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Anti-ERBB2 sh-RNA Suppress Cell Growth in ERBB2-Overexpressing Upper Gastrointestinal Adenocarcinomas

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Abstract

INTRODUCTION—ERBB2 is overexpressed in 15–25% of upper gastrointestinal adenocarcinomas. We use a stable lentiviral shRNA model to demonstrate that ERBB2 suppression in upper gastrointestinal adenocarcinomas with documented ERBB2 amplification effectively decreases ERBB2 protein levels and decreases cell viability. Further, we evaluate tumor growth of cells treated with the ERBB2 shRNA.

METHODS—Three upper gastrointestinal adenocarcinoma cells lines with varying ERBB2 levels were treated with one of three separate lentiviral GFP-labeled ERBB2 shRNA vectors or a nonsilencing control shRNA vector for 6 hours. Protein levels on Day 6 and cell viability was evaluated on Days 3–10. A xenograft *in vivo* experiment was performed using OE19 cells pretransduced with ERBB2 shRNA to evaluate tumor growth.

RESULTS—ERBB2 protein levels decreased by 80%. ERBB2 knockdown significantly decreased cell viability in cell lines with high ERBB2 levels. *In vivo* tumor growth was suppressed in ERBB2 shRNA treated groups.

CONCLUSION—ERBB2 suppression based on a stable lentiviral shRNA transfection system effectively decrease cell viability in cell lines with amplification of ERBB2 as compared to cell lines without overexpression. ERBB2 knockdown significantly decreases tumor growth *in vivo*. ERBB2-directed therapy may be of benefit in the subset of patients with gastrointestinal adenocarcinomas exhibiting overamplification of ERBB2.

Introduction

The incidence of esophageal adenocarcinoma and esophagogastric junction tumors has increased six-fold since 1970.^{1–4} As shown in a SEER (Surveillance, Epidemiology, and End Results) database review by Devesa et al, the annual rate of esophageal adenocarcinoma alone increased by more than 350% from 1974 to 1994.³ This rate of increase has made esophageal adenocarcinoma the fastest expanding type of cancer in Western countries when compared to all other cancers known to be increasing in incidence (e.g., two- to threefold increase for melanoma and prostate cancer). Unfortunately, the five-year survival of

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esophageal adenocarcinoma is dismal even in the subset of patients who undergo appropriate surgical intervention. The mainstay of treatment is surgery if the primary tumor is resectable. Chemotherapy, usually consisting of 5-fluorouracil, cisplatin, and epirubicin, does not change the overall poor prognosis. Improvements in the treatment of unresectable and late-stage disease remain insignificant. Therefore, the development of other treatment strategies is critical.

Several studies have evaluated genes that are commonly deleted or amplified in upper GI adenocarcinomas. Of all genes evaluated, reported proto-oncogenes in upper gastrointestinal adenocarcinomas include c-myc, ERBB2, CCNE1, mdm2, GATA-4, Cathepsin B, CMet, EGFR, and Ras.⁵⁶ Of these proto-oncogenes, ERBB2 is amplified in a much higher proportion of samples. In the case of esophageal adenocarcinoma, ERBB2 has been shown to be the most commonly amplified proto-oncogene and is overexpressed in approximately 15–40% of tumors evaluated.^{5–114} Similar amplification of ERBB2 has also been noted in GE junction and gastric cardia adenocarcinomas.¹²¹³⁴

As a member of the epidermal growth factor receptor family, ERBB2 (*c-erb-B2*, HER-2/*neu*) is a transmembrane tyrosine kinase receptor. Located on chromosome 17q12, the ERBB2 gene plays a key role in growth factor signal transduction and is also involved in the regulation of cell growth, survival and differentiation.¹⁴¹⁵ In its normal state, ERBB2 has no known ligand,¹⁶ and must pair with other EGFR receptors for activation. As a tyrosine kinase receptor, it is then autophosphorylated and initiates a phosphorylation cascade that results in activation of multiple intracellular pathways, including MAP kinase and Phosphoinositol 3 kinase. However, when ERBB2 is overexpressed as the result of a gene amplification event, it is hypothesized that the receptor homodimerizes. In this state, it remains constitutively active and promotes downstream signaling.

