Radioresistant laryngeal cancers upregulate IGF-1R and exhibit increased cellular dependence on IGF and EGF signalling.

Running title: IGF-1R associates with radioresistance in LSCC

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Abstract

Objectives: Patients failing radiotherapy for laryngeal squamous cell carcinoma (LSCC) often require salvage total laryngectomy which has major functional consequences, highlighting a need for biomarkers of radiotherapy resistance. In other tumour types, radioresistance has been linked to epidermal growth factor receptor (EGFR) and type 1 insulin-like growth factor receptor (IGF-1R). Here, we evaluated IGF-1R and EGFR as predictors and mediators of LSCC radioresistance.

Design: We compared IGF-1R and EGFR immunohistochemical scores in LSCC patients achieving long-term remission post-radiotherapy (n=23), patients treated with primary laryngectomy (n=22) or salvage laryngectomy following radiotherapy recurrence (n=18). To model radioresistance *in vitro*, two LSCC cell lines underwent clinically-relevant irradiation to 55 Gy in 2.75 Gy fractions.

Results: IGF-1R expression associated with tumour size, was higher in pre-treatment biopsies of radiotherapy-failures than those in long-term remission ($p=0.01$) and was upregulated post-radiotherapy. Patients undergoing primary laryngectomy had higher T and N stage ($p<0.05$) and higher tumour IGF-1R ($p=0.02$) than those achieving long-term remission. Pre-treatment EGFR did not associate with radiotherapy outcomes but showed a trend to upregulation post-irradiation. *In vitro*, radiosensitivity was enhanced by inhibition of EGFR but not IGF. Repeated irradiation upregulated IGF-1R in BICR18 and SQ20B cells and EGFR in SQ20B, and enhanced SQ20B radioresistance. Inhibition of IGF and/or EGFR did not reverse radioresistance, but co-inhibition suppressed cell survival more effectively than blockade of either pathway alone, and more effectively than in parental cells.

Conclusions: Radiation upregulates IGF-1R and may enhance IGF/EGFR dependence, suggesting that IGF/EGFR blockade may have activity in LSCCs that recur post-radiotherapy.

Key words: laryngeal squamous cell cancer, radiotherapy, radioresistance, type 1 IGF receptor, epidermal growth factor receptor, xentuzumab, afatinib.

Key points

1 There is a need for biological markers to guide treatment choice in patients with laryngeal squamous cell carcinoma (LSCC).

2 LSCC cells and tumours that persist through fractionated radiotherapy are radioresistant and have upregulated IGF-1R and EGFR.

3. Radioresistant LSCC cells are not radiosensitised by EGFR or IGF inhibition alone or in combination, indicating that these pathways are not drivers of radioresistance.

4. The survival of radioresistant LSCC cells is profoundly inhibited by IGF:EGF co-inhibition, suggesting that irradiation leads to enhanced dependence on IGF and EGF signalling.

5. These results suggest that there may be merit in testing whether IGF:EGFR co-inhibition delays progression in LSCC patients experiencing inoperable post-radiotherapy recurrence, or reduces the risk of recurrence after salvage surgery.

Introduction

The overall 5-year survival from laryngeal squamous cell carcinoma (LSCC) is ~65%, with only modest improvement over 20 years (1, 2). According to NICE guidelines, patients with glottic LSCC should be offered trans-oral laser microsurgery (TLM) for T1a glottic disease, and TLM or radiotherapy for T1b–T2 glottic or T1–T2 supraglottic LSCC (NICE guidelines [NG36]. https://www.nice.org.uk/guidance/ng36), with salvage laryngectomy or radiotherapy for recurrence (1). Rates of local control and 5-year survival in early stage tumours is similar following radiotherapy or TLM (~80%), meta-analyses in early glottic cancers suggests improved survival following TLM, with uncertainty regarding vocal quality outcomes (3, 4). Options for T3/T4 LSCC include radiotherapy, concurrent platinum-based chemo-radiotherapy, primary partial or total laryngectomy (2, 5). Post-radiotherapy recurrence is a major problem, with radiotherapy failure rates of 9%-21% in T1 glottic and 28%-37% in T2 glottic LSCC. Recurrance rates in supraglottic tumours are reported as 24%-30% in T1 and 25%-45% in T2 . In T3/T4 disease rates of 68-78%. When a recurrence does occur while laryngeal preserving surgery can be considered, for many tumours a salvage laryngectomy is required (6). Functional consequences of this include a tracheal stoma, communication and swallowing difficulties, complications including wound infection, haemorrhage, pharyngocutaneous fistula, stomal stenosis, and reduced overall survival (5, 6). There is an urgent need for predictors of radiotherapy failure, to guide treatment choice.

