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Studies on the inhibition of feline EGFR in squamous cell carcinoma

Enhancement of radiosensitivity and rescue of resistance to small molecule inhibitors

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Key words: EGFR, squamous cell carcinoma, gefitinib, resistance, RNA interference, animal model, feline

Abbreviations: EGFR, epidermal growth factor receptor; SCC, squamous cell carcinoma; HNSCC, head and neck cancer; TKI, tyrosine kinase inhibitor; RNAi, RNA interference; siRNA, small interfering RNA; BLAST, basic local alignment search tool; mAb, monoclonal antibody; MAPK, mitogen-activated protein-kinase pathway; PI3K, the phosphatidylinositol 3-kinase pathway; STAT, signal transducers and activators of transcription pathway; PLC- γ , phospholipase gamma pathway; NF κ B, nuclear factor kappaB; NSCLC, non-small-cell lung cancer; CFA, colony formation assay; gray (Gy) and Gab1 growth factor receptor binding 2 (Grb-2)-associated binding protein 1

This study investigated different methods of EGFR (epithelial growth factor receptor) targeting in feline squamous cell carcinoma with the ultimate aim of establishing a large animal model of human head and neck cancer. Both small molecule receptor tyrosine kinase inhibitor (TKI) and RNA interference (RNAi) techniques were employed to target the feline EGFR. We demonstrated that the human drug gefitinib caused a reduction in cell proliferation and migration in a feline cell line. However, we also document the development of resistance that was not associated with mutation in the kinase domain. RNAi caused a potent reduction in EGFR activity and was able to overcome acquired gefitinib resistance. In addition, RNAi targeting of EGFR, but not gefitinib, caused an additive effect on cell killing when combined with radiation. These results support the use of feline SCC as a model of head and neck cancer in man in the search for novel and effective treatments for both tumors.

Introduction

Squamous cell carcinoma (SCC) is common in cats affecting the oropharynx, trachea and larynx,^{1,2} nasal cavity and paranasal sinuses³ and skin.⁴ SCC accounts for up to three quarters of oral tumors reported in cats.⁵ Several treatment options are available including surgery,⁶ radiotherapy,⁷ chemotherapy⁸ or a combination of these,⁹ but overall survival remains poor.¹⁰ Treatment is often hampered by the locally aggressive nature of these tumors¹⁰ and the high morbidity that may be seen with treatment.⁶

Feline oral SCC shares many similarities with human head and neck cancer (HNSCC), which is also highly invasive and carries a poor prognosis.¹¹ Patients that present with locoregional advanced disease have a median survival of 19.1 months with chemoradiation treatments.¹¹ Multimodality treatments combining surgery, radiation and chemotherapy are often curative but associated with treatment-related morbidity, such as impairment in speech, swallowing and taste.^{11,12} The etiopathogenesis

of HNSCC is considered to be multifactorial, with contributions from lifestyle and environmental factors such as tobacco smoke, genetics and papillomavirus infections.¹¹ Cats share the same environments as their owners and may act as sentinels of environmental carcinogens.¹³ Environmental and life-style factors have also been implicated in the aetiopathogenesis of feline oral tumors including grooming behavior, diet, exposure to environmental carcinogens including tobacco smoke^{14,15} and papillomavirus infections.¹⁶

Key signaling pathways involved in oncogenesis have also been identified in HNSCC. EGFR belongs to the superfamily of proteins known as receptor tyrosine kinases. Upon ligand binding EGFR dimerizes and activates several downstream pathways including the mitogen-activated protein-kinase (MAPK) pathway, the phosphatidylinositol-3-kinase (PI3K) pathway and the signal transducers and activators of transcription (STAT) pathway.^{17,18} In addition, EGFR activation of the phospholipase gamma (PLC- γ) pathway can indirectly activate the PI3K and

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the MAPK pathways and induces nuclear factor kappaB (NFκB) upregulation, a pro-survival transcriptional regulator protein.¹⁹ These pathways are important activators of cell proliferation, survival, angiogenesis and cell migration.¹⁷

Overexpression of EGFR has been reported in up to 80%^{11,20} of HNSCC tumors and EGFR is a major oncogenic driver in a subset of these tumors.¹¹ Developing strategies to target EGFR in HNSCC has been the main focus of research in this area and recently the first drugs have been approved for clinical use.²¹ EGFR has also been reported to be widely expressed in feline oral SCC.^{22,23}

RNA interference (RNAi) is a highly specific method of gene silencing which utilizes small interfering RNA (siRNA) molecules complementary to the target sequence.²⁴ These bind to gene transcripts targeting them for early destruction before translation can occur.²⁴ RNA interference techniques are extensively used in research but also carry promise for the development of drugs capable of targeting specific proteins^{25,26} including EGFR.²⁷

The aims of this study were to investigate the role of EGFR in feline SCCs. Initial results supported the use of small molecule TKI in these tumors, but resistance to therapy developed. An alternative approach to EGFR targeting utilizing RNAi was developed, and was shown to rescue acquired TKI resistance in the cell line. Furthermore, RNAi used in combination with conventional therapies produced an additive effect. These results support using feline SCC as a model of HNSCC, an approach that could benefit drug development and treatment of both tumors.

Results

The feline *Egfr* tyrosine kinase region shows high degree of homology to the canine and human sequence. The sequences obtained with the primers designed from the known human, canine and murine *Egfr* sequences had a 100% sequence homology with the feline genome according to a basic local alignment search tool (BLAST) search and high homology with other mammalian *Egfr* sequences (data not shown). The amino acids making up the ATP binding pocket in the human EGFR were conserved in the feline EGFR (GenBank accession number HQ185236).

Inhibition of EGFR signaling causes reduced cellular proliferation and migration in the SCCF1 cell line. Small molecule TKIs like gefitinib bind to the ATP binding pocket and blocks phosphorylation.²¹ Since the ATP binding pocket was preserved in the feline cell line, human drugs should be effective against the feline receptor. In order to test this, cell proliferation assays were performed, and they showed a dose dependent decrease in proliferation rate in response to gefitinib treatment (Fig. 1A). Average inhibitory concentration causing 50% reduction in proliferation in the SCCF1 cell line was approximately 5 μM (Fig. 1A).

To investigate the effect on inhibition of EGFR signaling on cellular migration, scratch assays were performed on cells treated with gefitinib (Fig. 1B and C). The SCCF1 cell line showed a dose-dependent reduction in migration following gefitinib treatment.

