy.

The lower border of the lower neck field will be just below the clavicular head with appropriate margins when there are positive nodes in the supraclavicular fossa. The lateral borders of the lower neck field will extend to the intersection of the sternocleidomastoid muscle and the clavicle.

For all patients with clinically positive nodes greater than 6 cm, or with clinically positive supraclavicular nodes, a mediastinal “T” field may be used. The lateral limbs of the T field will extend below the clavicular heads with margin, and the central portion of the field extends 5 cm more inferiorly to include the upper mediastinum.

5.3.6 Boost Doses

Additional boost doses may be given through reduced field to persistent tumor and/or clinically positive nodes, or to compensate for significant interruptions in radiation therapy treatments (i.e., > 57 elapsed days). The additional boost dose should not exceed 4 Gy (i.e., 70 Gy maximum). The spinal cord must be excluded from any boost field.

5.3.7 Dose Calculations

Photon Beam Portal Arrangements

Dose specification for two parallel-opposed coaxial equally-weighted beams is to be on the central ray at mid-separation of the beams.

Dose specification for two or more intersecting beams is at the intersection of the central ray of the beams.

For other complex treatment arrangements, dose is specified at the center of the clinical target volume.

Note: There may be several target volumes.

For a single anterior AP lower neck field, the prescribed does will be delivered at a depth of 3.0 cm as determined by an off-axis “supraclavicular point”. A deeper prescription point may be required if distance to the supraclavicular fossa is greater or with clinically involved nodes. When AP-PA fields are used to treat the lower neck, the dose shall be prescribed at the mid-separation of the two beams along the central ray.

5.3.8 Isodose Distributions

Isodose distribution at central axis is required. All treatment volumes and critical structures should be indicated. Complete isodose curves are required. Attempts should be made to keep the dose variation within the planning target volume to be within +/- 5% of the target dose.

5.3.9 Electron Beam Dose Specifications

The target dose shall be prescribed at the depth of maximum dose. The energy and field size shall be chosen so that the target volume is encompassed within 90% of the prescribed dose.

5.3.10 Time and Dose Modifications

Treatment interruptions are strongly discouraged. Treatment breaks must be clearly indicated in the treatment record when they occur. If the total treatment interruptions exceed ten treatment days, the treatment will be considered a protocol deviation. The interruption of radiation therapy for grade 4 mucositis/dermatitis/dysphagia is at the discretion of the treating oncologist.

The maximum permitted spinal cord dose is 45 Gy.

Mandibular osteoradionecrosis will rarely occur if pretreatment dental evaluation is conducted prior to radiation therapy treatment. Pretherapy extractions of badly diseased teeth should be carried out with conservation of restorable teeth when possible. If an extraction site in the mandible has to be included in the irradiated field, 10 to 14 days should be allowed for healing before the initiation of irradiation.

Radiotherapy Guidelines/IMRT

5.3.11 Intensity Modulated Radiation (IMRT) is allowed in the study.

5.3.11.1 Following are the proposed radiotherapy guidelines:

All patients will have immobilization devices with treatment planning based on Computerized Tomography (CT) information in treatment position. The CT slice thickness through target will be 3 mm.

CT, PET, and MRI data will be used to define the various targets and contour normal structures. MRI information will be used when indicated. Image fusion will be used to relate CT/MRI data when necessary.

The field sizes and arrangements will be at the discretion of the attending Radiation Oncologist. ICRU 50 guidelines will be used for various Tumor and Target Nomenclature.

The Gross Tumor Volume (GTV): All gross disease determined from clinical examination and radiographic studies (CT/PET/MRI).

The Clinical Target Volume (CTV): The area that potentially contains microscopic disease. Lymph node groups at risk of microscopic disease will be outlined as part of corresponding CTV. The margin between each GTV and corresponding CTV can vary from 0.2 cm to 2.5 cm depending upon the proximity to the critical and uninvolved structures.

The Planning Target Volume (PTV): Provides a margin around CTV to compensate for internal motion and set up errors. Typically a 5 mm margin may be used around CTV.

5.3.11.2 Radiation Target and Dose Specifications

The following is the definition of targets and their dose specifications.

Primary Target (PTV3): Includes PTV's of primary tumor and the lymph nodes containing disease. The dose will be 70 Gy at 2 GY/ fractions.

Secondary Target (PTV2): Includes PTV's of area at moderate to high risk of microscopic disease i.e. first echelon nodes. The dose will be 60 Gy.

Tertiary Target (PTV1): Includes PTV's of areas that are at low risk of microscopic disease i.e. second echelon lymph nodes. The dose will be approximately 46-50 Gy.

Low Neck

The midjugular, low jugular, and supraclavicular nodes can be treated with IMRT or alternatively with an AP field that is beam split to the IMRT fields. Dose Volume Histograms (DVH) will be generated for each plan. Radiation doses will be as outlined above.

Treatment Planning

Either forward or inverse planning can be used in order to achieve the following:

The radiation dose will be prescribed to the isodose line that encompass 95% of PTV.

No more than 15% of the PTV will receive >110% of the prescribed dose.

The maximum dose within PTV does not exceed >25% of the prescribed dose.

No more than 1% of the tissue outside PTV will receive >110% of the prescribed dose.

No more than 1% of any PTV will receive < 93% of the prescribed dose.

Tissue heterogeneity correction is mandatory.

The planning priorities include critical normal structure constraints followed by prescription goals.

Normal Tissues: The appropriate normal organs will be contoured. Dose Volume Histograms (DVH) will be generated. An attempt will be made to keep the maximum radiation doses to the following organs as follows:

Brain Stems:	54Gy
Optic Nerve/Chiasm:	55 Gy
Spinal Cord:	45 Gy
Mandible/TM Joint:	70 Gy or 1 cc of the PTV not to exceed 75 Gy
Larynx	70 Gy

Parotid Glands: Attempt will be to achieve mean dose ≤ 26 Gy in at least one gland or at least 20cc of the combined volume of both parotid glands will receive < 20 Gy or at least 50% of the gland will receive < 30 Gy (should be achieved in at least one gland).

Other Normal Structures: Submandibular/sublingual glands, inner and middle ears, eye, lens will be considered low priority. The aim should be to decrease the doses as much as possible without compromising target.

5.3.12 Amifostine is not permitted.

5.3.13 Pilocarpine (Salagen) is allowed as per physician discretion. If used, all details must be recorded on the data forms.

5.4 Salvage Surgery

Salvage surgery may be performed after radiation, if there is local and/or regional progression of disease.

Elective (planned) neck dissection or other surgery required for residual but not progressive disease will NOT be considered treatment failure.
Surgical removal (salvage) of the primary tumor: Directed biopsies at the site of the index lesions should not be performed in the absence of suspicion for relapse. Criteria for biopsy after radiation includes a persistent mucosal abnormality or imaging studies that are suspicious for persistent or recurrent disease.

Surgical removal (salvage resection) of the primary tumor should be performed if biopsy-proven cancer remains more than two months after completion of chemoradiotherapy. This will be considered progression of disease for the evaluation of the primary endpoint. The nature of the surgical resection should be dictated by the extent of tumor at the initial evaluation. The operation should be conducted using accepted criteria for primary surgical treatment of the cancer.

Neck dissection: A neck dissection is recommended if a palpable or worrisome radiographic abnormality persists in the neck 8 weeks after completion of therapy.

Cervical lymphadenectomy should encompass the original levels of lymph node involvement. Preservation of the accessory nerve, jugular vein, and sternomastoid muscle is encouraged if consistent with complete removal of all residual nodal disease; however, the extent of the neck dissection will be at the discretion of the surgeon.

5.5 Supportive Care

All supportive measures consistent with optimal patient care will be given throughout the study.

Concomitant Drugs

The use of G-CSF, erythropoietin or amifostine is not allowed during radiotherapy. No other antitumor agents will be given during protocol therapy.

Prophylactic placement of a gastrostomy tube before treatment begins is at the discretion of the treating physician. Gastrostomy tube placement is strongly recommended in patients with significant dysphagia and weight loss at baseline.

Aggressive oral and skin care and analgesics are recommended.

The use of amifostine is not permitted. Pilocarpine (Salagen) is not encouraged during treatment but is allowed. If used, it should be recorded in the treatment forms.

6. ADVERSE EVENTS

This study will utilize the **NCI CTCAE version 4.0** for toxicity and Adverse Event (AE) reporting (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm). All appropriate treatment areas should have access to a copy of the CTCAE version 4.0.

Infusion reactions should be graded according to allergic reaction/hypersensitivity. Caution must be exercised with every cetuximab infusion, as there were patients who experienced their first severe infusion reaction during later infusions.

6.1 Adverse Drug Reactions Due to Gene Therapy

Note: A patient has died while being treated on a gene therapy protocol. That protocol used very high amounts of virus directly injected into the liver and treated a very different disease with a different gene than the one used in this study. This gene therapy protocol does not use a virus. The underlying disease that this patient had also made him more susceptible to complications of the gene therapy.

Risk of Cancer

When retroviral vectors enter a normal cell in the body, the DNA of the vector inserts itself into the normal DNA in that cell; this process is called integration. Most integration is expected to cause no harm to the cell or to the subject. However, there is a chance that there may be some regions of the normal human DNA where integration of the viral vector's DNA may result in activation of neighboring genes. For example, if one of these genes were a growth factor, this may cause uncontrolled division of the cell, resulting in a cancer. This type of event has occurred in one animal study in mice where the vector integration site correlates with the occurrence of cancer in these mice.

More recently, the first reports of a similar event have been identified in two children who received a retroviral vector in an experimental gene transfer clinical trial for X-linked Severe Immunodeficiency (X-SCID) conducted in France, not under the jurisdiction of the United States Food and Drug Administration (FDA). While most of the children who participated in this clinical trial appear to have been cured of their disease, these two children developed leukemia (a form of cancer of the blood) approximately 30 and 36 months, respectively, after receiving the gene therapy treatment. These children had extensive testing done to determine the cause of the leukemia. A group of experts in this field looked at all the test results and concluded that the gene transfer caused the leukemia. These children appear to be responding to the treatment of his/her leukemia, but his/her long-term prognosis in unknown at this time.

The risk of another cancer, including leukemia, developing in subjects who participate in this study, is unknown; however, in contrast to the French study this protocol does not use a virus.

Soreness and/or bleeding at the injection site.

Local inflammation (redness or swelling and/or discomfort) at the injection site.

Fever, reactions at the injection site of the gene transfer, which could develop into local ulcers or sores that could get infected.

Note: Administration of cancer gene transfer has been used before, although this specific EGFR antisense study drug has not been previously given to cancer subjects. It is not known what impact this gene transfer will have on future conventional chemo/radiation treatments or future reproductive capabilities.

6.2 Reporting Adverse Events

All adverse events which are considered to be related to the research study must be reported to the UT Health Science Center Institutional Review Board. All serious adverse events that meet the UT Health Science Center Institutional Review Board's criteria for reporting can be found website at (http://research.uthscsa.edu/irb/).

6.2.1 Serious AE (SAE) means any untoward medical occurrence that at any dose:

- Results in **death**.
- Is **life-threatening**. (The term "life-threatening" in the definition of "serious" refers to an event in which the patient was at risk of death at the time of the event; it does not refer to an event, which hypothetically might have caused death if it were more severe).
- Requires inpatient **hospitalization or prolongation of present hospitalization** (see clarification in the paragraph below on planned hospitalizations).
- Results in **persistent or significant disability/incapacity**. Disability is defined as a substantial disruption of a person's ability to conduct normal life functions.
- Is a **congenital anomaly/birth defect**.