Most HNSCCs express epidermal growth factor receptor (EGFR), which associates with adverse prognosis, loco-regional recurrence and radioresistance (7, 8). Addition of anti-EGFR antibody cetuximab to radiotherapy for locally-advanced HNSCC prolongs progression-free and overall survival, although randomised comparison of cisplatin and cetuximab was discontinued prematurely for slow accrual, reporting similar efficacy but more toxicity in the cetuximab arm (8-10). Resistance to EGFR inhibition is an important issue, and may involve type 1 insulin-like growth factor receptor (IGF-1R), which engages in cross-talk with EGFR and like EGFR signals via PI3K-AKT and RAS-RAF-ERK (11-13). We previously reported detectable IGF-1R in 92% of 346 HNSCCs, with association between IGF-1R

overexpression and higher tumour T-stage, shorter overall and disease-specific survival, and HPV negativity (14), the latter property well-known to associate with resistance to chemo-radiation (15). IGF-1R overexpression associates with clinical radioresistance in prostate, breast and colorectal cancer, and in experimental models, IGF-1R inhibition enhances radiosensitivity (16-19). The aims here were to assess IGF-1R as a predictor of radioresistance in LSCC, and investigate whether IGF blockade alone or with EGFR inhibition enhances radiosensitivity in an induced radioresistance model.

Methods

Ethical considerations

Tissue use was approved by National Research Ethics Service Oxfordshire Committee C (reference 07/H0606/120). All patients gave written informed consent to use of tissue in research.

Patients and tumour specimens

From the Oxford Head and Neck Cancer Database we identified all patients who underwent salvage laryngectomy from January 2004 to January 2016 at Oxford University Hospitals NHS Foundation Trust (n=25) and selected at random equivalent cohorts achieving long-term remission after primary radiotherapy or undergoing primary total laryngectomy during the same period. We obtained formalin fixed, paraffin-embedded (FFPE) diagnostic biopsies, recurrent tumour biopsies and salvage laryngectomies. Blocks were reviewed by Head and Neck Pathologist KAS to confirm tumour presence. From case notes we recorded demographic details, tumour stage (TNM staging), American Society of Anaesthesiologists Performance Status (ASA), treatment and clinical outcomes.

Immunohistochemistry (IHC)

IHC was performed on 4µm FFPE tissue sections using antibodies to IGF-1R and EGFR (#9750 and #4267, Cell Signalling Technology) as (14). Two observers (AQ, KS, blinded to treatments) scored EGFR and IGF-1R in tumour cell membranes, cytoplasm and nuclei for intensity (0-3) and percentage positivity (0-4), which when multiplied gave immunoreactive scores for each subcellular compartment, and combined to give a total IGF-1R or EGFR score as (14).

Cell culture and treatments

LSCC cell lines SQ20B and BICR18 were obtained respectively from Dr Geoff Higgins, University of Oxford (20) and European Collection of Cell Cultures. Both tested negative for mycoplasma (MycoAlert, Lonza Rockland Inc, Rockland). STR genotyping (Eurofins Medigenomix Forensik GmbH) validated BICR18, but SQ20B is not represented in the DSMZ database (www.dsmz.de/de/service/services-human-and-animal-cell-lines/online-stranalysis). Cultures were

maintained in Dulbecco's Modified Eagle Medium with 10% foetal calf serum (Gibco), 1% penicillin/streptomycin, supplemented with 2mM Glutamine (Gibco) and 0.4 μg/ml hydrocortisone (Sigma-Aldrich) for BICR18, and 1% non-essential amino acids (Gibco) for SQ20B. Cells were treated with Long R3 IGF-1, EGF (both Sigma-Aldrich), EGFR inhibitor afatinib (Selleck), and/or xentuzumab (BI 836845, ref (21), provided by Drs Weyer-Czernilofsky and Bogenrieder, Boehringer Ingelheim Vienna. Cultures were irradiated in a caesium-137 irradiator (Gamma-Service Medical GmbH). Cultures at 80-90% confluence underwent repeated irradiation to 55 Gy in 20 2.75 fractions, 5 days/week over 4 weeks. Early and late passages of parental cell lines were maintained as controls.