Acquired resistance to TKI monotherapy commonly develops over time,²⁸ so a gefitinib resistant cell line (SCCF1G) was developed and the tyrosine kinase region was re-sequenced, but no mutations were found. Using proliferation assays, the SCCF1G cell line showed no significant inhibition of proliferation at the 5 μM drug dose (Fig. 1A), and was consistently and significantly more resistant at concentrations of 1–15 μM gefitinib ($p < 0.001$ by two sample t-test). Scratch assays performed on the gefitinib resistant cell line showed a comparatively faster migration in the resistant cell line (Fig. 1D and E), with the DMSO control treated cells having a statistically significant faster migratory rate at both time points when compared to the parent cell line ($p < 0.001$, $p = 0.024$ by two sample t-test for 9 and 24 h respectively). Treatment with 10 μM gefitinib produced different effects in the cell lines; at 9 h the migratory ability of the parent cell line was statistically significantly reduced compared to the resistant cell line ($p < 0.001$ by two sample t-test). The gefitinib treated SCCF1 cells had at 9 h migrated 42% of the distance of their controls compared to 72% of DMSO control treated cells for the resistant cell line. Gefitinib had no effect on migration in the resistant cell line at 24 h, with both the DMSO treated and the gefitinib treated cells having filled the gap completely. In the parent cell line, the gefitinib treated cells had migrated just over half the distance of the DMSO control treated cells (59% of DMSO treated cells, $p = 0.0002$ by Mann Whitney U test).

siRNA transfections cause reduced cell proliferation and EGFR knockdown in SCCF1 cell line. To target the receptor using RNAi six siRNAs against the feline *Egfr* were screened using the psiCHECK-2 vector system, and the most effective siRNA (siRNA6: sequence antisense strand 5'-3' AGU UCA UCA AGG AUU UCC UU) was used in the remaining experiments. SCCF1 cells transfected with siRNA6 exhibited reduced proliferation ability compared to mock and scrambled transfected cells as well as untreated cells for up to 72 h following transfection (Fig. 2A). Transfecting the cells with siRNA6 produced an effect equivalent to a 10 μM dose of gefitinib in the SCCF1 cell line (Fig. 2B). Real-time PCR revealed a 55% relative reduction in *Egfr* mRNA levels as compared to scrambled control transfected SCCF1 cells at 24 h following transfection (Fig. 3A). Protein gel blot analysis showed reduction in EGFR protein levels at 72 h following transfection when compared to untreated cells and scrambled control transfected cells (Fig. 3B). To investigate the effect of *Egfr* knockdown on downstream signaling pathways of EGFR, protein gel blot analysis was performed of markers downstream of the EGFR in cells transfected. Reductions in phosphorylated Erk1/2 and phosphorylated STAT-3 levels were seen following *Egfr* knockdown (Fig. 3C).

siRNA transfections overcame gefitinib resistance in cell line. Targeting of EGFR using siRNAs instead of gefitinib in the SCCF1G cell line overcame the resistance to EGFR targeting observed when using gefitinib. Transfection of the resistant SCCF1G cell line with siRNA6 produced a marked reduction in cellular proliferation at 72 h following transfections when compared to mock transfected cells. In comparison, gefitinib treatment at 5 μM of the resistant cell line caused no significant

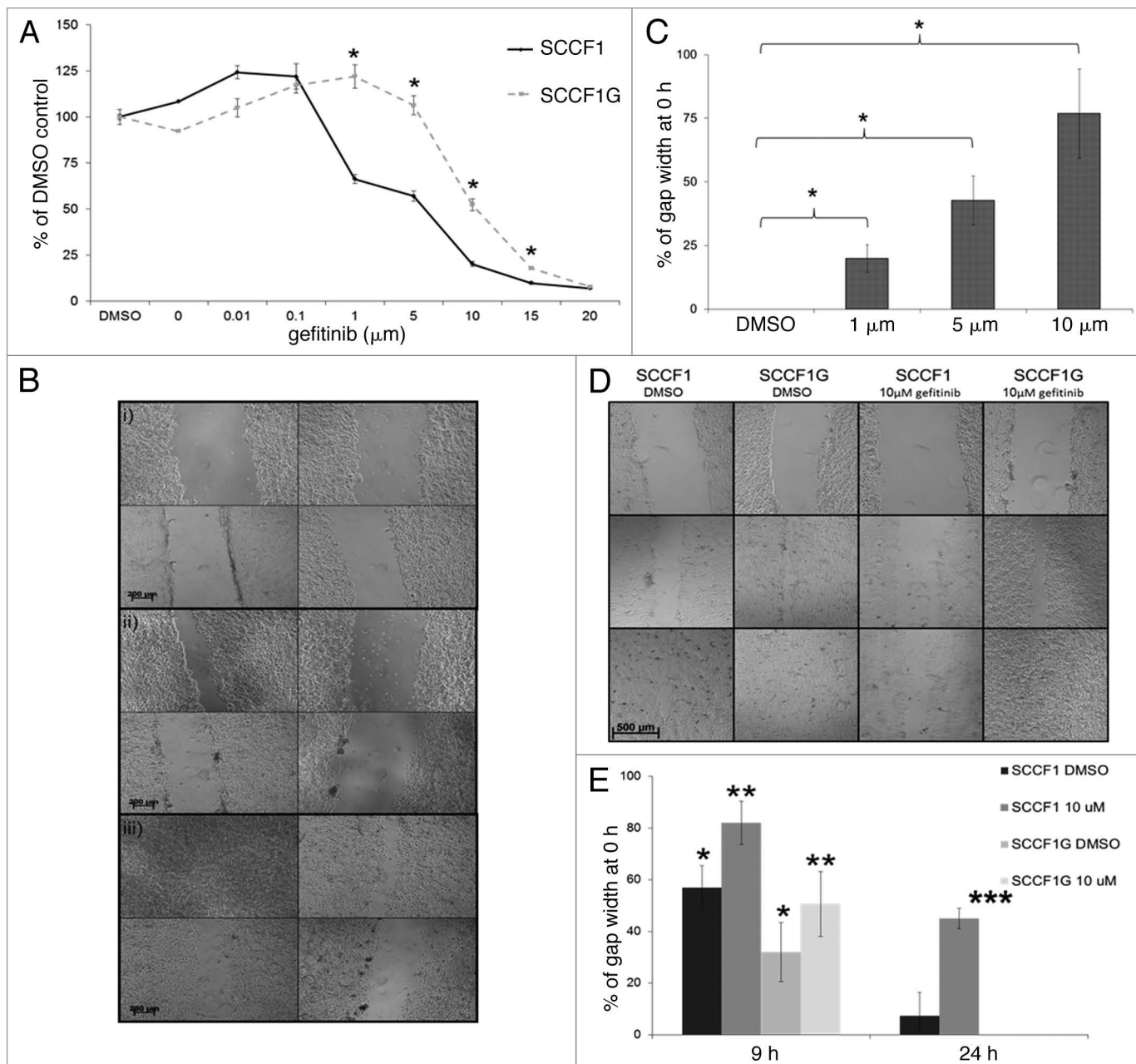


Figure 1. (A) Cell proliferation assays showing proliferation of SCCF1 and SCCF1G following 72 h of gefitinib treatment at a range of concentrations. X axis shows gefitinib concentrations in μM and Y axis shows % of DMSO control treated cells, error bars show standard deviations, * $p < 0.001$ by two sample t-test. (B) In vitro scratch assay of SCCF1 cell line showing effect of EGFR specific tyrosine kinase inhibitor gefitinib on cellular migration. Each box shows top left DMSO control, top right 1 μM gefitinib, bottom left 5 μM gefitinib, bottom right 10 μM gefitinib at (i) 0, (ii) 12 and (iii) 24 h. (C) Graphical representation of the relative migration of the SCCF1 cell line after 24 h of gefitinib treatment compared to DMSO control treated cells. The 10 μM gefitinib dose produced a 23% reduction in gap width after 24 h compared with the 100% reduction observed in DMSO treated cells (* $p < 0.001$ by one-way ANOVA). (D) In vitro scratch assay comparing migratory ability of the two cell lines. Top row shows 0 h, middle row 9 h and bottom row 24 h following gefitinib treatment. (E) Graphical representation of relative migration of cell lines shown in (D). The basal migratory rate of the cell lines differed, illustrated by the DMSO control treated cells. After 9 h, the DMSO control treated SCCF1 had closed the gap created for the assay by less than half while the SCCF1G cell line had reduced the width with over two thirds (* $p < 0.001$, two sample t-test). The 10 μM dose of gefitinib had different effect on the cell lines. At 9 h the resistant cell line had reduced the gap width with approximately half compared to only a fifth in the parent cell line (** $p < 0.001$, two sample t-test) and at 24 h the resistant cell line had migrated and filled the gap completely while the parent cell line had only migrated approximately half way across the gap (** $p = 0.0002$ by Mann Whitney U test).

reduction in proliferation when compared to DMSO control ($p = 0.898$, two sample t-test), and 10 μM of gefitinib only had a modest effect on proliferation (Fig. 3D).