Is a **medically important event** that may not be immediately life threatening or result in death or hospitalization but may jeopardize the patient or may require intervention to prevent one of the other outcomes listed in the definition above, or involves suspected transmission via a medicinal product of an infectious agent. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse; any organism, virus, or infectious particle (e.g., prion protein transmitting Transmissible Spongiform Encephalopathy), pathogenic or non pathogenic, is considered an infectious agent.

6.2.2 NIH guidelines for reporting serious adverse events for trials involving gene transfer agents or recombinant DNA molecules are found at http://www4.od.nih.gov/oba/rdna.htm.

6.2.3 The PI will report SAEs on trials involving gene transfer agents or recombinant DNA

molecules to the IRB according to their requirements.

6.2.4 FDA Guidelines for Adverse Event Reporting

All adverse events meeting the definition of serious, unexpected and associated to the research intervention will be reported to the IRB according to their guidelines found on the IRB's website and the IND file according to the following guidelines and FDA definition of events:

Associated with the use of the drug: There is a reasonable possibility that the experience may have been caused by the drug.

Disability: A substantial disruption of a person's ability to conduct normal life functions.

Lifethreatening adverse drug experience: Any adverse drug experience that places the patient or subject, in the view of the investigator, at immediate risk of death from the reaction as it occurred, i.e., it does not include a reaction that, had it occurred in a more severe form, might have caused death.

Serious adverse drug experience: Any adverse drug experience occurring at any dose that results in any of the following outcomes: death, a life threatening adverse drug experience, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant disability/ incapacity, or a congenital anomaly/birth defect. Important medical events that may not result in death, be life threatening, or require hospitalization may be considered a serious adverse drug experience when, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.

Unexpected adverse drug experience: Any adverse drug experience, the specificity or severity of which is not consistent with the current investigator brochure; or, if an investigator brochure is not required or available, the specificity or severity of which is not consistent with the risk information described in the general investigational plan or elsewhere in the current application, as amended. "Unexpected," as used in this definition, refers to an adverse drug experience that has not been previously observed (e.g., included in the investigator brochure) rather than from the perspective of such experience not being anticipated from the pharmacological properties of the pharmaceutical product.

6.2.5 IND Safety Reports

The sponsor shall notify FDA and all participating investigators in a written IND safety report of:

Any adverse experience associated with the use of the drug that is both serious and unexpected.

Any finding from tests in laboratory animals that suggests a significant risk for human subjects including reports of mutagenicity, teratogenicity, or carcinogenicity.

Each notification shall be made as soon as possible and in no event later than 15 calendar days after the investigators initial receipt of the information.

Telephone and facsimile transmission of safety reports. The sponsor/investigator shall also notify FDA by telephone or by facsimile transmission of any unexpected fatal or life threatening experience associated with the use of the drug as soon as possible but in no event later than 7 calendar days after the sponsor's initial receipt of the information.

6.3 Data Safety and Monitoring Plan (DSMP)

A Data and Safety Monitoring Plan is required for all an individual protocols conducted at CTRC. All protocols conducted at CTRC are covered under the auspices of the CTRC Institutional Data Safety Monitoring Plan.

The CTRC Institutional DSMP global policies provide individual trials with:

- Institutional policies and procedures for institutional data safety and monitoring,
- An institutional guide to follow,
- Monitoring of protocol accrual by the CTRC protocol review committee,
- Review of study forms and orders by the forms committee,
- Tools for monitoring safety events,
- Independent monitoring and source data verification by the CTRC QA monitor/auditor,
- Monitoring of UPIRSO's by the director of quality assurance and DSMC,
- Determining level of risk (priority of audit level score – pals),
- Oversight by the data safety monitoring committee (DSMC), and
- Verification of protocol adherence via annual audit for all investigator initiated studies by the CTRC quality assurance division.

Monitoring Progress and Safety

Due to the risks associated with participation in this protocol, the CTRC DSMB #2 in conjunction with the Principal Investigator will perform assessment of adverse events, adverse event trends and treatment effects on this study. The CTRC DSMB #2 acts as an independent DSMB for IIS conducted at CTRC. The CTRC DSMB #2 will monitor data throughout the duration of a study to determine if continuation of the study is appropriate scientifically and ethically. An additional layer of review is provided by the CTRC Data Safety Monitoring Committee (DSMC) who will review DSMB quarterly reports.

Baseline events and adverse events will be captured using the CTRC Master Adverse Events Document for each patient using CTCAE V. 4.0 for the grading and attribution of adverse events. Usage of the CTRC Master Adverse Events Document centrally documents:

- The event and grades the seriousness of the event,
- If the event was a change from baseline,
- The determination of the relationship between the event and study intervention,
- If the event was part of the normal disease process, and
- What actions were taken as a result of the event.

Safety Definitions:

For this study, the following safety definitions will be applicable:

Adverse Event Definition: An adverse event (AE) is defined as any untoward or unfavorable medical occurrence in a human subject, including any abnormal sign (for example, abnormal physical exam or laboratory finding), symptom, or disease, temporally associated with the subject's participation in the research, whether or not considered related to the subject's participation in the research for this study, all adverse events will be documented starting with *specify here when you want to start collecting adverse event information, generally this would be with the first dose of the study drug* and ending 30 days after the last dose of study drug is received.

Serious Adverse Event Definition: is any adverse event that:

1. Results in death;
2. Is life-threatening (places the subject at immediate risk of death from the event as it occurred);
3. Results in inpatient hospitalization or prolongation of existing hospitalization;
4. Results in a persistent or significant disability/incapacity;
5. Results in a congenital anomaly/birth defect; or
6. Based upon appropriate medical judgment may jeopardize the subject's health and may require medical or surgical intervention to prevent one of the other outcomes listed in this definition

Unanticipated Problems Involving Risk to Subjects or Others (UPIRSO) Definition: Unanticipated problem involving risk to subjects or others includes any incident, experience or outcome that meets all of the following criteria:

A. Unexpected (in terms of nature, severity, or frequency) given (a) the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol and informed consent document; and (b) the characteristics of the subject population being studied (note: the unfounded classification of a serious adverse event as "anticipated" constitutes serious non-compliance);

B. Definitely related or probably related to participation in the research; and

C. Suggests that the research places subjects or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized

Reporting Requirements

For this study, the Master Adverse Events Documents collected on patients for this protocol will be reviewed by the Principal Investigator on a quarterly basis to determine if a serious safety problem has emerged that result in a change or early termination of a protocol such as:

- Dose modification,
- Suspending enrollment due to safety or efficacy, or
- Termination of the study due to a significant change in risks or benefits.

6.4 PI Review

The PI will provide the DSMB #2 with the quarterly findings for discussion and review during their meetings.

As per the CTRC DSMP, any protocol modifications, problematic safety reports, unanticipated problems, and suspension or early termination of a trial must be reported to the DSMB #2 and all members of the research team. Furthermore, the PI of this study will promptly notify all study affiliates, the UTHSCSA IRB, the CTRC DSMC, and the FDA via a FDA Form 3500Aa written IND safety report of any adverse events that are either serious and/or unexpected. Suspension and early termination of a trial must also be reported immediately to the Director of Quality Assurance who will promptly notify the sponsor and the UTHSCSA IRB.

The PI will review the Master Adverse Events documents to determine the significance of the reported events and will provide findings using the Investigator Initiated Study Quarterly DSMC Report Form on a quarterly basis with the DSMB #2. The DSMB #2 will review the information provided by the PI and report to the CTRC DSMC on a quarterly basis, unless an emergent issue has been identified. The Investigator Initiated Study Quarterly DSMC Report Form includes information on adverse events, current dose levels, number of patients enrolled, significant toxicities per the protocol, patient status (morbidity and mortality) dose adjustments with observed response, and any interim findings. Any trend consisting of three or more of the same event will be reported to the CTRC DSMC for independent review outside of the quarterly reporting cycle, which begins three months following protocol start up. The DSMB #2 will also provide its findings to the CTRC's Regulatory Affairs Division so that it may be provided to the UTHSCSA IRB with the protocol's annual progress report. Conflict of interest is avoided by the independent reviews of the CTRC DSMB #2 and by ongoing independent review of UPIRSO trends by the Director of Quality Assurance.

All SAE and UPRISO's will be reported following CTRC, UTHSCSA institutional and FDA guidelines

UTHSCSA SAE/UPRISO REPORTING REQUIREMENTS For IIS that the PI holds the IND		
Type Event	Report to	Timeframe
All AE, SAE and UPIRSO	Regulatory Affairs and DQA	ASAP
All AE, SAE and UPIRSO	FDA on form 3500A	within 7 calendar days by telephone and 15 calendar days using the Form 3500A
SAE	PI at UTHSCSA	within 24 hours
SAE	UTHSCSA IRB	Annually
UPIRSO - all	PI at UTHSCSA	within 24 hours
UPIRSO - all	FDA	within 7 days
UPIRSO - life threatening	UTHSCSA IRB/UTHSCSA OCR	within 48 hours
UPIRSO - non-life threatening	UTHSCSA IRB/UTHSCSA OCR	within 7 days

Expedited Reporting for Phase II and III Studies (including hospitalization*)

UNEXPECTED EVENT		**EXPECTED EVENT**	
GRADES 2 - 3	**GRADES 4 - 5 Regardless**	**GRADES 1 - 3**	**GRADES 4 - 5 Regardless of**

Attribution of Possible, Probable or Definite	**of Attribution**		**Attribution**
Expedited report within 10 working days **Grade 1** - Adverse Event Expedited Reporting NOT required.)	Report by phone to IDB within 24 hrs. Expedited report to follow within 10 working days. This includes all deaths within 30 days of the last dose of treatment with an investigational agent regardless of attribution. Any late death attributed to the agent (possible, probable, or definite) should be reported within 10 working days.	Adverse Event Expedited Reporting NOT required.	Expedited report, including Grade 5 Aplasia in leukemia patients, within 10 working days. This includes all deaths within 30 days of the last dose of treatment with an investigational agent regardless of attribution. Any late death attributed to the agent (possible, probable, or definite) should be reported within 10 working days. Grade 4 Myelosuppression or other Grade 4 events that do not require expedited reporting will be specified in the protocol.

* For Hospitalization Only — Any medical event equivalent to CTC Grade 3, 4, 5 which precipitated hospitalization (or prolongation of existing hospitalization) must be reported regardless of requirements for Phase of study, expected or unexpected and attribution.

Expedited reporting may not be appropriate for specific expected adverse events for certain later Phase II and Phase III protocols. In those situations the adverse events that will not have expedited reporting must be specified in the text of the approved protocol. An expected Grade 3 event that is definitely related to the investigational agent is only to be reported if the patient is hospitalized using the generic reporting criteria. For instance, in a trial of an investigational agent where Grade 3 diarrhea requiring hospitalization is expected, only diarrhea requiring ICU care (Grade 4) might be designated for expedited reporting.

Serious adverse events on NCI sponsored trials utilizing a commercially available agent (with no IND's involved) will additionally be reported via the FDA's Medwatch program.

Assuring Compliance with Protocol and Data Accuracy

As with all studies conducted at CTRC, the PI has ultimate responsibility for ensuring protocol compliance, data accuracy/integrity and responding to recommendations that emanate from monitoring activities. Protocol compliance, data accuracy and reporting of events is further ensured by an annual audit conducted by the Data Safety Officer, whose audit report is shared with the PI, the research team, and will be reviewed by the CTRC DSMC.