To better understand the effect of ERBB2 knockdown in esophageal and gastric adenocarcinomas, we previously used a transient siRNA model to suppress ERBB2 levels in esophageal and gastric adenocarcinoma cell lines with known ERBB2 amplification. Our study reported that ERBB2 inhibition significantly decreases cell viability via an apoptotic pathway.¹⁷ Of note, there were no significant changes in cell cycle seen in our study. However, siRNA is a transient model in which the toxicity of the transfection reagent used as well as the toxicity of the siRNA itself prevents higher transfection efficiencies. Furthermore, siRNA is readily degraded by RNases. Therefore, *in vitro* work performed with siRNA technology cannot proceed to an *in vivo* model nor can it be translated into the clinical trial setting. Given these inherent limitations of siRNA, we proceeded to evaluate of ERBB2 knockdown on upper gastrointestinal adenocarcinomas using a stable transfection model, via lentiviral ERBB2 shRNA vectors. The purpose of this study was to generate a stable transfection model in order to analyze the effect of ERBB2 knockdown on (1) cell viability in upper gastrointestinal adenocarcinoma cell lines with different levels of ERBB2 expression, and (2) tumor growth in an *in vivo* model.

We believe that in cell lines with ERBB2 amplification there will be a more significant increase in cell death, correlating with our previous siRNA work. Further, we hypothesize that tumor growth of cells treated with the stable ERBB2 shRNA will be significantly

inhibited. We show that ERBB2 knockdown via lentiviral shRNA vectors in upper gastrointestinal (GI) adenocarcinoma cell lines significantly decreases cell viability in the cell lines known to have ERBB2 amplification. Further, tumor growth is inhibited in cell lines treated with shRNA. Our current data correlates with our previous siRNA study and further strengthens the potential benefit of ERBB2-directed therapies in the subset of patient with ERBB2-amplified upper GI adenocarcinoma tumors.

Materials and Methods

Cell Lines

In the present set of experiments, three upper GI adenocarcinoma cell lines were chosen due to their known varying amount of ERBB2 and compared. A gastric adenocarcinoma cell line, MKN45, was obtained from the Japanese Cancer Research Bank. MKN45 has approximately 10-fold amplification of ERBB2.¹⁸ An esophageal adenocarcinoma cell line, OE19, was obtained from the European Collection of Cell Cultures. This cell line has 100-fold amplification of ERBB2.¹⁸ Finally, a cell line with a baseline level of ERBB2, Seg-1 (a gift from Dr. David Beer, University of Michigan, Ann Arbor), was used as a control. All cell lines were cultured in DMEM, 5% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% amphotericin B. HEK293T cells (ATCC) were used for lentiviral viral titrating and maintenance. This cell line was maintained in DMEM, 5% FBS, and 1% penicillin-streptomycin. All cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Lentiviral shRNA generation

Three GFP-labeled ERBB2 shRNA sequences were purchased from OpenBiosystems (Huntsville, AL), as well as a non-silencing control shRNA sequence (to determine off-target effects). The referenced names as for the ERBB2 shRNA are V2LHS_17669, V2LHS_17671, and V2LHS_17672. Detailed sequence information for each shRNA used is shown in Table 2. The University of Minnesota RNAi Core Facility (Minneapolis, MN) then produced pGIPZ™ lentiviral shRNA vectors according to the manufacturer's instructions (OpenBiosystems, Huntsville, AL). The Trans-Lentiviral™ GIPZ Packaging System is based on lentiviral vectors developed by Kappes and Wu.¹⁹²⁰²¹²² In order to select out transfected cells, these lentiviral vectors not only include a GFP-label, but they also have a puromycin selection marker as shown in Figure 1. Treatment with puromycin after transduction eliminates all non-infected cells.