Western blotting and clonogenic survival assay

Subconfluent cultures were treated with afatinib and/or xentuzumab for 60 minutes and in the final 20 minutes with 50 nM IGF-1 and/or 20 nM EGF. Western blotting used antibodies to IGF-1R (#3027), phospho-Y1135/6 IGF-1R (#3024), EGFR (#2232), phospho-Y1068 EGFR (#2236), AKT (#9272), phospho-S473 AKT (#4060), ERK1/2 (#4695), phospho-T202/Y204 ERK1/2 (#9101), PTEN (#9556), β-tubulin (#T4026), all Cell Signaling Technology. Clonogenic assays were performed as (19), seeding 2000 or 10,000 cells/10 cm dish. After 24hr, cells were treated with solvent (control) or inhibitors for 24hr and irradiated. Next day, medium was replaced with fresh medium without drug(s), and dishes incubated until formation of colonies ≥50 cells. Colonies were counted on a GelCount (Oxford Optronics). Assays were performed ≥3 times, each with triplicate technical replicates.

Statistical Analysis

Statistical analyses used GraphPad Prism v7. Demographic factors were assessed by two-tailed Chi-squared test, and IHC scores by two-tailed Mann-Whitney-U test (two unpaired groups), pairwise Mann-Whitney-U test (≥3 groups), Pearson's correlation and least squares linear regression. Cell line data analysis used t-tests (two groups) and one-way Analysis of Variance (ANOVA, >2 groups), and $p \leq 0.05$ was considered significant. Radiosensitisation was assessed by calculating survival fraction 50% (SF_{50}, dose inhibiting survival to 50% of unirradiated controls) and dose enhancement ratios (DERs) at 2 Gy, ratio of % survival of control-treated cells over experimentally-treated cells.

Results

Locally-advanced LSCCs treated with primary laryngectomy contain more IGF-1R than lower T/N-stage tumours treated with radiotherapy

Tumours were available from 63/75 identified patients (Figure 1A), including 23 in remission post-radiotherapy (mean follow-up 49.2 months, range 12-60), 18 patients requiring salvage laryngectomy ('radiotherapy failure', mean time to recurrence 21.3 months, range 4-60), and 22 patients requiring primary total laryngectomy. There were fewer smokers in the long-term remission group, but no differences in other demographic factors or radiotherapy regimen (Table 1). All LSCCs contained EGFR with strong membrane and variable cytoplasmic signal (Figure 1B). IGF-1R was expressed by 70/77 (91%), consistent with our previous report, where IGF-1R was detected in 92% of LSCCs (14). IGF-1R showed variable membrane and cytoplasmic signal, and as previously (14) nuclear IGF-1R was undetectable. Figure 1B shows examples of EGFR and IGF-1R IHC. EGFR and IGF-1R were co-expressed in 53 samples, with evidence of weak positive correlation (Figure 1C). Patients undergoing primary total laryngectomy had higher T stage than the long-term remission group (T3/4 in 100% vs 26%, p=0.001), and higher N stage (N2/3 in 36.4% vs 4.3%, p=0.005; Table 1). EGFR content was similar in primary total laryngectomies and tumours of patients achieving long-term remission, while IGF-1R levels were higher in the primary laryngectomies (p=0.02; Figure 1D). This likely reflects their higher T/N stage, consistent with our report in HNSCC where IGF-1R expression associated with T-stage (14).