CTRC DSMB Membership

The CTRC has two DSMB's with a primary set of members specific to the histology of the study consisting of UTHSCSA faculty and staff. This Protocol will utilize DSMB#2 for Solid Tumor Studies.

DSMB #2 (for Solid Tumors)

Kevin Kelly, MD
Ting Lu, MD
Richard Meier, MD
Wendy Crabbe, PA, MSN, RN
Melissa Nashawati, MPA
Yuanyuan Liang, PhD

As per NCI guidelines and to eliminate conflict of interest (financial, intellectual, professional, or regulatory in nature), the CTRC DSMB specific to this study will not treat patients on this protocol. Usage of the DSMB specific to the histology has been created to ensure that experts in that histology are represented on the DSMB assembled for this protocol, but may be expanded, at the PI's discretion, to include other members which may include:

- Experts in the fields of medicine and science that are applicable to the study (if not currently represented on the DSMB),
- Statistical experts,
- Lay representatives,
- Multidisciplinary representation, from relevant specialties including experts such as bioethicists, biostatisticians and basic scientists, and
- Others who can offer an unbiased assessment of the study progress.

Additional or alternate membership of in the DSMB is selected by the DSMC chair, in conjunction with the PI of this protocol.

CTRC DSMB Charter and Responsibilities

The CTRC DSMB will provide information on the membership composition, including qualifications and experience to both the UTHSCSA IRB and CTRC PRC for review. The CTRC DSMB for this study will act as an independent advisory board to the PI and will report its findings and recommendations to the PI, the UTHSCSA IRB and the CTRC DSMC. CTRC DSMB reports will utilize the Investigator Initiated Study Quarterly DSMC Report Form and meetings will occur on a quarterly basis to review any updates from the prior meeting.

Once the protocol is activated, if not already established elsewhere in the protocol the CTRC DSMB will establish and provide:

- Procedures for maintaining confidentiality;
- Statistical procedures including monitoring guidelines, which will be used to monitor the identified primary, secondary, and safety outcome variables;
- Consider factors external to the study when relevant information becomes available, such as scientific or therapeutic developments that may have an impact on the safety of the participants or the ethics of the study;

- Plans for changing frequency of interim analysis as well as procedures for recommending protocol changes;
- Recommendation of dose escalation, MTD recommendation of early termination based on efficacy results;
- Recommendation of termination due to unfavorable benefit-to-risk or inability to answer study questions;
- Recommendation of continuation of ongoing studies;
- Recommend modification of sample sizes based on ongoing assessment of event rates; and
- Review of final results and publications.

7. MANAGEMENT OF CETUXIMAB ADVERSE REACTIONS AND DOSE MODIFICATIONS

All toxicities will be graded according to NCI **Common Toxicity Criteria AE version 4.0 (**http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm**)**.

7.1 Cetuximab Dose Modifications

Cetuximab will be held or its dose reduced to dose level -1 or -2 (see below) for the following toxicities (see sections 7.4 and 7.5).

Cetuximab will NOT be held or reduced for hematologic toxicities.

Dose levels for cetuximab are as follows:

Table 3: Cetuximab Dose Levels

	Weekly Cetuximab dose
Starting dose	250 mg/m^2
Dose Level -1	200 mg/m^2
Dose Level -2	150 mg/m^2

There will be no dose level reductions below a weekly dose of 150 mg/m^2.

7.2 Management of Infusion Reactions

Severe infusion reactions require the immediate interruption of cetuximab therapy and permanent discontinuation from further treatment. Appropriate medical therapy including epinephrine, corticosteroids, intravenous antihistamines, bronchodilators, and oxygen should be available for use in the treatment of such reactions. Patients should be carefully observed until the complete resolution of all signs and symptoms.

In clinical trials, mild to moderate infusion reactions were managed by slowing the infusion rate of cetuximab and by continued use of antihistamine pre-medications (e.g., diphenhydramine hydrochloride [Benadryl]) in subsequent doses. If the patient experiences a mild or moderate (Grade 1 or 2) infusion reaction, the infusion rate should be permanently reduced by 50%. For

grade 1 or 2 reactions manifesting only as delayed drug fever, see below.

Cetuximab should be immediately and permanently discontinued in patients who experience severe (Grade 3 or 4) infusion reactions.

7.3 Treatment of Isolated Drug Fever

In event of isolated drug fever, the investigator must use clinical judgment to determine if the fever is related to the study drug or to an infectious etiology.

If a patient experiences isolated drug fever, for the next dose, pre-treat with acetaminophen or non-steroidal anti-inflammatory agent (investigator discretion), repeat antipyretic dose 6 and 12 hours after cetuximab infusion. The infusion rate will remain unchanged for future doses.

If a patient experiences recurrent isolated drug fever following premedication and post-dosing with an appropriate antipyretic, the infusion rate for subsequent dosing should be 50% of previous rate. If fever recurs following infusion rate change, the investigator should assess the patient's level of discomfort with the event and use clinical judgment to determine if the patient should receive further cetuximab.

7.4 Management of Pulmonary Toxicity

In the event of acute onset (grade $\geq$ 2) or worsening pulmonary symptoms which are not thought to be related to underlying cancer, cetuximab therapy should be interrupted and a prompt investigation of these symptoms should occur. If ILD is confirmed, cetuximab should be discontinued and the patient should be treated appropriately.

7.5 Management of Dermatologic Toxicity

Patients developing dermatologic toxicities while receiving cetuximab should be monitored for the development of inflammatory or infectious sequelae, and appropriate treatment of these symptoms initiated. Dose modifications of any future cetuximab infusions should be instituted in case of severe (grade 3) acneiform rash. Treatment with topical and/or oral antibiotics should be considered; topical corticosteroids are not recommended.

If a patient experiences severe acneiform rash, cetuximab treatment adjustments should be made according to the following table. In patients with mild and moderate skin toxicity, treatment should continue without dose modification.

Table 4 Cetuximab Dose Modification Guidelines

Grade 3 Acneform Rash	Cetuximab	Outcome	Cetuximab Dose Modification
1st occurrence	Delay infusion 1 to 2 weeks	Improvement	Continue at 250 mg/m^2
		No Improvement	Discontinue cetuximab
2nd occurrence	Delay infusion 1 to 2 weeks	Improvement	Reduce dose to 200 mg/m^2
		No Improvement	Discontinue cetuximab
3rd occurrence	Delay infusion 1 to 2 weeks	Improvement	Reduce dose to 150 mg/m^2
		No Improvement	Discontinue cetuximab
4th occurrence	Discontinue cetuximab		

7.6 In-Field Toxicities During Radiation: Radiation Mucositis/Dysphagia/Dermatitis

No cetuximab dose adjustments for grade 3 or less radiation mucositis, dysphagia, dermatitis.

Grade 4 radiation mucositis/dysphagia or dermatitis that develops at any time will require permanent dose reduction of cetuximab by one dose level on all subsequent cycles. If radiation is given in the setting of grade 4 radiation mucositis/dysphagia/dermatitis, cetuximab will be administered with a dose reduction by one dose level.

Delays in radiation as strongly discourages. Short delays may be required for the management of grade 3 /4 toxicities. If RT is held because of in-field toxicities, hold cetuximab until RT is resumed.

EGFR AS will be held for grade 3 or 4 in-field toxicities (see section 5.2.3).

7.7 Retreatment Criteria for Cetuximab

Cetuximab may only be administered if all the following criteria are met regardless of cycle, providing no criteria for discontinuation are met:

- Acne-like rash is grade 2 or less
- All other grade 3 or 4 non-hematologic out-of-field toxicities (except fatigue, electrolyte abnormalities, anorexia, pain) have improved to grade 2 or less.
- For in-field toxicities see section 7.6

7.8 Duration of Therapy/Withdrawal from Study

Criteria for removal from study are:

- Disease progression,
- Study closure,
- Unacceptable adverse event(s) as defined in dose modification or other unacceptable AEs,
- Patient decision to withdraw from the study, or
- In the judgment of the investigator, further treatment would not be in the best interest of the patient.
- Pregnancy: Pregnant participants will continue to be followed for the duration of the pregnancy.

8. MEASUREMENT OF RESPONSE

Response will be evaluated in this study using the international criteria proposed by the Response Evaluation Criteria in Solid Tumors (RECIST v1.1) Committee.[36]

8.1 Methods of Measurement

Imaging based evaluation is preferred to evaluation by clinical examination. The same imaging modality must be used throughout the study to measure disease.

CT and MRI

CT and magnetic resonance imaging (MRI) are the best currently available and most reproducible methods for measuring target lesions. Conventional CT and MRI should be performed with contiguous cuts of 10 mm or less in slice thickness. Spiral CT should be performed by use of a 5 mm contiguous reconstruction algorithm. This specification applies to tumors of the chest, abdomen, and pelvis, while head and neck tumors, and those of the extremities require specific procedures.

Chest X-Ray

Lesions on chest x-ray are acceptable as measurable lesions when they are clearly defined and surrounded by an aerated lung. However, CT is preferable. A chest x-ray only needs to be done if clinically indicated by the treating physician. It is not required for study purposes.

Tumor Markers

Tumor markers alone cannot be used to assess response. If initially above the upper normal limit, a tumor marker must return to normal levels for a patient to be considered in complete clinical response when all tumor lesions have disappeared.

Clinical Examination

FOR THIS STUDY evaluation of measurable disease in the primary site by ENT examination is allowed.

Cytology and Histology

Cytologic and histologic techniques can be used to differentiate between complete and partial response in rare cases (e.g., after treatment to differentiate residual benign lesions and residual malignant lesions in germ cell tumors). Cytologic confirmation of the neoplastic nature of any effusion that appears or worsens during treatment is required when the measurable tumor has met response or stable disease criteria.

The pathologic response after biopsy or neck dissection post chemoradiotherapy will be recorded.

9. STUDY PARAMETERS

9.1 Evaluations at Screening/Baseline
(Performed within 4 weeks of registration unless otherwise indicated)

- Sign informed consent
- History and physical examination, including vital signs, weight and performance status determination (within 1 week of registration).
- Complete blood counts, including platelets (within 1 week of registration).
- Blood chemistry studies, including creatinine, electrolytes (K+, Na+, Cl-, CO2), calcium, Mg (within 1 week of registration), PT/PTT and liver function tests, including bilirubin, AST (SGOT), ALT (SGPT), and alkaline phosphatase (within 1 week of registration).
- Pregnancy (when applicable, within 3 days prior to treatment initiation) test.
- CT and/or MRI of the neck and CT of the chest and other imaging studies as indicated for staging and baseline tumor measurements. Any scans used to document measurable disease should be done as close to study entry as possible and within 4 weeks prior to registration.
- PET scan (optional)
- Bone scan (if clinically indicated)
- Dental evaluation (within 8 weeks of registration)
- ENT evaluation/endoscopy (within 8 weeks of registration)
- Swallowing assessment (within 8 weeks of registration)
- Pretreatment biopsy

9.2 Evaluations During Treatment
During treatment - weekly

- History and physical examination. A focused evaluation of toxicities on a weekly basis will be

performed at a minimum. A complete history and physical examination is needed at least every 3 weeks.