Treatment of cells with lentiviral shRNA vectors

Prior to starting treatments, viral titers of all vectors were routinely found to be 10⁶–10⁷ transforming units (TU)/mL. Optimization experiments were carried out with varying amounts of lentivirus (1–100 multiplicity of infection, MOI) to determine that the optimal MOI for transduction without toxicity for each lentiviral vector was a MOI of 10. Finally, a puromycin kill curve was performed with each cell line to determine the optimal amount of puromycin needed for selection without toxicity to transduced cells. In all cell lines, the optimal concentration of puromycin was 7.5µg/mL.

For generation of a stable transduced cell line, cells were plated in a 6-well plate at a density of 2×10^5 cells/well and incubated overnight at 37°C and 5% CO_2 . A transduction media was made, consisting of serum-free antibiotic-free DMEM media and $8\mu\text{g/mL}$ of Polybrene. Each lentiviral vector was diluted with transduction media to a MOI 10. Cells were washed with PBS and then 1 mL of diluted virus was added. After 6 hours, the lentiviral shRNA media was removed and replaced with DMEM containing serum and antibiotic. Cells treated with serum-free media served as the control and are referred to as nontreated cells. On post-transduction day 1, media was removed and replaced with 2mL of DMEM containing serum, antibiotic, and $7.5\mu\text{g/mL}$ puromycin. After 3–5 days, all non-infected cells were killed and remaining cells had GFP-expression, indicating 100% transduction efficiency. Once cells reached 80% confluence, they were then trypsinized with 0.25% trypsin and placed in a 100-mm culture dish for propagation of cell lines.

For cell viability assays, 1×10^3 cells per well were seeded in 96-well plates and then incubated overnight at 37°C . 50 μL of the appropriate diluted virus at MOI of 10 in transduction media (described above) was then placed on the appropriate wells for 6 hours. After 6 hours, the viral-containing media was removed and replaced with serum-containing DMEM. Cells treated with serum-free media served as the control and are referred to as nontreated cells. Cells were then incubated for 3–10 days at 37°C and 5% CO_2 until collection for cell viability.

Determination of ERBB2 by Western Blot Analysis

Cells were treated with shRNA lentivirus as described above. Once placed in 100-mm culture plate, cells were grown to 70% confluence and then collected by trypsinization (0.25% trypsin). Cells were washed with PBS thoroughly. Next, cells were lysed with lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, complete Protease Inhibitor (Roche, Indianapolis, IN)) for 2 hours at 4°C and cleared by centrifugation for 10 minutes at 10,000g twice. Supernatants were collected and then stored at -80°C . Total protein concentration was determined using the Bio-Rad DC protein assay (Bio-Rad Hercules, CA). ERBB2 protein expression was analyzed by western blotting by loading 75–100 μg samples of total protein from the supernatants onto a 10% SDS-PAGE gel. The separated proteins were transferred onto polyvinylidene difluoride membranes. Nonspecific binding to the membranes was blocked by 2-hour incubation in a buffer containing TBS-T (10mM Tris-HCl, pH 7.4, 150 NaCl, 0.05% Tween-20) and 5% nonfat dry milk. The ERBB2 protein was subsequently detected by overnight incubation at 4°C with a monoclonal anti-ERBB2 antibody (sc-7301; 1:250 dilution; from Santa Cruz, Santa Cruz, CA) and then 1-hour incubation with a peroxidase-conjugated sheep anti-mouse IgG antibody (1:4,000 dilution), both in TBS-T and 5% bovine serum albumin. Bound secondary antibody was visualized by ECL (Thermo Fisher Scientific, Rockford, IL) and autoradiography. Actin expression was used as an internal control.

Determination of cell viability

Cell viability was determined using MTS assay (CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation Assay from Promega, Madison, WI). Briefly, cells were seeded into a 96-well plate at a density of 1×10^3 cells/well and allowed to adhere overnight. After treatment

with lentiviral shRNA, cells were incubated for 3–10 days. Next, 20 μ L of MTS-PMS solution was added to each well. Plates were incubated at 37°C for 2 hours, after which the absorbance at 490nm was measured.