Hairpin expression vectors cause long term knockdown of EGFR and reduced proliferating ability and colony formation in the SCCF1 cell line. Naked siRNA transfections are

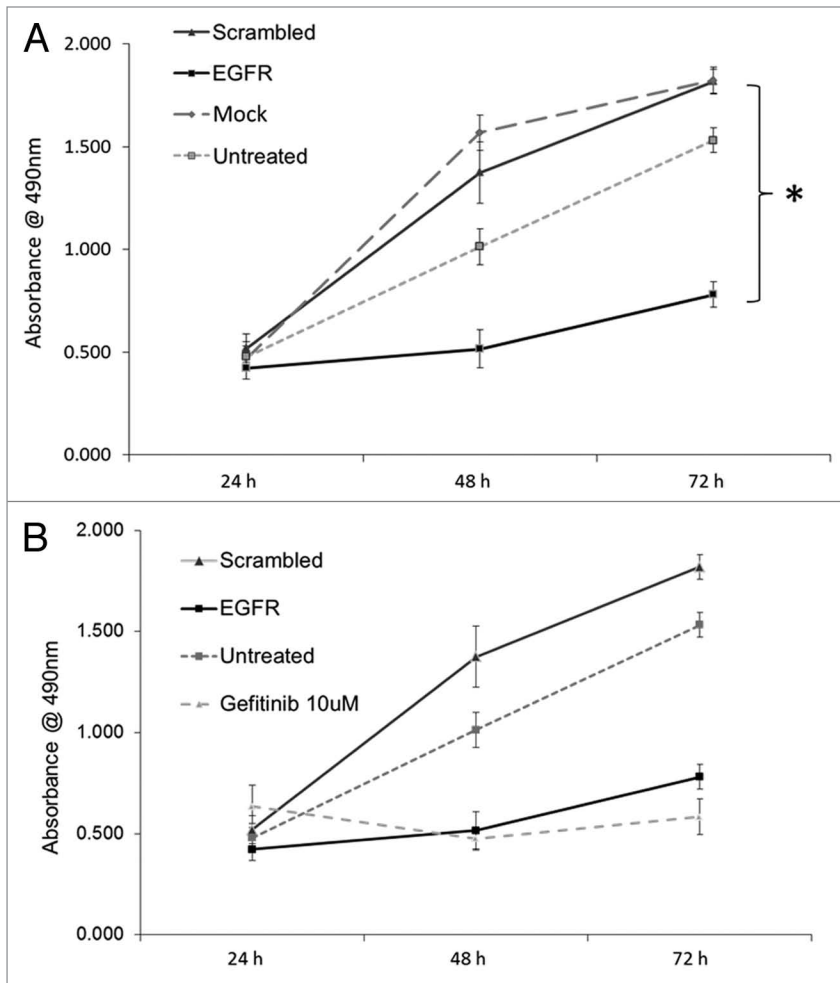


Figure 2. (A) Cell proliferation assay showing proliferation of SCCF1 cells up to 72 h post siRNA6 transfections compared to untreated SCCF1 cells, mock and scrambled control transfected cells, * $p < 0.0001$, at 48 and 72 h by one-way ANOVA. (B) Cell proliferation assay comparing the effect on proliferation of siRNA6 transfection and gefitinib at 24, 48 and 72 h. A single siRNA6 transfection produced an equivalent effect to a 10 μ M dose of gefitinib.

typically transient in nature, with observed effects lasting up to one week.²⁴ To investigate if longer term knockdown of the receptor could be achieved, the siRNA sequence was cloned into a hairpin expression vector. Transfection efficiencies achieved with the hairpin transfection vectors were lower than what was achieved with the naked siRNA transfections (53 vs. 69% for vector and siRNA transfections respectively, data not shown).

Real Time PCR analysis (Fig. 4A) showed variable reduction in EGFR mRNA levels in EGFR transfected cells compared to scrambled transfected cells with an average reduction of 35% (SD 18%, range 10–53%). Similarly, protein gel blot analysis (Fig. 4B) of cell lysates produced 20 days after transfection showed variable reduction in EGFR protein levels when compared to scrambled controls.

Colony formation assays were performed during antibiotic selection (Fig. 4C) and the cell lines ability to form colonies were reduced on average with 45% compared to scrambled control transfected cells. This difference, however, only showed a trend towards

statistical significance ($p = 0.06$ by Mann Whitney U test).

Cell proliferation assays performed on the cell line after 20 days of antibiotic selection (Fig. 4D) showed a statistically significant difference between the cell proliferation rates at all three time points. The effect on proliferation after 20 days was less pronounced compared to the effect observed in transiently transfected cells (Fig. 2A).

Radiation and EGFR knockdown show an additive effect in the SCCF1 cell line. To investigate the effect of *Egfr* targeting in combination with conventional therapies a radiosensitivity assay was performed. Ten days after radiation colony formation assays showed a dose dependent reduction in colony formation ability in the irradiated cells which was enhanced in the siRNA6 but not the scrambled transfected or gefitinib treated cells (Fig. 5A and B). An additive effect was observed when siRNA6 transfections were combined with irradiation according to the Bliss additivism model (Fig. 5C).

Discussion

This study demonstrated that the tyrosine kinase region of the feline *Egfr* gene has a high degree of homology with the human gene, including a preserved ATP binding pocket. In addition, a commercially available monoclonal antibody produced against the EGF binding region of the human protein (EGFR Ab-12, Thermo-Scientific) also binds specifically to feline EGFR epitopes showing species cross reactivity (protein gel blot analysis Fig. 4B). Although these results represent only one cell line, and further studies should be performed investigating a panel of feline oral SCC cell lines and preferably compar-

ing them to human HNSCC cell lines, these findings do support using feline oral SCC as a natural model for HNSCC as specific EGFR targeting strategies used in man are likely to also target feline EGFR. Specific therapeutics targeting EGFR, like TKIs and monoclonal antibodies (mAbs), have been approved for clinical use in a range of solid tumors including HNSCC.¹⁷ TKIs block the ATP binding pocket of the tyrosine kinase domain of EGFR preventing activation of downstream targets,²¹ while mAbs block the ligand binding region of the receptor preventing activation and target it for antibody dependent cell-mediated cytotoxicity.¹⁷ This study aimed to test currently available human EGFR targeting strategies in the feline model, and a TKI was chosen as mAbs used in human medicine are either humanised or chimeric antibodies. If used in veterinary species they are likely to cause anti-human antibody responses similar to the human anti-mouse antibody responses seen in man to murine antibodies.^{29,30}