- CBC, platelets and creatinine/electrolytes
- Liver function tests: Bilirubin, AST (SGOT), ALT (SGPT), and alkaline phosphatase. LFTs are required every 3 weeks or more frequently if indicated.
- Vitals signs will be checked every time cetuximab is administered.
- Tumor biopsy at baseline and after 2 weeks (prior to the start of radiation).

9.3 Evaluations at Follow-Up

2 weeks after completion of radiation treatment

- History and physical examination
- Complete blood counts, including platelets
- Blood chemistry studies, including creatinine, electrolytes (K+, Na+, Cl-, CO2), Ca++, Mg++, and liver function tests, including bilirubin, AST (SGOT), ALT (SGPT), and alkaline phosphatase

After completion of radiation (10 +/- 2 weeks after)

- History and physical examination
- Complete blood counts, including platelets
- Blood chemistry studies, including creatinine, electrolytes (K+, Na+, Cl-, CO2), Ca++, Mg++ENT evaluation/endoscopy
- Liver function tests: Bilirubin, AST (SGOT), ALT (SGPT), and alkaline phosphatase
- CT and/or MRI of neck
- CT of chest
- Response assessment by clinical and by radiographic evaluation (reported separately) in primary and lymph nodes
- PET scan (optional)

After completing treatment, patients will be seen every 3 months for 2 years and then every 6 months for 3 years (i.e. until 5 years from radiation completion), and subsequently annually for a total of 10 years from study initiation (follow up evaluation will include assessment of late toxicities, such as dysphagia, G-tube feedings requirements, xerostomia, and others)

- CT scans of chest and head and neck will be performed every 3 months for 1 year and then every 6 months until 3 years from the completion of radiation. Then, CT of the chest will be done on an annual basis

- Swallowing assessments will be performed 3 months post radiation completion, and if problems persist, at 12 months post radiation

9.4 Study Parameters Table

	Baseline	During treatment and 2 weeks post RT	10 +/- 2 weeks after RT	Off therapy Follow-Up[8]
Study treatment (See Section 5)		X	X	
History and physical exam	X	X[9]	X	X
Height	X			
Weight	X	X[9]	X	
Vital signs	X	X[9]		
Toxicity assessment		X	X	X
Performance Status	X	X[9]	X	
CBC, Differential, Platelets[1]	X	X[9]	X[9]	
Creatinine, Electrolytes (K^+, Na^+, Cl^-, CO_2), Mg^{++}, Ca^{++}	X	X[9]	X[9]	
Liver enzymes[2]	X	X (every3 weeks)	X	
PT/PTT[10]	X			
Chest X-ray	X			
Pregnancy test[3]	X			
HIV status obtained via history	X			
CT or MRI of the head/neck	X		X	X[4,8]
CT of the chest	X		X	X[4,8]
Bone scan, if clinically indicated	X			
Tumor measurements[4,9]	X		X	X[4,8]
PET scan (optional)[5]	X		X	X
Swallowing assessment[6]	X			X[6]
Dental evaluation	X			
Tumor Biopsy and correlatives[7]	X	X (after 2 weeks)		
ENT evaluation/endoscopy	X		X	

1. Complete blood counts with differential and platelet count should be performed.
2. Liver function tests should include: Bilirubin, AST (SGOT), ALT (SGPT), and alkaline phosphatase. LFTs are required at least every 3 weeks during treatment.
3. All females of childbearing potential must have a negative pregnancy test done within 3 days of initiation of treatment to rule out pregnancy.
4. Tumor measurements will be made using physical examination, including endoscopic examination (clinically) and CT scans or MRI scans (radiographically). Clinical and radiographic assessments will be reported separately. Response in the primary and the neck will be reported separately as well. Tumor assessments will be performed 6-8 weeks after completion of radiotherapy. Subsequently, repeat imaging with CT scans of chest and head and neck will be performed every 3 months for 1 year and then every 6 months for a total of 3 years from the time of radiation completion. Then, CT of the chest or chest x-ray will be done on an annual basis.
5. Positron emission tomography (PET) scan or combined PET/CT scanning is optional. Results will be reported separately.
6. Swallowing assessments will be performed at baseline and 3 months post radiation completion, and if problems persist, at 12 months post radiation.
7. The following materials will be submitted: fresh frozen and paraffin-embedded tumor specimens for molecular studies.
8. Patients will be followed every 3 months for 2 years and then every 6 months for 3 years, and subsequently annually for a total of 10 years from study initiation.

9. During radiation, in the immediate 2 week period following RT completion, and the 6-10 week follow-up after RT completion; patients need a focused evaluation of toxicities on a weekly basis. However, a complete history and physical examination is only needed every 3 weeks.
10. PT/PTT will be performed at baseline while the patient is receiving intratumoral injections or more frequent, if clinically indicated.
11. All patients should be seen by a Medical Oncologist, an ENT surgeon, and a Radiation Oncologist prior to initiating therapy. Unresectability should be determined by a multidisciplinary team.

10. DRUG FORMULATION AND PROCUREMENT

10.1 EGFR Antisense

EGFR antisense DNA is plasmid DNA and will be prepared under conditions consistent with cGMP as described in CFR 210 and 211. Production will occur in the Center for Biomedicine and Genetics, the National Gene Vector Laboratory (NGVL) designated site at the Beckman Research Institute of the City of Hope in Duarte, California, under the direction of Dr. Larry A. Couture. Manufacturing and quality release testing will be performed as described in BB-MF 9778 (see cross reference authorization letter provided as an attachment).

Drug Information

Production of Plasmid DNA

Plasmid DNA was produced under GMP conditions at the Center for Biomedicine and Genetics (CBG) at the City of Hope according to City of Hope's Master File BB-MF-9778. Briefly, a bacterial Master Cell bank was produced in the STBLII competent cell line. Bacteria from the tested and released Master Cell Bank was used to inoculate "starter" and expansion cultures to seed 6 liter batches in a New Brunswick BioFlo3000 fermentor using proprietary bacterial media optimized for plasmid DNA production. The 6 liter culture was grown at 35°C for 20 ·4 hours with automated (PID loop) temperature, aeration and pH adjustment. Following fermentation the culture was harvested and bacterial pellets stored at -20°C for further processing.

Bacteria were processed using a modified alkaline lysis method (Birnboim, H.C. and Doly, J.(1979) Nucl. Acids. Res. 7,1513-1522). Briefly, bacterial pellets are resuspended in 50mM Tris-Cl, 10mM EDTA and then lysed with an equal volume of 200mM NaOH, 1% SDS at room temperature. The lysate is neutralized with 3.0M potassium acetate and clarified by centrifugation to remove the bulk of proteins, lipid and bacterial genomic DNA. The clarified lysate is passed through a series of filters (50 · ·0.2) and then concentrated 10-fold to 15-fold over a 30,000 NMWCO PES ultrafiltration device (Millipore ProFlux M12/Pellicon system). At this stage the concentrated lysate was held for 24 hours at 4°C.

Concentrated lysate was further clarified using depth filtration in the presence of filter aid and RNA removal was achieved by size exclusion chromatography over Sepharose 6 Fast Flow · (GE Healthcare). Plasmid DNA devoid of RNA contaminants was treated with an endotoxin adsorbent to remove trace endotoxin contamination. The plasmid DNA was concentrated and reformulated into PBS using tangential flow filtration. Samples of the bulk material were taken for release testing with the balance of the material stored at -20 C. The bulk material that met the defined specifications for protein, endotoxin, RNA and contaminating bacterial genomic

DNA were thawed and pooled for fill and finish.

Final fill of the plasmid DNA was preceded by end-point sterilization through a 0.2 ㎛ filter. Final product vials were transferred to Quality Assurance who coordinated final product testing and shipment of released material.

EGFR Antisense Shipping

EGFR antisense DNA is plasmid DNA, and is considered to be Biohazard I (BSL-1) since it does not contain viral elements, toxic proteins or oncogenes and will be shipped and handled as a research reagent as per biohazard material guidelines. The product will be supplied vialed and formulated in 1-2 mg/mL concentrations, stored at -80 ℃, and shipped on dry ice. Based on ICH recommendations, the product must be used within 3 hours from thawing of the product. Any/all unused product will be returned to the NGVL. EGFR antisense DNA will be sent from the University of Pittsburgh Pharmacy to our site's Investigational Drug Section (IDS). It will be shipped on dry ice for overnight delivery (Monday-Thursday). Upon arrival at IDS, drug will be inspected to ensure its intact/undamaged during shipping.

10.2 Cetuximab (C225, ERBITUX)

Cetuximab is an anti-EGFR human-to-murine chimeric antibody. Cetuximab is expressed in SP2/0 myeloma cell line, grown in large scale cell culture bioreactors and purified to a high level purity using several purification steps including protein A chromatography, ion exchange chromatography, low pH treatment and nanofiltration. Cetuximab is not known to be a vesicant.

Supplier/How Supplied
Cetuximab is commercially available. The product is a sterile, clear, colorless liquid of pH 7.0 to 7.4, which may contain a small amount of easily visible, white, amorphous cetuximab particulates. Each single-use 50-mL vial contains 100 mg of cetuximab at a concentration of 2 mg/mL and is formulated in a preservative-free solution containing 8.48 mg/mL sodium chloride, 1.88 mg/mL sodium phosphate dibasic heptahydrate, 0.42mg/mL sodium phosphate monobasic monohydrate, and Water for injection, USP.

Packaging and Labeling
Cetuximab is supplied as a 50mL, single-use vial containing 100mg of Cetuximab at a concentration of 2mg/mL in phosphate buffered saline. The solution should be clear and colorless and may contain a small amount of easily visible white amorphous Cetuximab particulates.

Storage Requirement/Stability
Store vials under refrigeration at 2°C to 8°C (36°F to 46°F). DO NOT FREEZE. Increased particulate formation may occur at temperatures at or below 0°C. This product contains no preservatives. Preparations of cetuximab in infusion containers are chemically and physically stable for up to 12 hours at 2°C to 8°C (36°F to 46°F) or up to 8 hours at controlled room temperature (20°C to 25°C; 68°F to 77°F). Discard any remaining solution in the infusion container after 8 hours at controlled

room temperature or after 12 hours at 2 ·to 8 Ċ. Discard any unused portion of the vial.

Preparation and Administration
Cetuximab must not be administered as an IV push or bolus.

Cetuximab must be administered with the use of a low protein binding 0.22-micrometer in-line filter.

Cetuximab is supplied as a 50-mL, single-use vial containing 100 mg of cetuximab at a concentration of 2 mg/mL in phosphate buffered saline. The solution should be clear and colorless and may contain a small amount of easily visible white amorphous cetuximab particulates. DO NOT SHAKE OR DILUTE.

Cetuximab can be administered via infusion pump or syringe pump.

Infusion Pump:

- Draw up the volume of a vial using a sterile syringe attached to an appropriate needle (a vented spike or other appropriate transfer device may be used).
- Fill cetuximab into a sterile evacuated container or bag such as glass containers, polyolefin bags (eg, Baxter Intravia), ethylene vinyl acetate bags (eg, Baxter Clintec), DEHP plasticized PVC bags (eg, Abbott Lifecare), or PVC bags.
- Repeat procedure until the calculated volume has been put in to the container. Use a new needle for each vial.
- Administer through a low protein binding 0.22-micrometer in-line filter (placed as proximal to the patient as practical).
- Affix the infusion line and prime it with cetuximab before starting the infusion.
- Maximum infusion rate should not exceed 5 mL/min.
- Use 0.9% saline solution to flush line at the end of infusion.