***In vivo* Antitumor effect in EAC Xenograft Model**

To study the effect of ERBB2 shRNA on tumor growth of esophageal adenocarcinoma *in vivo*, the cell line with 100-fold ERBB2 amplification, OE19, was used. OE19 cells were pretransduced with lentiviral shRNA vectors as described above. After puromycin treatment, all remaining cells expressed GFP, indicating 100% transduction efficiency. Once cells were infected with the appropriate lentiviral ERBB2 shRNA vector, cells were maintained and grown as described above.

Female *ncr/nu* nude mice (Frederick Cancer Research, Frederick, MD; 6–8 weeks of age) were used to establish EAC xenografts. The mice were randomized into four groups of five mice each prior to inoculation. The pretransduced lentiviral shRNA OE19 cells were harvested, resuspended in cold PBS, and kept on ice until injected. 4 $\times$ 10⁶ cells per injection site were inoculated into both flanks of each animal. Therefore, each treatment group had a total of ten tumor sites. The condition of the mice was monitored daily, and the tumor diameter was measured twice a week with calipers. The tumor volume was calculated using the formula: tumor volume=(width² $\times$ length)/2. In accordance with institutional approved animal experimental protocol, the mice were euthanized when tumors ulcerated or at time of overgrowth of the tumors. All of the animals received humane care based on the guidelines set by the American Veterinary Association. All of the experimental protocols involving live animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Subcutaneous tumors rapidly excised at the end of the treatment and evaluation period. Tumors were then snap-frozen with dry ice and stored at –80°C for protein extraction and analysis of ERBB2 by Western blot, as described above.

Statistical Analysis

Statistical analysis of lentiviral shRNA treatment *in vitro* and *in vivo* was performed with a two-tailed *t* test. Data are expressed as a mean $\pm$ SD of at least three sets of results. Results were considered statistically significant when *P* < 0.05.

Results

Transduction efficiency was measured after puromycin selection by the amount of GFP-expression and was consistently 100% for each cell line.

ERBB2 shRNA Treatment of Gastrointestinal Adenocarcinoma Decreases Protein Expression

Transduction with lentiviral ERBB2 shRNA decreased protein levels significantly at 72 hrs.. ERBB2 protein levels decreased by 80% with siRNA treatment in OE19, with similar results

seen in both MKN4 and seg-1 (Figure 2). Results were reproduced with all three ERBB2 shRNA.

ERBB2 shRNA Treatment of Gastrointestinal Adenocarcinoma Decreases Cell Viability

The effect of ERBB2 shRNA on the viability of the upper GI adenocarcinoma cancer cells was examined after 6 hour incubation with shRNA and incubation with serum containing media for 3–10 days hours post-infection. The treatment with ERBB2 shRNA significantly reduced cell viability of the two cell lines with known ERBB2 amplification as shown by cell death of 55% by day 7 in MKN45 cells and up to 80% in OE19 cells on day 7 (Table 3; Figure 3). The cell line with a normal ERBB2 levels, Seg-1, exhibited no significant reduction in cell viability when compared to non-treated cells and to cells treated with control siRNA (Table 3; Figure 3).

ERBB2 shRNA Treatment of Gastrointestinal Adenocarcinoma *In Vitro* Decreases Tumor Growth *In Vivo*

The cell line with the highest amount of ERBB2 amplification, OE19, was chosen for the *in vivo* model. Tumors were palpable in the control group (mice injected with nonsilencing control shRNA) by day 4. All mice developed tumors. However, given that one animal in the control group developed tumor only in one injection site, the total number of tumors evaluated in the control group was nine. On day 14 after injection, the nonsilencing control shRNA group (relative tumor volume 33.85 ± 14.26 ; $n=9$) had significantly larger tumors in comparison to the ERBB2 shRNA treated groups (Table 4; Figure 4). All ERBB2 shRNA tumors had a significantly slower rate of tumor growth as compared to the control group. By day 25, tumors in the control (nonsilencing shRNA) group were 2.5 times larger than all ERBB2 shRNA treated tumors. Of the tumors evaluated, ERBB2 protein levels were suppressed in five of the six ERBB2 shRNA tumors as compared to the nonsilencing control shRNA tumors (Figure 4). This indicates that ERBB2 protein continued to be suppressed in the majority of ERBB2 shRNA tumors. Thus, the *in vivo* data indicate that ERBB2 suppression does inhibit tumor growth of upper GI adenocarcinomas.