In this study gefitinib effectively reduced cellular migration and proliferation in the feline SCC cell line, but at the relatively

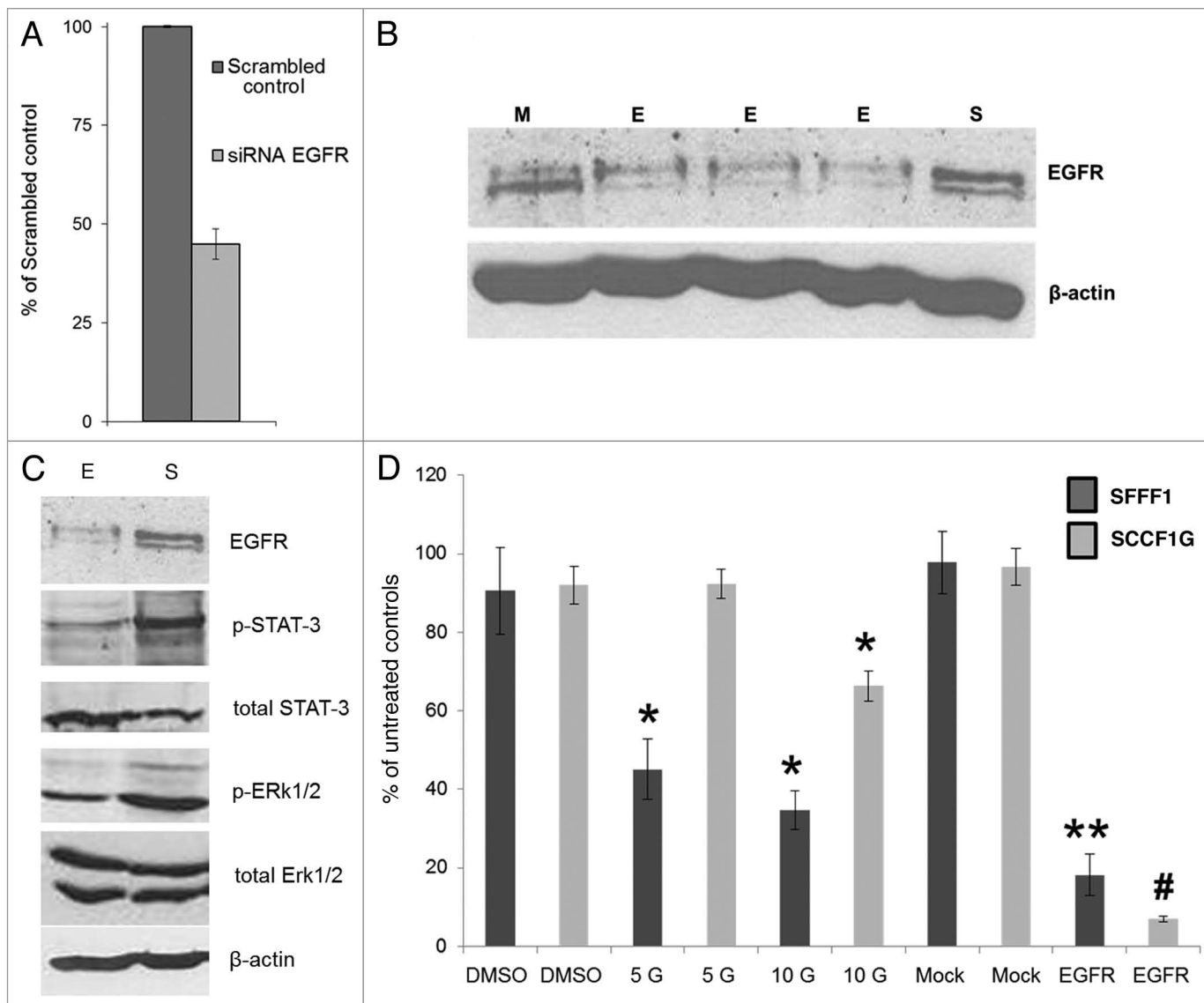


Figure 3. (A) Diagram showing real time PCR result of *Egfr* mRNA levels in SCCF1 cells transfected with siRNA6 expressed as percentage of scrambled control levels. Results shown are an average of triplicate transfections and each transfection was run in three technical replicates for the real time PCR. Similar results were obtained in separate experiments. (B) Protein gel blot showing reduction in EGFR protein levels 72 h post siRNA transfection of SCCF1 cell line. Lysates from mock transfected, M, and scrambled control transfected cells, S, are shown next to lysates from SCCF1 cells transfected with siRNA6 against the feline *Egfr* in triplicates, E, while lower band show β -actin levels for loading control. (C) Protein gel blot showing levels of phosphorylated downstream targets of EGFR in siRNA6 transfected SCCF1 cells, E, compared to scrambled control transfected cells, S. A reduction was seen in phosphorylated STAT-3 and Erk1/2 levels in the EGFR transfected cells. Total Erk1/2, total STAT-3 and β -actin levels are shown for loading control. (D) Mock transfections and DMSO treatments of both cell lines had minimal effect on proliferation, while gefitinib treatment and *Egfr* siRNA6 transfections caused marked effects on the SCCF1 cell line. The SCCF1G cell line exhibited resistance to doses of gefitinib of up to 10 μ M while siRNA6 transfections against the feline EGFR still caused reduction in proliferation in the cell line. Bars show standard deviations, * $p < 0.001$, ** $p < 0.001$ compared to DMSO control and mock transfected respectively by two sample t-test, # $p = 0.005$ compared to mock transfected by Mann Whitney U test.

high dose rate of 10 μ M. An in vitro dose of gefitinib of 1 μ M is equivalent to a clinical dose of 250 mg per day used in non-small-cell lung cancer (NSCLC).³¹ In NSCLC in vitro studies doses above 2 μ M of gefitinib would class the cell line as insensitive to the drug.³¹ Early studies on HNSCC cell lines used much higher doses than this³² while more recent studies all have used doses below 2 μ M gefitinib.^{33,34} The relative insensitivity of the cell line to gefitinib in combination with the development of

acquired resistance in the cell line prompted the search for an alternative approach to EGFR targeting.

The cell line in this study developed resistance to gefitinib treatment following chronic exposure. In man, the T790M mutation in the tyrosine kinase domain has been associated with the development of resistance to gefitinib in NSCLC.³⁵ It results in a substitution of methionine for threonine in the ATP binding site increasing the receptor affinity for ATP.³⁵ In this study, however,