Tumours recurring after radiotherapy contain more IGF-1R than tumours of patients remaining in long-term remission

We took three approaches to assess associations of IGF-1R and EGFR with post-radiotherapy outcomes. First, in patients experiencing recurrence post-radiation, we found no correlation between IGF-1R or EGFR expression and time to recurrence (Figure 2A). Secondly, comparing IHC scores in diagnostic biopsies, we found a trend to increased IGF-1R content in the radiotherapy-failures (n=7, mean score 5.43 ± 1.95) compared with patients experiencing long-term remission (n=23, 3.17 ± 0.53;

Figure 2B left), lack of significance likely relating to small numbers. However, total IGF-1R scores were significantly higher in biopsies of post-radiotherapy recurrences, compared with pre-treatment biopsies in the long-term remission group (6.43 ± 1.23; p=0.01, Figure 2B right). Conversely, EGFR scores were higher in biopsies of patients achieving long-term remission compared with diagnostic biopsies in the radiotherapy-failure group (16.14 ± 0.95 vs 12.29 ± 1.55, p=0.07), with no change in biopsies taken post-radiotherapy recurrence (15.00 ± 0.89, p=0.44, Figure 2C). Because comparison of cohorts could mask changes in individual tumours, as a third approach we analysed receptor content in six patients where we accessed biopsies at diagnosis and post-radiotherapy failure, finding significant increase in post-radiotherapy IGF-1R scores (Figure 2D). Membrane EGFR also increased post-radiotherapy, with variable/no changes in cytoplasmic EGFR (not significant, Figure 2E).

LSCC cells are radiosensitised by EGFR but not IGF inhibition

Following these studies in clinical LSCCs, we investigated effects of IGF and EGFR blockade on response to ionising radiation (IR) in LSCC cell lines. SQ20B and BICR18 expressed IGF-1R, with IGF-responsive AKT and ERK activation, and SQ20B also expressed EGF-responsive EGFR. EGFR was undetectable in BICR cells but EGF did activate ERKs (Figure 3A), suggesting EGFR expression below detection limits or response of alternative ErbB receptors, although HER2 was undetectable by western blot (not shown). To assess effects of receptor inhibition, we used IGF-neutralising antibody xentuzumab, and afatinib, inhibitor of ErbB family kinases (22). Xentuzumab blocked IGF-induced IGF-1R activation in both cell lines, and AKT/ERK phosphorylation in BICR18. Afatinib suppressed EGF-induced EGFR, AKT and ERK activation in both cell lines (Figure 3B). Together, these agents suppressed phosphorylation of IGF-1R and AKT, and ERKs only in BICR18 (Figure 3C). With this evidence of signalling inhibition by 100-1000 nM xentuzumab and afatinib, we tested these concentrations in survival assays. SQ20B cell survival was suppressed by afatinib but not xentuzumab, although xentuzumab enhanced inhibition by 100 nM afatinib (p=0.007), but in BICR18 neither agent reduced survival (Figure 3D). In radiation assays, SQ20B cells were radiosensitised by afatinib alone and with xentuzumab, with reduction in SF_{50} and increase in DER at 2Gy, but BICR18 cells were not radiosensitised by either drug alone or in

combination (Figure 3E-F). Radiosensitisation by EGFR inhibition was previously reported although not universally; radiosensitivity was enhanced by cetuximab or IGF-1R blocking antibody cixutumumab only in a minority of HNSCC cell lines, with no additive effect upon combination treatment (23).

Radioresistant LSCC cells upregulate IGF-1R with profound cell survival inhibition upon IGF/EGFR co-inhibition.

To derive clinically-relevant radioresistance models we irradiated cultures to 55 Gy in 20 2.75 Gy fractions, as received by most patients in the clinical study (Table 1). Assessing cell signalling in repeatedly-irradiated and parental cultures of equivalent passage, SQ20B cells exhibited ligand responses consistent with previous results (Figure 3A). Repeatedly-irradiated subline SQ20B_55 had upregulated EGFR and IGF-1R, with enhanced phospho-receptor response to EGF and IGF-1, and increased phospho-ERK1/2 (Figure 4A, left). BICR_55 also manifest increased IGF-1R and phospho-IGF-1R, and despite remaining EGFR negative showed EGF-induced AKT/ERK phosphorylation (Figure 4A, right). This could relate to altered expression/activation of other ErbB receptors or downstream effectors, although we found no evidence of PTEN loss, which would activate AKT (Figure 4A).