Syringe Pump:

- Draw up the volume of a vial using a sterile syringe attached to an appropriate needle (a vented spike may be used).
- Place the syringe into the syringe driver of a syringe pump and set the rate.
- Administer through a low protein binding 0.22-micrometer in-line filter rated for syringe pump use (placed as proximal to the patient as practical).
- Connect up the infusion line and start the infusion after priming the line with cetuximab.
- Repeat procedure until the calculated volume has been infused.
- Use a new needle and filter for each vial.
- Maximum infusion rate should not exceed 5 mL/min.

- Use 0.9% saline solution to flush line at the end of infusion.
- Cetuximab should be piggybacked to the patient's infusion line.

Following the cetuximab infusion, a 1-hour observation period is recommended.

Safety Precautions:
Appropriate mask, protective clothing, eye protection, gloves, and Class II vertical-laminar-airflow safety cabinets are recommended during preparation and handling. Opened vials must be disposed of at the investigational center as chemotherapy or biohazardous waste provided documented procedures for destruction are in place. For questions regarding cetuximab destruction please contact BMS at (866) 339-4267 or (203) 677-7017.

Cetuximab therapy should be used with caution in patients with known hypersensitivity to Cetuximab, murine proteins, or any component of this product.

It is recommended that patients wear sunscreen and hats and limit sun exposure while receiving cetuximab as sunlight can exacerbate any skin reactions that may occur.

Cetuximab Records at Investigational Site(s)

It is the responsibility of the Investigator to ensure that a current record of investigational product disposition is maintained at each study site where investigational product is inventoried and disposed. Records or logs must comply with applicable regulations and guidelines, and should include:

- Amount received and placed in storage area.
- Amount currently in storage area.
- Label ID number or batch number.
- Dates and initials of person responsible for each investigational product inventory entry/movement.
- Amount dispensed to and returned by each patient, including unique patient identifiers.
- Amount transferred to another area for dispensing or storage.
- Non-study disposition (e.g., lost, wasted, broken).

Drug Ordering and Accountability
The ordering and drug accountability for Cetuximab will be handled per institutional Standard Operating Procedures (SOPs).

It is the responsibility of the Investigator to ensure that a current record of study drug disposition is maintained at each study site where the study drug is inventoried and

disposed. Records or logs must comply with applicable regulations and guidelines.

Nursing Implications
Patients need monitoring for infusion reactions. Premedication with diphenhydramine hydrochloride [Benadryl] will be used as described in Sections 5.0 and 5.1.

Diarrhea is a possible side effect of cetuximab. It can be managed with loperamide (Imodium).

11. STATISTICAL CONSIDERATIONS

11.1 Primary Endpoint

This is a 2-stage clinical trial. In the first stage, toxicity will be the primary endpoint. In the second stage (phase II component), the primary endpoint will be the locoregional progression free survival (PFS) at 1 year. Toxicities will also be evaluated during the second stage using continuous Bayesian monitoring as described below.

Sample size for the primary objective: 11 evaluable patients (stage 1); 31 evaluable patients (stage 2)

First stage: 11 evaluable patients. The safety-evaluable population for stage 1 consists of all patients who either complete the treatment regimen or withdraw from study by reason of toxicity.

Toxicities due to EGFR AS in our previous phase I trial were negligible, and consisted of grade 1 injection site pain and swelling. Because we will combine EGFR AS with radiation and cetuximab in the proposed clinical trial, a separate safety evaluation will be conducted. In the first stage (safety component), 11 patients will be treated and observed for serious adverse events (SAEs) that are due to EGFR AS (possibly, probably, or definitely), not cetuximab or radiation. In the first stage, after three (3) subjects are enrolled and have received all EGFR AS injections, there will be a two-week waiting period to assess adverse events. After the two-week period, additional subjects may be enrolled. Eligible patients will include stage IV or recurrent patients who would not be eligible for the subsequent study of efficacy, and their accrual to study is only for the assessment of safety. We seek to rule out an SAE rate of 20% or greater due to EGFR AS. If no grade 3/4 toxicities due to EGFR AS are observed among 11 patients, then an upper 90% confidence bound for the SAE rate is less than 20%.

Second stage (phase II component): 31 evaluable patients. The efficacy-evaluable population consists of all patients who complete the treatment regimen.

Sample size for the second stage of study is based on the primary endpoint: 1-year locoregional PFS. We use a historical control rate of 63% for 1-year locoregional PFS in patients on radiation and cetuximab, as reported by Bonner et al. To account for the inherent variability in this estimate, and to improve the likelihood that we can detect a true improvement with the addition of EGFR AS, we use the reasoning of Fazzari et al.[45] which advocates setting the null hypothesis to the upper bound of the 75 percent confidence interval for the historical-control endpoint. By assuming that 1-year locoregional PFS as reported by Bonner et al follows an exponential distribution from a

sample of 211 patients, the upper 75th percent confidence bound for 1-year locoregional PFS is 68%. Targeting a 50% reduction in the hazard rate for 1-year locoregional PFS under our treatment regimen implies that 1-year locoregional PFS would be improved to 83%. To achieve this objective, we plan a study with 2 years of patient accrual, 1 year of additional follow-up, and assume that 1-year locoregional PFS will have an exponential distribution. Under these assumptions, a 1-sided exponential test at $\alpha = 0.10$ will provide 90% power with 31 patients. In addition, we plan to evaluate tumor response and locoregional progression in injected and non-injected lymph nodes. The rates of progression in injected versus non-injected regions will be compared by an exact test for two rates, implemented in StatXact 8.

Total sample size

A total of 42 evaluable patients will be accrued to study.

Eleven patients will be treated in the first stage, which will assess the safety of the combination. Patients failing to complete treatment for reasons other than toxicity will be replaced.

In the second stage, 31 efficacy-evaluable patients will be enrolled, provided safety is acceptable.

11.2 Secondary Clinical Endpoints

Clinical secondary endpoints in phase II include adverse events, in-field and out-field, objective response rate, distant PFS, overall PFS, and overall survival.

11.3 Accrual

The study will be conducted at the CTRC at UTHSCSA. We anticipate that 42 evaluable patients (11 in the first stage and 31 in the second stage) will be accrued over two years, and that the monthly accrual rate will be approximately 1-2 patients per month.

11.4 Safety Evaluation During the Second Stage

In the phase II component of study, safety will be monitored continuously using Bayesian methods. The toxicities of the combination of RT and cetuximab have been characterized by a randomized study by Bonner et al [18] in which 208 patients treated with RT and cetuximab were evaluated for toxicity. Grade 3/4 radiation mucositis and radiation dermatitis were common toxicities occurring with high frequencies. The proportion of grade 3/4 events in the RT+cetuximab arm were 56% for mucositis and 23% for radiation dermatitis. While the intratumoral injection of EGFR AS is not expected to increase the rate of SAEs, we will nonetheless monitor patients for unexpected increases in mucositis and radiation dermatitis that may be attributable (possibly, probably, definitely) to the addition of EGFR AS, and will suspend the trial if there is evidence that the occurrences of these toxicities are greater than what would be expected from radiation and cetuximab without EGFR AS.

We use informative prior distributions of SAEs to conform in part to the reported SAE frequencies in Bonner – probabilities of grade 3/4 mucositis of 56% and grade 3/4 radiation dermatitis of 23%. Our assumed prior probabilities are not as concentrated as the nominal uncertainty in the

estimates from the Bonner study of 208 patients, but instead allow for greater variability, which can arise from different treatment populations. Specifically, we specify a 90% credibility interval for the probability of mucositis as 56% 15%, and 23% 10% for the probability of radiation dermatitis; these are wider intervals than suggested by the results of the study by Bonner et al.

Assuming that the prior probability of an SAE follows a beta distribution, the stopping rule is based on the updated posterior probability of an SAE. As each patient is observed for SAEs, the posterior probability, which follows a beta distribution, of SAE can be calculated. If at any time during the trial the posterior probability is ≥ 0.80 that the rate of treatment-related grade 3/4 mucositis exceeds 56%, the study will be suspended pending review by Data and Safety Monitoring Committee (DMSC). Similarly, if the posterior probability is greater than 0.80 that the rate of treatment-related grade 3/4 radiation dermatitis exceeds 23%, the study will be suspended for review by the DSMC. To permit continuous monitoring of SAEs, a table (see below) based on the cumulative number of patients experiencing SAEs at any point during the trial can be used to signal when the study should be suspended and reviewed by the DSMC.

Table 6 Number of Patients Observed to Have Treatment-Related SAEs and Corresponding Posterior Probabilities Needed to Suspend the Study in Phase II

	Treatment-Related Grade 3/4 Mucositis		Treatment-Related Grade 3/4 Radiation Dermatitis	
Patients	SAEs	Pr($\pi \geq 0.56$)*	SAEs	Pr ($\pi \geq 0.23$)
4	---	---	4	.825
5	---	---	4	.804
6	6	.814	5	.861
7	7	.848	5	.843
8	8	.877	5	.823
9	8	.832	5	.802
10	9	.862	6	.858
11	9	.815	6	.840
12	10	.848	6	.821
13	11	.875	6	.801
14	11	.833	7	.856
15	12	.862	7	.838
16	12	.818	7	.820
17	13	.848	7	.800
18	13	.803	8	.854
19	14	.834	8	.837
20	15	.862	8	.819
21	15	.821	9	.868
22	16	.849	9	.852
23	16	.807	9	.835
24	17	.837	9	.818
25	18	.863	10	.866
26	18	.824	10	.850
27	19	.851	10	.834
28	19	.811	10	.817
29	20	.839	11	.864
30	21	.864	11	.849
31	21	.827	11	.833

* π is the posterior probability of a treatment-related SAE. For example, given the observed number of patients treated and the number of patients observed with SAEs, Pr(π ≥0.56) is the posterior probability that treatment-related grade 3/4 mucositis exceeds 0.56, the expected value prior to study.

The table above presents the minimum number of patients experiencing treatment-related SAEs (i.e., those SAEs judged to be possibly, probably, or definitely related to the study regimen) that would dictate suspension of the trial in accordance with the stopping rule. For example, after the first 10 patients have completed treatment and been observed for toxicity, 9 patients experiencing treatment-related grade 3/4 mucositis, or 6 patients experiencing treatment-related grade 3/4 radiation dermatitis, would trigger trial suspension pending review by the DSMC.

11.5 Hypothesis Testing and Operating Characteristics for Correlative-Study Endpoints

Correlative studies will assess antitumor activity measured by levels of biomarker expression. This will involve measurement of the following biomarkers from tumor tissue using RPPA: EGFR, pEGFR, Src, pMAPK, STAT3, pSTAT3, pSTAT5, pSTAT1, pAKT, p38, p21, p27, PARP, E-cadherin, p-ErbB3, and Ki67. In some instances immunohistochemical staining of selected proteins will be performed to verify or validate RPPA results. Biomarkers will be tested for treatment-associated modulation (i,e., change from baseline) as well as for association with 1-year locoregional PFS (primary endpoint), and other efficacy parameters, such as objective response rate, PFS, and overall survival. These analyses will be exploratory in nature.