Discussion

In the present study, ERBB2 shRNA treatment significantly decreased protein levels of ERBB2 in all cell lines, indicating that the lentiviral shRNA model is an effective tool to evaluate ERBB2 suppression. Further, ERBB2 shRNA treatment significantly decreased cell viability in our two upper GI adenocarcinoma cell lines with known ERBB2 overexpression (OE19 and MKN45), though it did not affect that of a cell line with a normal level of ERBB2 (Seg-1). These results correlate with results in our earlier work with ERBB2 siRNA in which we found that ERBB2 siRNA significantly decreases cell viability by up to 60% in three days.¹⁷ Given that a decrease in cell viability can be the result of an increase in apoptosis, an increase in cell cycle arrest, or a combination of both mechanisms, we also reported that decreased cell viability in this model occurred via an apoptotic pathway. Of note, cell cycle did not significantly change in cell lines treated with ERBB2 siRNA.¹⁷ When evaluated *in vivo*, we find that treating the cell line with the highest level of ERBB2 amplification with shRNA, tumor growth is significantly slower and less than in tumors of

cells treated with a nonsilencing control shRNA. These results also correlate with a similar model by Yang et al in which breast cancer cells were treated with ERBB2 siRNA and found to have decrease cell viability, increased apoptosis, and decreased tumor growth.²³

The most well-documented studies involving ERBB2-directed therapy are using the breast cancer model.^{24, 25} As seen in these breast cancer models, ERBB2 amplification is documented in 25–40% of tumors evaluated.^{24, 25} ERBB2 overexpression in breast cancer tumors has been found to be an independent prognostic predictor of overall survival and time to relapse, perhaps due to the increased association with estrogen receptor negativity and early metastasis of these tumors. In this subset of patients, ERBB2-targeted therapies have significantly improved overall survival and disease-free rates of survival.^{24, 25}

The ERBB2 gene is amplified in several cancers other than breast and upper gastrointestinal adenocarcinomas, including cancers of the prostate²⁶, pancreas²⁷, lung^{28, 29}, and ovary¹⁴. Trastuzumab (Herceptin), a humanized monoclonal antibody which binds to the extracellular domain of the HER2 protein, has been used clinically to treat patients with ERBB2-amplified metastatic breast cancers.²⁵ In previous studies by our lab, this drug also inhibited growth of OE19, an esophageal adenocarcinoma with 100-fold amplification of ERBB2. However, in cell lines with normal ERBB2 levels, cell growth was not inhibited.⁵ No clinical trials have yet tested trastuzumab in the subset of patients with known ERBB2-amplified upper GI adenocarcinomas. Unfortunately, there are many obstacles to overcome with the use of herceptin, including the significant side effects (in particular the cardiotoxicity) secondary to systemic administration as well as the cost of treatment (ranging from \$20,000–80,000/year). Although herceptin therapy may be an effective strategy in a high-risk population, development of treatment with local administration could potentially bypass these obstacles. Therefore, the future direction of our lab is to generate a cost-effective stable transfection model with the potential of local administration.

Advanced upper gastrointestinal adenocarcinomas are highly aggressive cancers that portray poor long-term prognoses.^{30,31,3} Curative treatment consists of surgical resection for early-stage disease. Unfortunately, only 20–30% of patients are eligible for surgery secondary to metastases at time of diagnosis, invasion of surrounding structures, or due to multiple comorbidities of the patients.^{32, 33} Under current standards of clinical care, there are few treatment options for the majority of patients who cannot undergo surgical resection of their tumors. Neoadjuvant and adjuvant chemotherapy, in the form of 5-fluorouracil, cisplatin, or epirubicin provides patients with little improvement in overall survival rates. Therefore, new therapeutic approaches must be investigated to offer a more effective treatment for unresectable disease. Given that upper gastrointestinal adenocarcinoma is easily accessible via endoscopy, direct injection of ERBB2-directed therapies could provide a beneficial treatment strategy without the systemic toxicities.