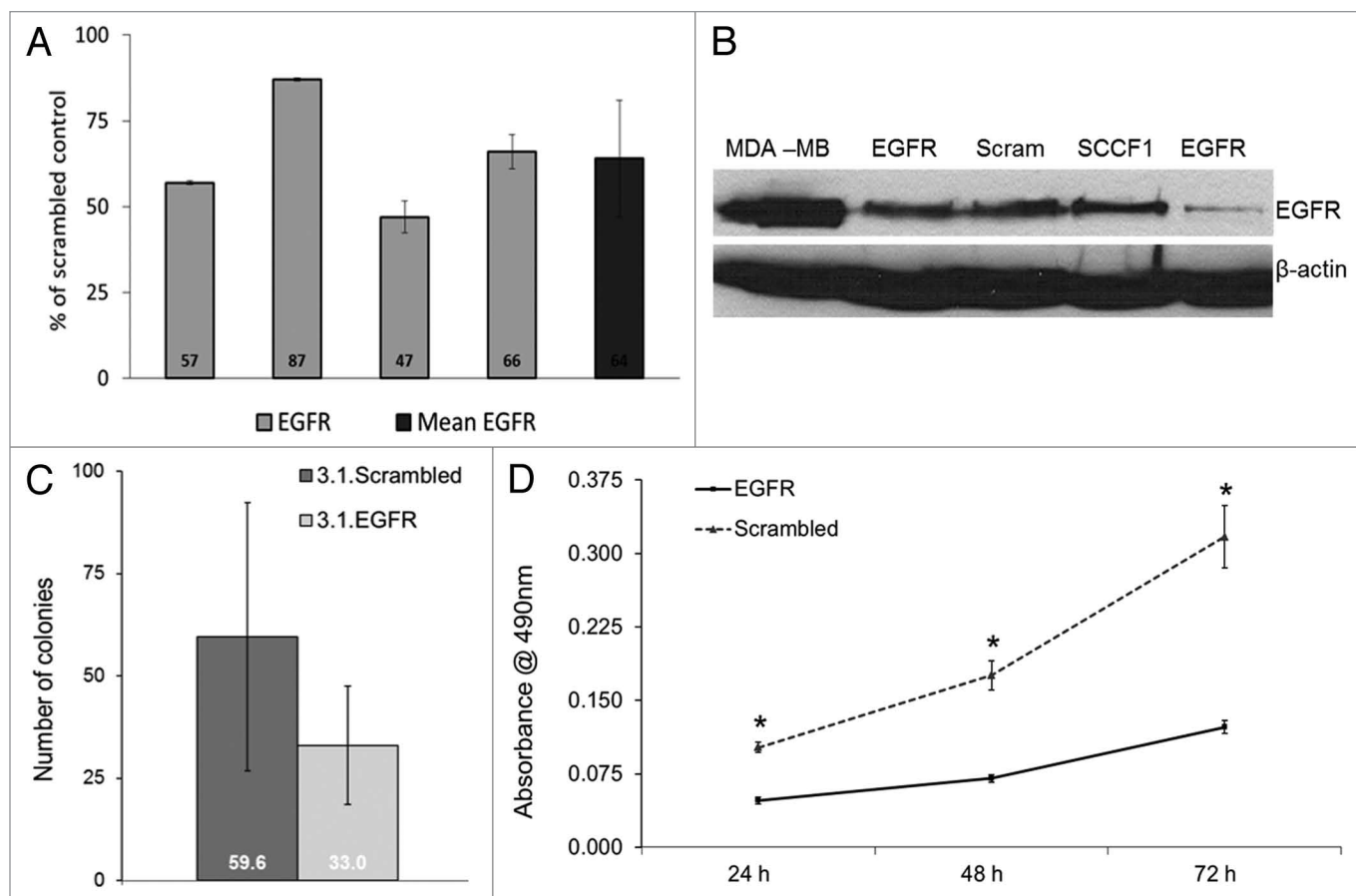


Figure 4. (A) Real time PCR results showing *Egfr* mRNA levels post 3.1pSilencer™ neo Kit transfections of SCCF1 cell line after culturing cell line for 20 days in selective media. Each bar is an average of three technical replicates run from separate transfection experiments. (B) Protein gel blot showing variable reduction in EGFR protein levels in two lysates from different pSilencer neo Kit transfections. MDA-MB (lane 1) contains lysate from the MDA-MB-468 cell line (MD Anderson Cancer Center, TX), a human breast cancer cell line that overexpresses EGFR. It was used as positive control to validate the antibody for the use on feline protein lysates. Lanes 2–5 contain lysates from SCCF1 cell line: EGFR, transfected with vector expressing shRNA against EGFR; Scram, transfected with vector with scrambled control sequence; SCCF1, untransfected cells. Protein loaded: 30 μ g MDA-MB, 60 μ g remaining samples. (C) Colony formation assay performed during 20 days of G418 selection post hairpin expression vector transfection. A 45% reduction in colony formation ability was seen in the EGFR transfected cells when compared to scrambled control transfected cells. The histogram shows average number of colonies counted with error bars showing standard deviations. Bars is average of eight (EGFR) and nine (Scrambled) 10 cm plates for each sample and represents transfections performed in triplicate. The difference showed a trend towards statistical significance ($p = 0.06$ by Mann Whitney U test). (D) Cell proliferation assay showing SCCF1 cell lines proliferating ability after 20 days of selection with G418 containing media. EGFR transfected cells showed a statistically significant lower proliferation rate when compared to scrambled transfected cells at 24, 48 and 72 h (* $p = 0.001$, $p = 0.003$ and $p < 0.001$ respectively, two sample t-test). Each point show an average absorbance value of four wells with the background subtracted, bars show standard deviation. Similar results were obtained in two separate experiments.

similar mechanisms were not responsible for the development of resistance in the cell line as no mutations were found in the ATP binding pocket of the receptor when the region was re-sequenced in the resistant cell line. This is in accordance with reports in the literature on the development of resistance to TKIs in HNSCC in which tyrosine kinase mutations of *Egfr* are rare events.²⁸ Cancer cells become resistant to EGFR targeted therapies through several mechanisms. The nature of the gefitinib resistance in the SCCFIG cell line in this study is not known, but some of the previously reported mechanisms of resistance could be consistent with an apparent resistance to tyrosine kinase inhibition but not to siRNA. Nuclear translocation has been implicated in resistance to the monoclonal antibody cetuximab.³⁶ Although it has been suggested that small molecule TKIs affect nuclear EGFR,³⁶

their relative efficiencies in the nucleus are unknown. siRNAs exert their effect mainly in the cytoplasm of cells,³⁷ but if efficient will reduce nuclear as well as membranous and cytoplasmic receptor levels. Increased expression of receptor ligands³⁶ would have limited effect on transfected cells as the receptor levels would be markedly reduced. If the cells upregulated other members of the receptor family like for example ErbB3,³⁸ the siRNA would not be expected to be effective. An alternative resistance pathway relies on transactivation of EGFR by the cytosolic tyrosine kinase Src.³⁹ siRNAs targeting *Egfr* would still be effective in this scenario as the reduction in receptor protein levels would counteract the increased phosphorylation. Likewise, if the receptor ubiquitination and degradation pathways were dysregulated,⁴⁰ siRNAs targeting *Egfr* would still be effective. Regardless of the