To estimate the power of our study to assess whether significant modulation of biomarkers occurs from therapy, we assume that 80% of patients in phase II will have a 2nd biopsy after 2 doses of cetuximab with EGFR AS, thereby providing paired tumor tissue samples in 24 patients total. Based on data from a prior phase I clinical trial, the standard deviation was 0.7 on average for paired differences ($\log_2$[post/baseline]) in proteins measured pre and post therapy. Assuming this standard deviation will apply, a two-tailed signed rank test at $\alpha = 0.01$ (to control the false-positive rate) in 24 patients will provide 80% power to detect a change of 0.55 ($\log_2$ scale), which corresponds to a change of ·46% from the baseline-expression level. Although the search for individual biomarkers that change with treatment is exploratory and hypothesis generating, we will report all nominal p-values along with those that remain significant using a 10% false discovery rate.

It is also of interest to assess whether biomarker modulation within the treatment window is associated with PFS. To estimate power for these analyses, we use Cox proportional hazards regression to evaluate whether PFS depends on change in biomarker expression, considered for each biomarker individually. We assume that 25% of treated patients will have loco-regional progression within 1 year, i.e., PFS = 0.75, based on expectation averaged over the null and alternative hypotheses. We also assume that among 31 patients enrolled in phase II, 24 will have adequate tissue and biomarker assessments before and after therapy. We further assume that accrual in phase II will take 18 months, with 12 months of follow-up beyond the last patient enrolled. Under exponential survival, these assumptions imply that 9 of the 24 patients with pre-post biomarker measurements will have loco-regional progression within our study time frame. A two-tailed Cox-regression test of trend at $\alpha = .05$ will therefore have 80% power to detect a hazard ratio of 3.8, which corresponds to a one-unit change (i.e., 2-fold increase or decrease) in the biomarker tested.

With respect to assessing PFS in relation to baseline measurements of biomarkers, we assume that all 31 patients enrolled in study will have tissue available for analysis, in which case 12 patients are expected to have loco-regional progression within the study time frame. For this

situation, the minimum detectable hazard ratio would be 3.2 under the conservative assumption that variability in biomarker expression among patients is no greater than that within patients (greater variability would provide increased opportunity to detect biomarker effects).

The analyses of the correlative data are exploratory. A less restrictive test (α = .05) is used for PFS to avoid missing important leads. Nominal p-values for all biomarkers associated positively or negatively with PFS will be reported along with a designation of those that fall within a 10% false discovery rate.

11.6 Additional Analyses

The data from all evaluable patients will be used in the analyses of safety (phase I and phase II), tumor response (phase II), and survival (phase II).

Baseline Characteristics

Baseline descriptive statistics on all evaluable patients will be provided on demographic variables (age, sex, and race/ethnicity), performance status, laboratory parameters, treatment regimens that were previously used, and disease characteristics, including tumor size, number of nodes involved, and metastatic sites.

Safety Profile

Using NCI CTCAE (v4.0), the number of patients experiencing AEs over their course of treatment, and for 30 days beyond the last protocol treatment administered, will be characterized by type of AE and grade, and by the time of onset in relation to the first day of therapy. Attribution of AEs to treatment (unrelated, unlikely, possible, probable, or definite) will be recorded, and the details of all treatment-related toxicities will be reported.

Survival

PFS will be measured from the initial date of treatment to the date of documented progression, or the date of death (in the absence of progression).

Overall survival will be measured from the initial date of treatment to the recorded date of death or for a total of 5 years follow up (See Section 12.3).

PFS and overall survival will be estimated by the Kaplan-Meier method. The corresponding median survival times (with 90% confidence limits) will be determined, as will the cumulative percentage of patients remaining progression-free at selected time points after initial treatment.

Tumor Response

Response rates will be estimated by the proportion of patients with a best response of complete response (CR), partial response (PR), or stable disease (SD) by RECIST criteria, with corresponding exact 90% confidence limits being reported. The distribution of response duration (time to progression among patients

achieving CR, PR, or SD) will be characterized by median and quartiles, with the corresponding Kaplan-Meier estimate being made of PFS among patients responding to treatment.

12. RECORDS TO BE KEPT

12.1 In addition to the regular hospital or clinic chart, a separate patient folder will be kept which includes:

- The patient's signed, dated and witnessed consent
- Pathologic documentation of squamous cell carcinoma of the head and neck
- The Completed Patient Registration Form and all other study forms

12.2 Within 7 days after patient's entry on study, copies of the following must be received by the coordinating nurse at the CTRC at the UTHSCSA:

Ofelia Romero, RN
Clinical Research Nurse III
Department of Clinical Investigations
Institute for Drug Development
Cancer Therapy and Research Center at UTHSCSA
Mail Code 8229
7979 Wurzbach Road, Suite Z413
San Antonio, Texas 78229
Office: (210) 450-1810
Fax: (210) 614-4418
Email: romeroo@uthscsa.edu

12.2.1 Completed Patient Registration Form. Eligibility criteria will be clearly stated. Baseline information will include: age, gender, race, feeding tube placement, smoking and alcohol history, performance status, TNM staging, and sites of disease involvement.

12.2.2 Flow Sheet reflecting pretreatment test results and the first therapy.

12.2.3 Measurement Form showing baseline measurement (measurable disease patients).

12.2.4 Pathology Report (pathologic confirmation of disease.)

12.3 Thereafter, while the patient is on study, forms will be completed according to the following schedule:

12.3.1 Evaluation forms after completion of radiation. Clinical and radiographic response will be reported. Also, response in the primary and lymph nodes will be reported

separately. Measurement forms must give serial measurements or evaluations as required by protocol to assess disease response to therapy. Follow-up forms will report the presence of feeding tube and late toxicities.

12.3.2 A final Treatment Summary Form is to be submitted when the patient progresses, dies, or goes off study for any other reason. Any subsequent head and neck surgery will be reported.

12.3.3 Follow-Up Form is submitted at the time the patient goes off study at any time prior to treatment completion as scheduled and thereafter, every 3 months for the first 2 years, then every 6 months for the next 3 years, and subsequently annually at the discretion of the treating physician.

12.3.4 Death must be reported, using the Follow-Up Form

13. CORRELATIVE STUDIES

13.1 Reverse Phase Protein Microarrays (RPPA)

The last decade has witnessed a relative explosion in the development and availability of therapeutic reagents that block specific signaling pathways. While gene microarrays and transcriptional profiling can provide information regarding coordinate gene expression and transcriptional control mechanisms, they cannot capture fluctuating signaling events that occur at the protein and particular the post translational level. In addition, mRNA levels do not necessarily correlate with protein abundance. To fully realize the potential of molecular targeted therapy, it is critical to apply tools that can profile the cancer cell proteome in terms of both total levels and functional modifications. Only recently has technology been developed to measure the activation state of kinase driven signal transduction networks. Using antibodies that specifically recognize the phosphorylated and total isoforms of kinase substrates, reverse phase protein microarrays (RPPA) can multiplex and quantify a large array of activated proteins.[37] The amount of target is determined by comparison to standard curves of peptide and phosphopeptide target. Approaches that rely on immobilized antibodies to capture their analytes from solution (forward phase approach) are limited by the lack of antibodies that can function in this format and the need for a relatively large amount of starting material as well as using a single condition for multiple antibodies. In contrast, the reverse phase approach (RPPA) is accomplished by depositing small volumes of cell lysates onto a high protein-binding substratum such as a nitrocellulose slide using a robotic microarrayer requiring small amounts of input protein which has been boiled in SDS mimicking the rigorous conditions of western blotting. Each slide can accommodate up to 160 different samples with titration curves and peptide controls. Arrays are then probed with signal generating detection antibodies, which can be dependent or independent of the phosphorylation state. Hierarchical clustering analysis facilitates the determination of distinct molecular subgroups according to their protein signature. New antisera continue to be validated and over 100 different signaling related antibodies to date have been validated for their use in RPPA.

We have acquired experience with RPPA in collaboration with Dr. Gordon Mills at MD Anderson Cancer Center (MDACC). Using RPPA, we are currently analyzing biomarker expression levels in

tumor biopsies obtained before, during and after intratumoral gene therapy treatment using an EGFR antisense gene in patients with recurrent HNSCC. As shown in Figure 1, this technique provides quantitative information on the expression of critical proteins.

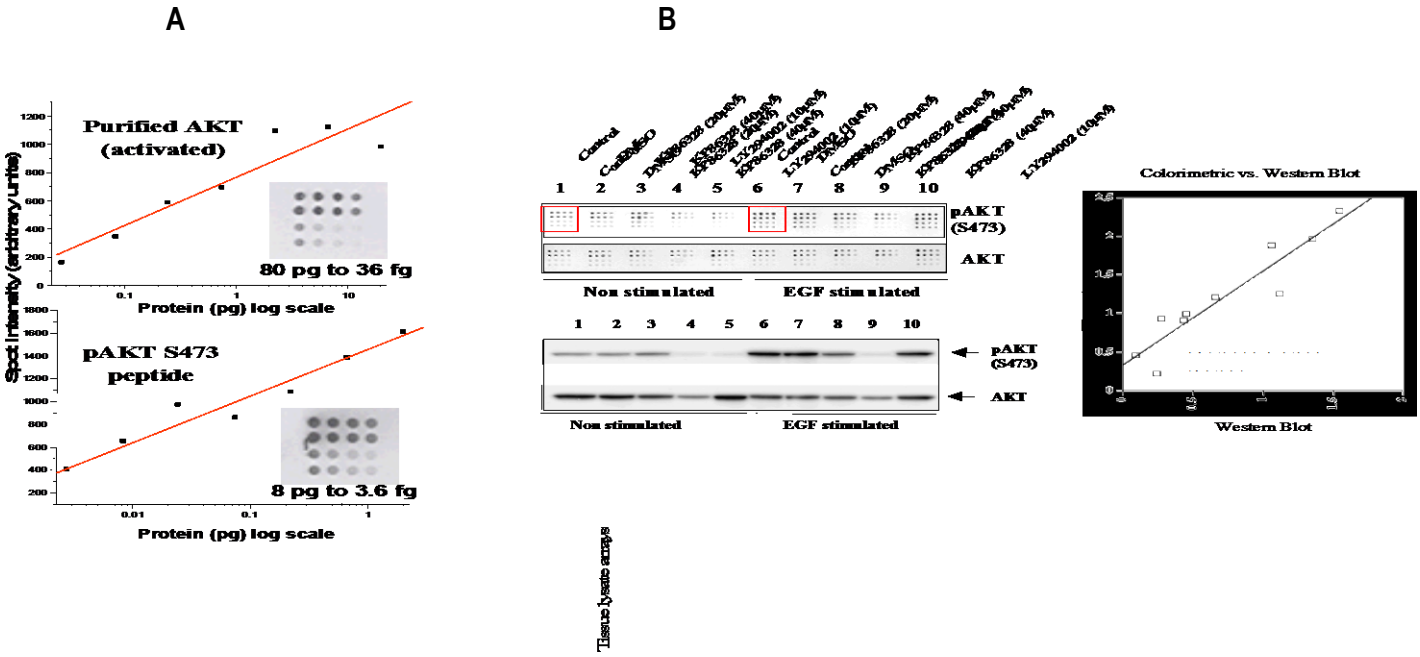

Figure 1. Validation and sensitivity of tissue lysate arrays. Panel A. Serial dilutions of activated purified AKT, Phospho-AKT peptide were arrayed in duplicate and quantitated. The linearity and sensitivity (femtograms of purified protein) is represented in the plot. **Panel B.** Tissue lysate arrays are compared to Western blots of the same cell lysate treated with the KP86328 (AKT inhibitor) and LY294002 (PI3 kinase inhibitor). The squares highlight the detection of phospho-AKT upon EGF stimulation by tissue lysate arrays. Quantitative results from tissue lysate arrays and Western Blot demonstrated remarkable concordance.