Lentiviral shRNA vectors provides several advantages over siRNAs. Unlike siRNA, lentiviral vectors are more stable, and are not degraded by RNase. Unlike siRNAs in which expression of suppression is transient, lentiviruses integrate into the host genome as part of their life cycle, and thus their genomic backbone provides a means for life-long expression of ERBB2 shRNA. Further, lentiviruses infect all cells, including actively dividing cells as

well as resting and differentiated cells. Given that the lentivirus used is non-replicative, ERBB2 shRNA is continually expressed but infectious virus is not generated.

However, there are limitations to this model. For example, endogenous expression of shRNA can cause side effects such as the activation of innate immunity via induction of an interferon response. As with siRNA, lentiviral shRNA vectors also have the potential of off-target gene silencing though by evaluating several ERBB2 shRNA in comparison to a non-silencing control shRNA the potential of off-target effects is essentially eliminated. Additionally, shRNAs have the risk of competition with cellular miRNAs given that they use some of the miRNA machinery for their generation and export.

A stable cost-effective transfection model for ERBB2 suppression of upper GI adenocarcinomas. Our lab is currently generating replication-incompetent adenoviral ERBB2 shRNA vectors to repeat all experiments. There are several benefits in an adenoviral system, including providing us with a much higher level of initial transient expression. Further, as with the lentiviral model, replication-incompetent adenoviruses are limited in their ability to spread beyond the local injection site thus generating little systemic effects.

In summary, we have shown that ERBB2 suppression significantly decreases cell viability via an apoptotic pathway¹⁷, and inhibits tumor growth in upper gastrointestinal adenocarcinomas. ERBB2-directed therapy may be of benefit in the subset of patients with gastrointestinal adenocarcinomas exhibiting overamplification of ERBB2. Further studies are clearly warranted to generate improved treatment strategies for patients with unresectable disease.

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Figure 1. Schematic of Lentiviral shRNA vectors

All of the lentiviral ERBB2 shRNA vectors contain a GFP-label and a puromycin selection marker. This allows for the preferential selection of cells infected with the appropriate lentiviral shRNA vector. Abbreviations are shown in Table 1.

Figure 2. ERBB2 protein levels after treatment with lentiviral ERBB2 shRNA

All cell lines showed protein suppression with shRNA treatment. Membranes were stripped and reprobed for actin to show equal protein loading.

Figure 3. Cell Viability assay of upper GI adenocarcinoma after ERBB2 shRNA

Effect of ERBB2 shRNA treatment on upper GI adenocarcinoma cell viability. After 6 hour transduction with ERBB2 shRNA, cell viability was significantly reduced in OE19 and MKN45, with no significant change in Seg-1. n=4. **p<0.001 when compared to nonsilencing control shRNA

Figure 4. Tumor growth *in vivo* of OE19 pretransduced cells

A. Tumor growth was significantly slower and less in the ERBB2 shRNA treated cells as compared to cells treated with nonsilencing control shRNA. Tumors were measured every 3–4 days with calipers. Tumor volume was calculated as [tumor volume = (width² × length)/2]. * $p < 0.01$ when compared to nonsilencing control shRNA. ** $p < 0.001$ when compared to nonsilencing control shRNA **B.** ERBB2 protein levels were decreased in

five of the six ERBB2 shRNA treated tumors as compared to the nonsilencing control tumors.

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Table 1
Abbreviations of structures included in the lentiviral vectors

As shown, each lentiviral vector is under a CMV promoter, and includes a poly-A tail

CMV	RNA polymerase II promoter
Ψ (Psi)	Region of viral RNA responsible for directing packaging.
PPT	Purine-rich sequence cleaved during reverse transcription to produce RNA primer for viral DNA synthesis
wpre	Woodchuck hepatitis virus post-transcriptional regulatory element
5' LTR	Long terminal repeat
SIN	Deletion of the transcriptional enhancers & promoter in the U3 region of the 3' LTR.
RRE	Binding site for the Rev protein
TurboGFP	Green fluorescent protein utilized to track shRNAmir expression.
Puro	Puromycin-N-acetyl transferase, mammalian drug selectable marker.
polyA	Polyadenylation site
pUC Ori	Permits high-copy replication and maintenance in E. coli.
Amp	Allows selection of the plasmid in E. coli.