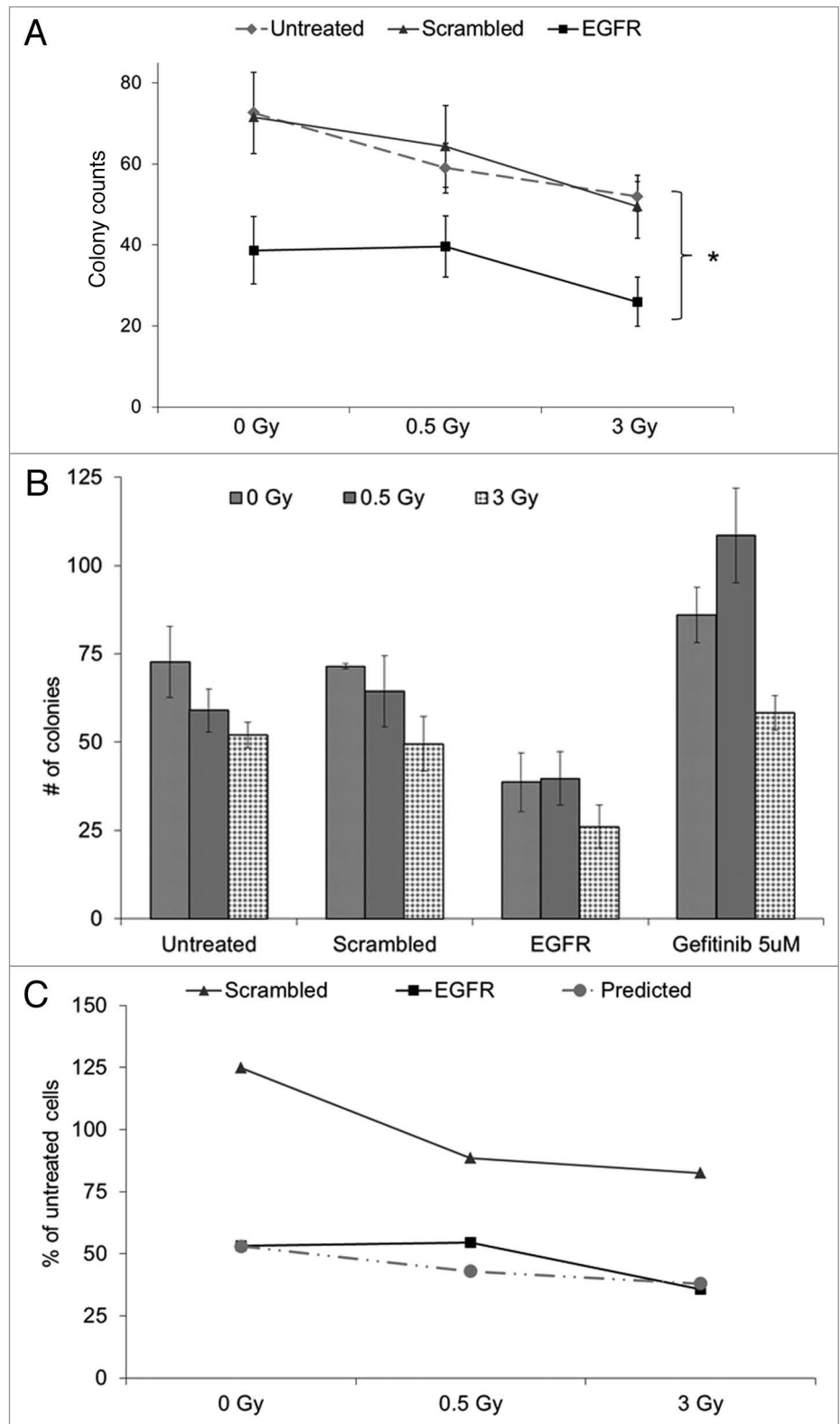
Figure 5. (A) Graphs show results of colony formation assays post radiation and EGFR knockdown in SCCF1 cell line ten days following radiation. Cells transfected with siRNA6 showed statistically significant reduction in colony formation ability as compared to untreated and scrambled control transfected cells at 0, 0.5 and 3 Gy radiation doses (* $p = 0.007$, $p = 0.022$ and $p = 0.028$ respectively, one-way ANOVA). (B) Histogram showing colony formation assay following EGFR targeting by either siRNA6 or gefitinib at 5 μ M and radiation at 0, 0.5 and 3 Gy. Gefitinib at a dose of 5 μ M showed no effect on colony formation ability compared to siRNA6 transfection. (C) Graph showing additive effect of combining siRNA6 transfection and radiation. The predicted graph gives the E_{bliss} values for radiation and EGFR targeting causing an additive effect according to the Bliss additivity model.

mechanism used by the cells in this study, the net effect was that resistance to gefitinib could be overcome by using RNAi to target *Egfr*. This study illustrated how the resistant cancer cells were still dependent on EGFR for proliferation, despite acquiring resistance to an EGFR targeting drug.

This study demonstrated that *Egfr* siRNA effectively reduced both *Egfr* transcripts and EGFR protein in the SCCF1 cell line. RNAi was used in this study as it is a highly selective method of evaluating the effect of gene silencing of a target gene.²⁴ Silencing the feline *Egfr* in the cell line had a profound effect on cell proliferation and colony formation ability and this effect could be sustained over time. This demonstrates that EGFR is an important oncogenic driver in this model of feline oral SCC and that targeting EGFR in feline oral SCC has potential as a therapeutic strategy.

This study also investigated the effect of EGFR targeting on the pathways downstream of the receptor and demonstrated that some of the pathways involved in cell proliferation and angiogenesis including MAPK and STAT-3 were downregulated. A marked reduction in proliferation was seen in the cells following EGFR knockdown in this study, which is consistent with a reduction in phosphorylation in these two pathways.^{17,31} Although EGFR has been shown to directly activate the PI3K pathway mediated by growth factor receptor binding 2 (Grb-2)-associated binding protein 1 (Gab1),⁴¹ ErbB3, a closely related member of the *Egfr* gene family, is the major activator of the

PI3K pathway with six separate phosphorylation sites for the p85 subunit of PI3K.⁴² EGFR targeting may be less likely to cause a major reduction in PI3K pathway activation if ErbB3 receptor remains active. The clinical responses to EGFR targeting therapies in human trials have so far been modest¹¹ and this may be because tumors have an intrinsic resistance to EGFR-targeted



therapy.^{11,36} These problems may be overcome by using combination therapies that target these alternative resistance pathways and by improved selection of patients that are more likely to respond to EGFR targeting.¹¹

Part of effective clinical application of *Egfr* RNAi is likely to include inducing prolonged EGFR inhibition to have a sustained effect. Direct transfection of siRNA molecules produces only transient inhibition of *Egfr* gene expression as siRNA molecules become depleted or destroyed.²⁴ To evaluate the effect of sustained *Egfr* gene knockdown, a plasmid that expressed short-hairpin RNA molecules was developed. This study demonstrated that a hairpin expression vector can be effectively taken up by cells and lead to sustained production of shRNAs. The short hairpin expression vector transfection efficiency was lower than the naked siRNA transfection efficiency. To counter this effect, antibiotic selection was used to enrich for cells incorporating the expression vector. Despite selection, the efficacy of gene knockdown and the variability between transfection replicates were greater when using hairpin expression vectors compared to naked siRNAs. Possible explanations include that some clones may have developed resistance to the shRNAs by acquiring mutations rendering them insensitive to interference by the shRNA. Alternatively, as experiments have shown, between 1–10% of vectors integrated into the genome during stable transfections are not intact.⁴³ These clones may have acquired the antibiotic resistance gene without acquiring functionally intact shRNA expression. Considering that EGFR knockdown is deleterious to cell proliferation, these clones would have a growth advantage over the cells expressing shRNAs against the feline *Egfr*.