Preliminary results from tumor biopsies collected from SCCHN patients treated on the first two dose tiers of a phase I clinical trial to determine the toxicity and biologic efficacy of EGFR AS demonstrates that expression of the target gene (EGFR) as well as its phosphorylated form and downstream targets can be measured using RPPA (Figure 2).

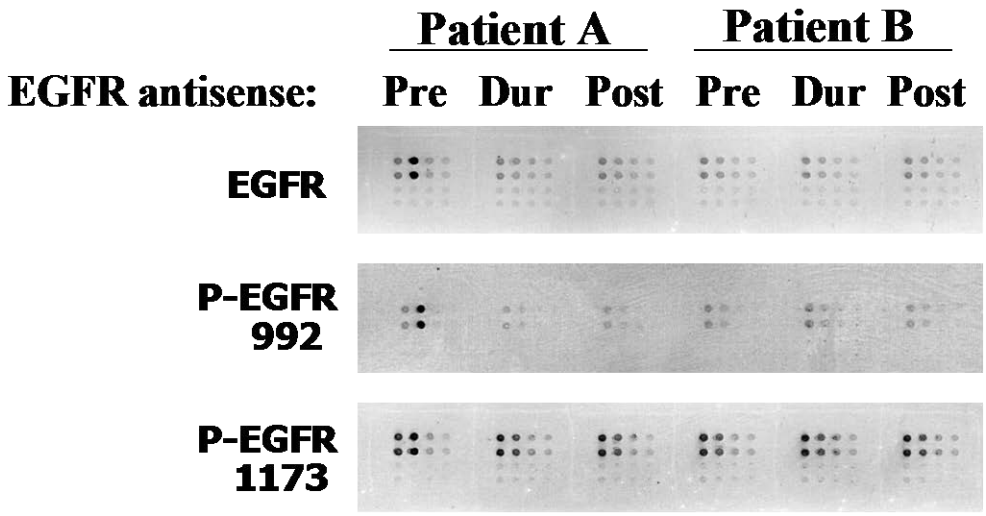

Figure 2. Detection of total EGFR and phosphorylated forms of EGFR (p-EGFR 992, p-EGFR 1173) in SCCHN tissues by RPPA. Tissue lysates are prepared from SCCHN patients treated on an EGFR antisense gene therapy trial. Tissue is harvested before (Pre), during (Dur) and after (Post) treatment. Each sample is analyzed in duplicate serial dilutions. The RPPA panels represent results from two patients (patients A and B) treated on the first 2 dose tiers.

To date, over 100 monospecific antibodies have been validated for use in RPPA (see http://home.ccr.cancer.gov/ncifdaproteomics). MDACC and NCI/FDA maintain a joint database of validated antibodies for RPPA. These antibodies detect a variety of proteins involved in phosphorylation, apoptosis (activated and cleaved proteins) and cell cycle progression (cyclins, phosphohistones p21, p27). Although up to 100 antibodies have been validated, we will prioritize pathways and antibodies for analysis. Blockade of EGFR in head and neck cancer cell lines leads to downregulation of several downstream signaling proteins including pSrc, pMAPK, pSTAT3, pSTAT5, pSTAT1, and pAKT. The first tier of proteins/phosphoproteins would include EGFR, pEGFR, Src, pMAPK, STAT3, pSTAT3, pSTAT5, pSTAT1, pAKT, p38, p21, p27, PARP, E-cadherin, p-ErbB3, and Ki67. Additional pathways/proteins can be evaluated as other antisera are validated and/or new hypotheses emerge from ongoing experiments.

Frozen tumor will be homogenized with lysis buffer. Lysates will be boiled with 1% SDS, mimicking the stringent conditions used in immunoblotting. After centrifuging, the protein-rich supernatant can be stored at –80°C and transferred to the Mills laboratory at MDACC for RPPA. The cost of setting up a RPPA facility (~$500,000) requires that the lysates be arrayed in the Mills lab. Serial dilutions with additional lysis buffer are generated from each lysate immediately prior to array preparation using a Tecan liquid handling robot. A robotic arrayer then creates a 640 spot array on nitrocellulose-coated glass slides where each spot on the array represents a precise dilution of a particular sample lysate. As many as 160 different samples with 4 serial dilutions can be studies on a single slide and with robotics, 24 identical slides can be printed at a time. The Mills lab has successfully transferred lysates from center to center with retention of signals. Each slide is then probed with a specific validated primary antibody under optimal blocking conditions to minimize

background noise. Multiple controls are placed on each slide to facilitate quantitation. The signal is amplified using a DAKO catalyzed system and antibody to β-actin and GAPDH can be used as controls. Slides are then scanned and analyzed by ImageQuant software (Molecular Dynamics). The serial dilution "dot" intensities are quantitated using Microvigene software (VigeneTech, Inc.) The Mills lab has demonstrated that this technique is sensitive (to femtograms of protein) and reproducible. Serial dilutions allow an accurate determination of protein quantity using concentration curves of synthetic phospho- and non-phosphopeptides of the antibody-binding site for each antibody.

13.2 IHC Analysis of Tissue Microarrays

Because RPPA is an emerging technology, which cannot identify the cells expressing the target protein, we will also carry out IHC analysis of the proteins identified by RPPA of TMA generated from the biopsy samples. For high throughput immunohistochemical analysis and standardization of staining conditions, tissue microarrays (TMAs) will be constructed from formalin fixed paraffin embedded tissue blocks from the tumor biopsies. Using a manual tissue arrayer (MTA-1, Beecher Instruments, Sun Prairie, WI) 0.6 mm tissue cores will be extracted from each pre and post treatment tumor in triplicate and arrayed on 1-2 recipient paraffin blocks along with normal tonsillar controls. The newly constructed array block will then be warmed to 35-37°C for 10 minutes to allow annealing of donor cores to the paraffin wax of the recipient block and minimize core loss. Donor cores will range from 2-4 mm in length. Hence each TMA will yield an estimated 100 to 200 tissue sections (4 μm thickness). For TMA quality assessment morphologic confirmation of tumor, throughout the entire thickness of the block, one hematoxylin and eosin stained slide will be prepared from every five tissue sections. Immunohistochemical staining will be performed to assess various biomarkers in the EGFR signaling pathway including EGFR, pEGFR, Src, pMAPK, STAT3, pSTAT3, pSTAT5, pSTAT1, pAKT, p38, p21, p27, PARP, E-cadherin, p-ErbB3, and Ki67 on paraffin sections cut from the TMAs using standard methods. Sections will be deparaffinized with successive ethanol and xylene baths. These sections were then subjected to an optimized antigen retrieval method individualized to each antibody. Signal amplification will be performed using a proprietary micropolymer peroxidase (ImmPRESS™, Vector, Burlingame, CA) conjugated to an anti mouse antibody. Immunoreactive cells will be visualized with the brown color resulting from incubation with diaminobenzidine (DAB) chromogenic substrate at room temperature for 5 minutes. Sections will be counterstained blue with hematoxylin for 15 seconds and lithium carbonate for 5 seconds to provide morphologic detail on paraffin sections cut from the TMAs using commercially available antibodies and standard methods. Sections will be deparaffinized with successive ethanol and xylene baths. These sections will then be subjected to an optimized antigen retrieval method individualized to each antibody. Signal amplification will be performed using a proprietary micropolymer peroxidase (ImmPRESS™, Vecto

r, Burlingame, CA) conjugated to an anti mouse antibody. Immunoreactive cells will be visualized with the brown color resulting from incubation with diaminobenzidine (DAB) chromogenic substrate at room temperature for 5 minutes. Sections will be counterstained blue with hematoxylin for 15 seconds and lithium carbonate for 5 seconds to provide morphologic detail. Immunohistochemical staining will be scored semiquantitatively for each core. The percentage of immunoreactive cells will be recorded, rounded to the nearest 10 percentile. Additionally, intensity will be scored as

follows: 0 (none), 1+ (weak), 2+ (moderate) 3+ (strong). A composite score will be derived from the product of the percentage and intensity of staining. When appropriate, separate scores for each case will be assigned for cytoplasmic and nuclear staining.

Table 7. Summary Table Of Material To Be Collected And Timepoints

Method	Specimen Type	Timepoints	
		Baseline biopsy	Week 2 biopsy (after week 2 of cetuximab and EGFR AS but prior to radiation)
RPPA	Tumor Tissue, fresh frozen and/or paraffin-embedded	X	X
IHC	Tumor Tissue, paraffin-embedded	X	X

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APPENDIX I

ECOG PERFORMANCE STATUS

Grade	ECOG
0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work
2	Ambulatory and capable of all selfcare but unable to carry out any work activities. Up and about more than 50% of waking hours
3	Capable of only limited selfcare, confined to bed or chair more than 50% of waking hours
4	Completely disabled. Cannot carry on any selfcare. Totally confined to bed or chair
5	Dead

APPENDIX II

Template for Reporting Adverse Events In Human Gene Transfer Trials

This template is intended to facilitate the reporting of adverse events in human gene transfer trials. You may download this as a Word document and the fields will expand according to the amount of text entered. Use of this template is not required and other formats (e.g. AdEERS reports, MedWatch forms) may be acceptable provided that they include all the information specified in M-I-C-4-a of the *NIH Guidelines for Research Involving Recombinant DNA Molecules.* (http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html)

Submitting this completed template to the NIH Office of Biotechnology Activities alone does NOT fulfill the reporting requirements of other agencies. However, some agencies may accept submission of a duplicate copy of this completed template. You should verify with the other parties to whom you report whether the use of this template is acceptable.

Completed reports may be sent via U.S. mail, courier service, e-mail, or facsimile to:
NIH Office of Biotechnology Activities
6705 Rockledge Drive, Suite 750
Bethesda, Maryland 20892-7985
(For all non-USPS deliveries use Zip Code 20817)
Telephone 301-496-9838
Fax 301-496-9839
E-mail address for Reporting Adverse Events: GeMCRIS@od.nih.gov
General E-mail: oba@od.nih.gov

Website: www4.od.nih.gov/oba/ Gene Transfer Adverse Event Reporting Template Version 4.29.05 **PROTOCOL AND EVENT TYPE**	
NIH/OBA (RAC) Protocol Number	
FDA IND number	
Date this report completed:	
Seriousness of the AE (choose one)	Death Life-threatening Initial or prolonged hospitalization Disability Congenital anomaly Required intervention to prevent permanent impairment/damage Other medically important condition Non-serious
Severity of Event	Minimal Moderate Severe Life- Threatening Fatal
Was this event expected in terms of its severity?	Yes No
Was this event expected in terms of its specificity?	Yes No
Relationship of Event to gene transfer product	Unrelated Unlikely Possible Probable Definite
Attribution of AE Attribution of AE, continued	Concomitant medication Product Intervention Underlying disease Route of administration

	Other suspected cause (describe)
Type of report	Initial Follow-up

DEMOGRAPHICS

PI Name	
Name of Clinical Trial Site/Organization	
PI Telephone Number	
PI E-mail Address	
Reporter name	
Reporter Telephone number	
Reporter E-mail address	
Research Participant's study identification number	
Research Participant's gender	
Research Participant's date of birth	
Research Participant's date of death	
Research Participant's weight in kgs	
Research Participant's height in cms	
Which Arm/Cohort/treatment group was the subject assigned to?	
Was subject dosed?	Yes No Information Not Available
What study agent was received:	IND agent Placebo Blinded Study Agent
Were there any Protocol Deviations/Violations/Exceptions for this participant?	Yes:_____ _____ _____ No