Table 2
Details of ERBB2 shRNA sequences

Each shRNA sequence is preceded and is followed by a 30-mir sequence which increases subsequent Dicer recognition and specificity. The loop sequences for all shRNAs are identical. ERBB2 shRNA sequences vary slightly in the sense and antisense sequences.

	Sense Sequence	Loop Sequence	Antisense sequence
V2LHS_17669	AGCGCAGATGCGGATCCTGAAA	TAGTGAAGCCACAGATGTA	TTTCAGGATCCGCATCTGCGCC
V2LHS_17671	CGCCCTGGCCGTGCTAGACAAT	TAGTGAAGCCACAGATGTA	ATTGTCTAGCACGGCCAGGGCA
V2LHS_17672	CGCTGAACTGGTGTATGCAGAT	TAGTGAAGCCACAGATGTA	ATCTGCATACACCAGTTCAGCA

Table 3
Cell Viability assay of upper GI adenocarcinoma after ERBB2 shRNA treatment Day 7

Effect of ERBB2 shRNA treatment on upper GI adenocarcinoma cell viability. After 6 hour transduction with ERBB2 siRNA, cell viability was significantly decreased in OE19 and MKN45, with no significant change in Seg-1. n=4.

Cell Line	Nontreated Cells	Nonsilencing Control shRNA	ERBB2 shRNA #1	ERBB2 shRNA #2	ERBB2 shRNA #3
Seg-1	100(±11.13)%	94.93(±9.42)%	103.08(±2.21)%	100.79(±5.88)%	93.26(±5.51)%
OE-19	100(±8.00)%	91.50(±5.57)%	29.09(±9.33)% **	31.10(±12.57)% **	21.80(±1.14)% **
MKN45	100(±8.61)%	88.17(±9.07)%	52.83(±7.11)% **	45.19(±2.95)% **	52.40(±8.40)% **

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p<0.001 when compared to nonsilencing control shRNA

Table 4
Tumor growth *in vivo* of OE19 pretransduced cells

Tumor growth was significantly slower and less in the ERBB2 shRNA treated cells as compared to cells treated with nonsilencing control shRNA. Tumors were measured every 3–4 days with calipers. Tumor volume was calculated as [tumor volume =(width²×length)/2].

Day	Nonsilencing control shRNA	ERBB2 shRNA #1	ERBB2 shRNA #2	ERBB2 shRNA #3
0	0	0	0	0
4	6.22(±7.91) mm ³	0.25(±0.42)mm ³	6.80(±6.93)mm ³	2.75(±4.40)mm ³
7	20(±11.22) mm ³	8.82(±5.67)mm ³ *	10.41(±6.54)mm ³ *	6.36(±7.08)mm ³ *
11	31.66(±15.65)mm ³	13.01(±7.82)mm ³ *	12.38(±5.79)mm ³ *	9.58(±8.86)mm ³ *
14	33.85(±14.26)mm ³	15.83(±6.40)mm ³ **	16.03(±9.40)mm ³ **	21.08(±17.20)mm ³ **
18	80.54(±19.06)mm ³	26.44(±10.40)mm ³ **	20.19(±8.33)mm ³ **	32.75(±19.73)mm ³ **
21	155.26(±42.75)mm ³	49.63(±27.29)mm ³ **	44.81(±20.94)mm ³ **	53.77(±26.81)mm ³ **
25	241.83(±32.00)mm ³	98.48(±62.12)mm ³ **	72.79(±51.69)mm ³ **	89.66(±59.17)mm ³ **

* p<0.01 when compared to nonsilencing control shRNA.

** p<0.001 when compared to nonsilencing control shRNA