In HNSCC the role of chemoradiation is well established,¹¹ and a monoclonal antibody targeting the EGFR, cetuximab, has been given FDA approval for use in combination with radiotherapy for HNSCC.^{11,17} Combination of EGFR targeted therapy and radiotherapy for management of feline oral squamous cell carcinoma is attractive as EGFR-mediated therapy may be able to augment the efficacy of radiotherapy, an established treatment option.⁷ Combined therapy with radiation and EGFR targeting produced an additive effect similar to that observed in human HNSCC cell lines^{32,44} and xenograft models.³² A study investigating the effect of cetuximab in combination with cisplatin and irradiation in HNSCC cell lines reported an additive effect in five cell lines while two cancer cell lines exhibited a synergistic effect.⁴⁴ When comparing EGFR basal and phosphorylated levels between the cell lines exhibiting additive versus synergistic effects, they found that the cell line that exhibited synergy had a much higher basal level of EGFR and that the receptor became phosphorylated upon irradiation.⁴⁴ This supports the theory that overexpressing EGFR cell lines are more likely to respond to EGFR targeting therapy.⁴⁵ In these cell lines combination treatments are thought to act synergistically because the cell lines utilize EGFR pathways like the PI3K and MAPK pathways to counteract the effect of radiation.¹⁹ When these pathways are blocked, the cells become more sensitive to radiation.¹⁹

The SCCF1 cell line had a relatively low basal level of EGFR when compared to known EGFR overexpressing human cancer cell line MD-MBA-468. HNSCC cell lines with low basal levels

of EGFR showed additive rather than synergistic effects when cetuximab was combined with radiation,⁴⁴ which is similar to the findings in this study. EGFR-targeted therapy may be of clinical value in augmenting radiotherapy in feline oral SCC but tumors expressing higher EGFR levels may be more sensitive to combined treatment.

In this study we utilized RNAi for EGFR targeting, and it proved to efficiently reduce the target protein levels and cell proliferation in the cell line. RNAi is a promising technique that has wide applications in research but with emerging uses in therapeutics.²⁴ RNA interference may prove to be a powerful tool for the new generation of targeting therapies where acquired resistance to drugs has developed over time. In a recent study, transfection of cetuximab resistant HNSCC cell lines with siRNA against EGFR reversed the acquired resistance in the cell lines.³⁸ This is similar to the findings in this study, where transfection of the gefitinib resistant cell line caused similar reduction in cell proliferation as in the non-resistant parent cell line SCCF1. The major obstacle to wider clinical use of RNAi based drugs is the lack of a good in vivo delivery system²⁴ but this area is advancing and the first siRNA-based clinical trial using a nanoparticle delivery system has been reported.²⁵ If a suitable delivery vector can be found, RNAi may become an attractive option for achieving EGFR-targeted therapy.

Feline SCCs are naturally occurring tumors that have developed in pet animals which possess an intact immune system and which share their environment with humans. The aetiopathogenesis of the disease in cats is not fully characterised, but environmental factors similar to those in man have been postulated as risk factors.⁴⁶ Evaluating drug strategies in naturally occurring disease in pets may help bridge the gap between in vitro research and clinical application in man.⁴⁷ This study supports the use of feline SCC as a model for HNSCC, an approach that could potentially benefit both felines and humans alike. During the development of a TKI against KIT (SU11654, toceranib)⁴⁸ the human field benefited greatly from the canine studies,⁴⁷ and the development of acquired resistance to single-agent TKIs in dogs with mast cell tumors is of particular interest.⁴⁷ Further studies in cats with SCCs using EGFR targeting siRNA based therapies in combination with radiotherapy would potentially advance the treatment of the disease in both species.

Materials and Methods

Cell culture. Cell culture reagents were obtained from Gibco® Invitrogen unless otherwise specified. The SCCF1 cell line⁴⁹ is a previously characterised feline cell line derived from a laryngeal squamous cell carcinoma. The cells were grown in William's E Medium with GlutaMAX supplemented with 10% FBS, 0.05 mg/ml Gentamicin and 10 ng/ml EGF. For post-transfection selection of cells 0.4 mg/ml Geneticin (G418) was added to the medium.

For the siRNA screening protocol, the 293FT cell line (Invitrogen) was used. The 293FT cell line was grown in DMEM supplemented with 10% FBS, 0.1 M Non-Essential Amino Acids, 6 mM L-glutamine, 1 mM Sodium Pyruvate, 1%

Penicillin-Streptomycin and 0.5 mg/ml G418. During transfections, cells were grown in DMEM media as above without antibiotics and, immediately following electroporation, cells were resuspended in RPMI media containing 10% FBS before plated in normal DMEM growth media with antibiotics.

Buffers and reagents. Reagents were obtained from Sigma Aldrich unless otherwise specified. The following buffers were used: Urea lysis buffer: 7 M Urea, 0.1% DTT, 0.05% v/w Triton X-100, 25 mM NaCl and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5). Tris-buffered saline Tween-20 (TBST): 20 mM Tris-HCl, 0.15 M NaCl and 0.1% Tween-20. TBST blocking buffer: 5% Bovine Serum Albumin (BSA) in TBST. Phosphate-buffered saline (Gibco) Tween-20 (PBST): 0.01 M Phosphate, 0.154 M NaCl and 0.1% Tween-20, pH 7.4. PBST blocking buffer: 5% Skimmed Milk (Oxoid) in PBST. 2x Laemmli Sample Buffer: 120 mM Tris-HCl pH 6.8, 4% (w/v) sodium dodecyl sulphate (SDS), 20% (v/v) glycerol, 200 mM DTT, 0.04% (w/v) bromophenol blue.

Cloning and sequencing of feline EGFR tyrosine kinase region. Total RNA was isolated using RNeasy Mini Kit (Qiagen). First strand cDNA was synthesised from 2 µg RNA using Qiagen Omniscript® Reverse Transcriptase Kit and OligoT primers (Qiagen). Three primer sets were designed to span the putative tyrosine kinase region of the feline *Egfr* gene using areas with a high degree of homology between the human, murine and canine published sequences (www.ncbi.nlm.nih.gov and www.ensembl.org/index.html) (5' to 3' primer pair A: *for* GGA GAA GCT CCC AAC CAG GCT, *rev* GAT AGG CAC TTT GCC TCC TTC, B: *for* GAA TAT CAC CTG CAC AGG AC, *rev* GCC ATC ACG TAA GCT TCA TC and C *for* TGC GAA GGG CAT GAA CTA C, *rev* ACT CAT CGG CAT CTA CGA C). All DNA oligonucleotides were purchased from Eurofins MWG Operon and PCR reactions were performed using HotStar® DNA polymerase kit (Qiagen) on iCycler thermal cycler (BioRad) using the following conditions: one cycle of initial denaturation at 95°C for 15 min followed by 25 cycles of one min each at 94°C (denaturation), 52°C (annealing) and 72°C (extension) before a final extension step of 72°C for 10 min.

Samples were sequenced in both directions at The Gene Pool (The University of Edinburgh, Edinburgh, UK) using primer pairs A, B & C, and were then checked against the feline genome using the Basic Local Alignment Search Tool (BLAST) nucleotide search (www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9685).

Gefitinib treatment of cell line. Gefitinib (Tocris Bioscience 184475-35-2) was used at a range of concentrations from 1 nM to 20 µM. To produce a gefitinib resistant cell line SCCF1G, the SCCF1 cell line was maintained in its normal media supplemented with 5 µM of gefitinib for 5 months.