DETAILED ADVERSE EVENT INFORMATION

Adverse Event Date	
Description of Event	
Relevant tests (e.g. x-rays) and results	
Treatment (s) of Adverse Event (Include medications used to treat this event.)	
Name of Concomitant Medications (Do not include medications used to treat this event.)	
Pre-existing conditions/ relevant clinical history (if this is an oncology trial, please designate primary disease, e.g. ovarian cancer)	
Date(s) of treatment(s) of the adverse event	
Was autopsy performed?	Yes No
Date of autopsy	or Not Applicable _____
Outcome of the event	Recovered/resolved Recovering/resolving Not recovered/not resolved

	Recovered/resolved with sequelae Fatal Unknown
Documentation accompanying the report (e.g., H& P, Progress Notes, Discharge Summary, Lab or Autopsy Reports, Other, etc.)	
Description of any "other" documentation	

PRODUCT AND DOSING INFORMATION

Name of gene transfer product
Vector type (e.g. adenovirus)
Vector sub-type (e.g. type 5, also include relevant deletions)
Lot number
Was the agent manufactured at an NGVL?
Route of administration
Site of administration
Did subject receive the dose specified in the protocol?
If not, what dose was given?
Date of first exposure to study agent?
Date of most recent exposure to study agent?
Total dose received prior to this event?
Total dose quantity administered to subject to date
Unit of measure for a single dose
Dose quantity in a single administration
If courses used, how many were given prior to this event?
How many doses on the last course were given?
Was the administration of this product stopped because of this adverse event?
Name of other treatment (s) (medications, radiation, surgery) received by research participant as required by the protocol

APPENDIX III

Supplemental Efficiency and Distribution Data

We performed tumor volume studies in our athymic nude mouse xenograft model using the same EGFR antisense construct that will we are proposing to adminimister to the head and neck cancer patients. This construct was administered either by intratumoral inoculation (Figure 1A) or by intravenous administration (Figure 1B). In each case, tumor growth inhibition was observed compared with treatment using the control EGFR sense DNA construct.

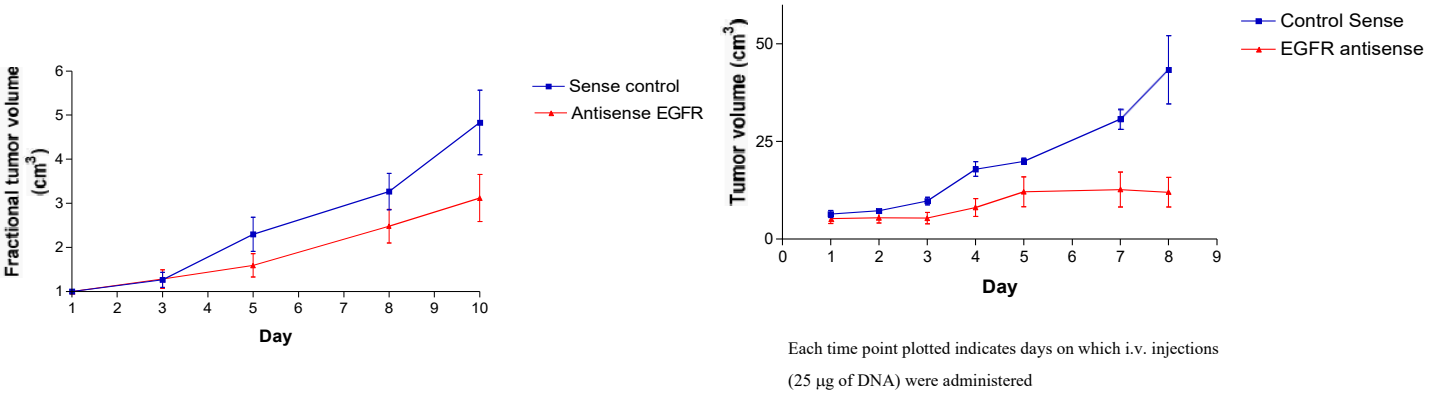
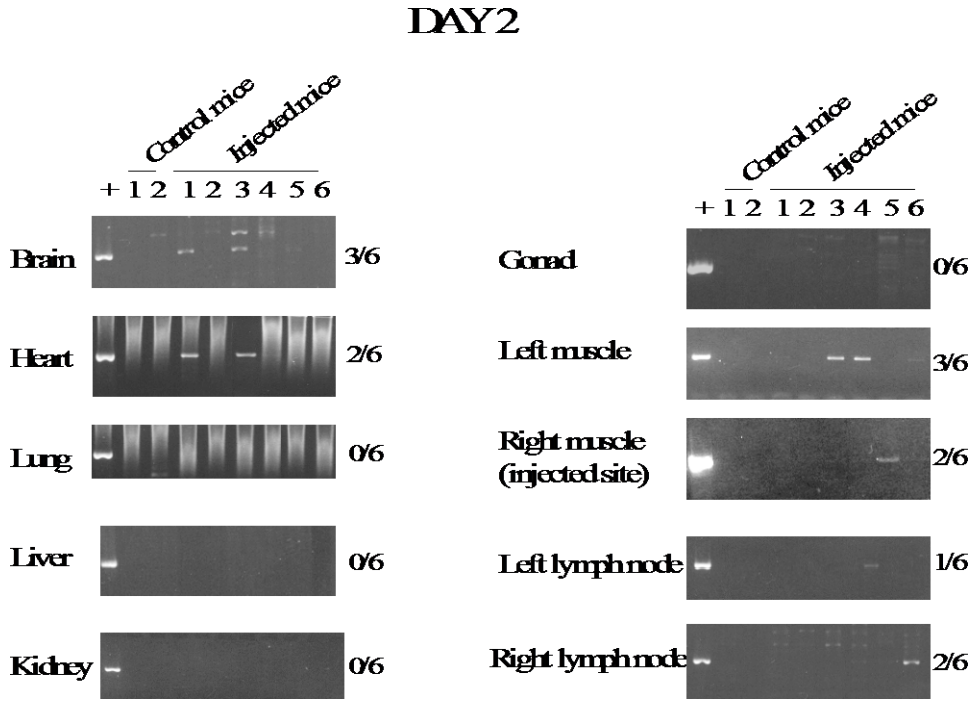


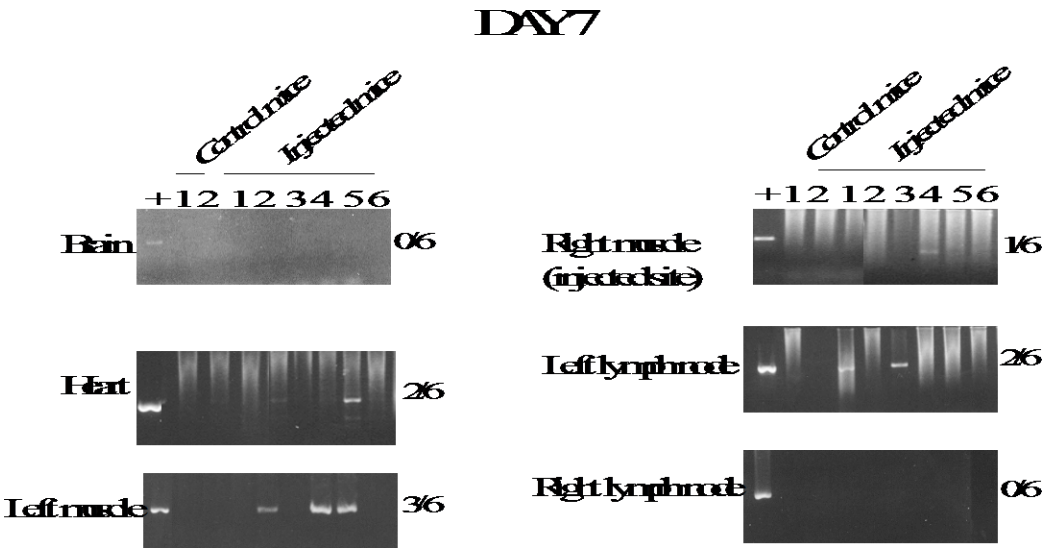
Figure 1. Tumor growth inhibiton of SCCHN xenografts treated with (**A)** intratumoral (left above) or **(B)** intravenous administration of EGFR antisense DNA (pNGVL-EGFR-AS) compared with administration of the control EGFR sense DNA. The intratumoral DNA (25 µg) was given three times a week and the intravenous DNA (25 µg) was administered daily after the formation of palpable tumor nodules (~10-14 days) (right above).

In addition to the efficacy data, we performed DNA distribution studies using the pNGVL-EGFR AS construct. As shown in the summary Table and figures below, the DNA was less widely distributed without co-administration of liposomes.[38]

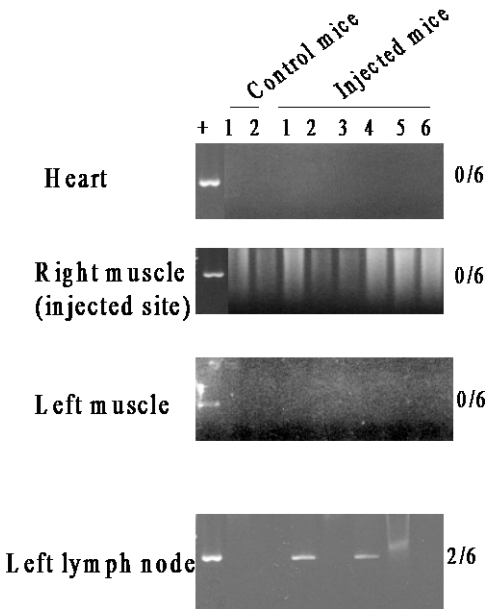
Table: Summary of EGFR antisense DNA tissue distribution at each time point

Organ	Day 2	Day 7	Day 14	Day 21	Day 28
Blood	-	-	-	-	0
Brain	3	0	-	-	-
Heart	2	2	0	-	-
Lung	0	-	-	-	-
Liver	0	-	-	-	-
Kidney	0	-	-	-	-
Gonad	0	-	-	-	-
Left muscle	3	3	0	-	-
Right muscle (Inj)	2	1	0	-	-
Left Lymph node	1	2	2	1	0
Right lymph node	2	0	-	-	-

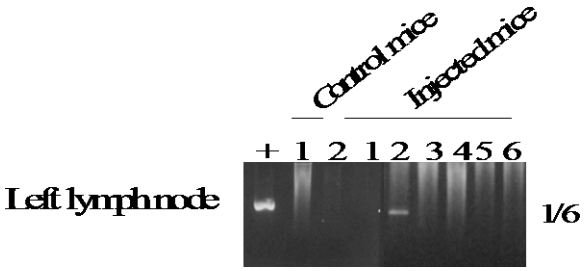

DAY2
Control mice
Injected mice
+ 1 2 1 2 3 4 5 6
Brain
3/6
Heart
2/6
Lung
0/6
Liver
0/6
Kidney
0/6
Gonad
0/6
Left muscle
3/6
Right muscle (injected site)
2/6
Left lymph node
1/6
Right lymph node
2/6


DAY7
Control mice
Injected mice
+ 1 2 1 2 3 4 5 6
Brain
0/6
Heart
2/6
Left muscle
3/6
Right muscle (injected site)
1/6
Left lymph node
2/6
Right lymph node
0/6

DAY 14



DAY21



DAY28