Cell proliferation assays. Cells were seeded into 96 well plates at 1,500–3,000 cells per well and allowed to attach overnight before treatment. Cellular proliferation was measured using the CellTiter 96® AQ_{ucous} One Solution Cell Proliferation Assay (Promega) according to manufacturer's protocol and absorbance at 490 nm was determined on a plate reader (Perkin Elmer 1420 Multilabel Counter Victor³™).

In vitro scratch assay. Cells were grown to confluency in six well plates and a scratch was created with a one ml pipette tip in the monolayer as previously described in reference 50. The gap width was measured at ten points for each photograph and the mean was expressed as percentage of gap width at 0 h and compared between treatment groups.

siRNA design, construction and screening. Six siRNAs against the feline sequence were designed using the Ambion siRNA finder (www.ambion.com/techlib/misc/siRNA_finder.html). They were produced using Silencer® siRNA Construction Kit (Ambion) following manufacturer's recommendations and screened using the psiCHECK-2 vector system (Promega). The psiCHECKTM-2 vector has two reporter genes. The Renilla primary reporter gene was fused with the directionally cloned feline *Egfr* gene sequence. This allowed for monitoring of target gene expression following transfections with the different siRNAs. The Firefly secondary reporter gene was used to normalise the Renilla readings to correct for variations due to different transfection efficiency between samples. Following electroporation, the luminescence was read on a plate reader (1420 Multilabel Counter Victor³™, Perkin Elmer, Germany) using the Dual-GloTM Luciferase System (Promega) following manufacturer's protocol.

Vector cloning. DNA oligonucleotide templates (top strand 5'-GAT CCG GAA ATC CTT GAT GAA GCT TTC AAG AGA AGC TTC ATC AAG GAT TTC CTT TTT TGG AAA-3' and bottom strand 5'-AGC TTT TCC AAA AAA GGA AAT CCT TGA TGA AGC TTC TCT TGA AAG CTT CAT CAA GGA TTT CCG-3') was ligated into the BamH I or Hind III restriction sites of the 3.1pSilencerTM neo Kit (Ambion) and the 500 base pairs sequence produced by primer pair A with the sequences of restriction enzyme sites Not I and Xho I added was ligated into the psiCHECK-2 vector (Promega) by T4 DNA ligase (BioLine). The ligation reactions were used directly in One Shot Chemically Competent *E. coli* (Invitrogen) transformation reactions. Colonies were selected and expanded overnight before performing plasmid purifications using QIAfilter Plasmid Midi Kits (Qiagen) according to manufacturer's protocol. The vectors were sequenced to ensure the correct sequences had inserted.

Transfections. For the siRNA screening, transfections of the 293FT cell line by electroporation were performed using the Nucleofector® 96-well shuttle (Amaxa, Lonza, Switzerland) and Cell Line 96-well Nucleofector® Solution SF Kit (Amaxa) following manufacturer's protocol. In brief, cells were seeded at 2 x 10⁵ cell per well into a NucleocuvetteTM 96-well plate and 1 µg psiCHECKTM-2 vector was co-transfected with 250 nM siRNA per well in triplicate using program DS-150. Cells were cultured for 24 h before luminescence were assessed using the Dual-GloTM Luciferase System (Promega).

The SCCF1 and SCCF1G cell lines were transfected with 50 nM RNA or with 4 µg DNA using LipofectamineTM 2000 (Invitrogen) following manufacturer's protocol. Cells were transfected with siRNA against the feline *Egfr*, scrambled sequence Silencer® Negative Control #1 siRNA (Ambion) or with pSilencerTM neo Kit hairpin expression vectors containing the sequence directed against the feline *Egfr* or a scrambled control sequence.

Cells transfected with the hairpin expression vectors were trypsinated and counted 24 h following transfection, seeded at 3×10^5 cells per well into six-well plates before adding selective G418 containing media for 20 days.

Real time PCR. Total RNA was isolated using RNeasy Mini Kit (Qiagen). First strand cDNA was synthesised from 1.0 μg RNA using Roche Transcriptor High Fidelity cDNA Synthesis Kit and random hexamers (Roche). Primers and FAM labeled probes for feline EGFR and feline β -actin were designed using Roche Universal Probe Library (www.roche-applied-science.com/sis/rtPCR/upl/index.jsp). Real Time PCR reactions were set up using Roche LightCycler[®] 480 Probes Master kit according to manufacturer's protocol and 2 μl of cDNA synthesis reaction diluted 1:10. The thermal conditions were one pre-incubation step at 95°C for ten min followed by 45 cycles consisting of 10 s of denaturing at 95°C, 30 s of annealing at 54°C and 1 s of extension at 72°C before cooling. VIC labeled probes and primers for human 18S rRNA (Applied Biosystems) was used as a reference gene. Relative mRNA expressions were determined according to the Pfaffl method.⁵¹

Protein gel blot analysis. Cells were lysed in urea lysis buffer 72 h after transfections, cleared by centrifugation and quantified using a standard Bradford absorbance assay. The proteins were resolved on 8% SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, blocked and incubated with primary antibodies overnight. The membranes were then incubated with HRP-conjugated secondary antibodies (Dako) diluted 1:2,000 and developed using Immun-StarTM westernCTM Kit (BioRad). The antibodies used were EGFR (MS-400-P0, Thermo-Scientific), phospho-MAPK/Erk1/2 (D13.14.4E, Cell Signalling Technologies), total MAPK/Erk1/2 (137F5, Cell Signalling Technologies), phospho-STAT-3 (SC-81523, Santa Cruz), total STAT-3 (SC-7179, Santa Cruz) and β -actin (ab6276, abcam).

Colony formation assays (CFAs). Cells were seeded at a density of 300–500 cells per 10 cm plate 24 h following transfection with naked siRNA or gefitinib treatment into normal media

(radiosensitivity assay) or 24 h following shRNA expression plasmid transfection into G418 containing selective media. The media was changed and the plates were checked for colony formation twice weekly. After 10–20 days the colonies were fixed in cold methanol, stained by adding 10% Giemsa stain and manually counted.

Radiosensitivity assay. SCCF1 cells were transfected or treated with 5 μM gefitinib in six-well plates and incubated for 24 h before being irradiated. All cells were irradiated in culture medium using a Faxitron[®] cabinet X-ray system 43855D (Faxitron X-ray Corporation) at a central dose rate of 2 Gray (Gy)/min. All irradiation was given as a single fraction at room temperature. Cells were trypsinated and counted 24 h after transfections and 300 cells were seeded in triplicate into ten-cm plates and irradiated in suspension at 0, 0.5 and 3 Gy prior to performing CFAs as previously described.

Statistical analyses. All experiments with the exception of the radiosensitivity assay were repeated at least on two separate occasions with similar results. All quantitative analysis is based on a minimum of three replicates for 6-well plates and ten cm plates or a minimum of four wells for 96-well plates for each separate experiment. One-way ANOVA was used to compare differences between more than two samples. Two sample t-tests or the non-parametric equivalent Mann-Whitney U test were used to compare differences between two samples. Results were considered significant when $p < 0.05$. All statistical analysis was performed using Minitab[®] 15 Statistical software (Minitab Ltd.) and all graphs were generated using Microsoft[®] Office Excel 2010 software. To assess the effect of combining radiation with EGFR knockdown in the SCCF1 cell line the Bliss additivism model was used as previously described in reference 52.

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