We assessed baseline survival in the absence of irradiation, also checking effects of passage, as SQ20B_55 and BICR_55 had undergone >20 passages during repeated irradiation. In parental cultures clonogenic survival was relatively stable from passage 14 to 23-24, while survival was significantly reduced to ~30% and 70% of parental levels in SQ20B_55 and BICR_55 respectively (Figure 4B). Next, we assessed radiosensitivity, seeding SQ20B_55 at 10,000 cells/10 cm dish (increased from 2000) to allow sufficient survival to assess IR response. We first checked relative radioresistance in early and later (+10) passage parental SQ20B and BICR18, finding no change (not shown). There was clear evidence that repeated irradiation induced radioresistance in SQ20B_55, with significant increase in relative cell survival at 2-8Gy, increased SF_{50} and reduced DER at 2 Gy (Figure 4C). Differences in BICR_55 were less marked, with radioresistance significant only at 8Gy, and minor changes in SF_{50} and DER (Figure 4D). To assess the contribution of IGF-1R/EGFR upregulation and amplified ligand

response (Figure 4A) to radioresistance, we tested afatinib and xentuzumab alone and in combination, finding minor changes in response but no significant radiosensitisation in SQ20B_55 or BICR_55 (Figure 4E-F). This negative finding indicates that IGF-1R and EGFR are not radioresistance drivers.

Finally, we tested effects of IGF and/or EGFR blockade on basal cell survival, without IR. Here, afatinib caused significant inhibition of SQ20B_55 cell survival, with further inhibition upon afatinib/xentuzumab co-treatment ($p<0.01$). Compared with parental cells, the SQ20B_55 subline was significantly more sensitive to 100nM afatinib (mean survival 18.0 ± 1.4% vs 44.6 ± 5.9% in the parental cells, $p<0.001$). SQ20B_55 cells were also more sensitive than parental cells to afatinib:xentuzumab co-treatment (8.4 ± 0.6% vs 25.6 ± 2.5%, $p<0.05$). As before (Figure 3D), parental BICR18 cells were not inhibited by xentuzumab and afatinib alone or together (Figure 4G). The BICR_55 subline showed minor survival inhibition upon afatinib/xentuzumab co-treatment (74.9 ± 8.4% of control, $p<0.05$, Figure 4H), but the effect was minor compared with responses in SQ20B_55 cells.

Discussion

Our study was motivated by the need to inform treatment choice in early stage disease, and had three principal findings. Firstly, we identified a trend to higher IGF-1R content in pre-treatment biopsies of LSCCs that relapsed post-radiotherapy compared with those achieving long-term remission. While our study was in progress, Matsumoto and colleagues published results of IGF-1R IHC in early glottic LSCC, reporting local recurrence in 9/25 patients with high IGF-1R and 1/18 with low IGF-1R, and IGF-1R associated independently with adverse outcome (24). Together with our data, these results suggest that IGF-1R overexpression may be predictive of radioresistance, and supports assessment in larger cohorts, ideally prospectively. If IGF-1R is confirmed as a radioresistance biomarker, patients with high IGF-1R LSCCs could be offered TLM rather than radiotherapy.

Secondly, IGF-1R was significantly upregulated following radiotherapy. This effect was apparent when comparing biopsies of patients experiencing post-radiotherapy recurrence vs long-term remission (Figure 2B), in paired biopsies (Figure 2D), and in repeatedly-irradiated LSCC cells *in vitro* (Figure 4A). This could represent IR-induced upregulation, or selective survival of a pre-existing high IGF-1R subpopulation. We also found evidence of EGFR upregulation in paired biopsies (Figure 2E) and repeatedly-irradiated SQ20B cells (Figure 4A), suggesting that SQ20B_55 is a useful radioresistance model. Finally, while IGF-1R and EGFR appeared not to mediate radioresistance induced in LSCC cells by repeated irradiation, combined IGF:EGFR inhibition inhibited their basal survival more profoundly than inhibition of IGF or EGFR alone, and more effectively than in parental cells (Figure 4G). We infer that irradiation induced dependence on IGF and EGF signalling, which could favour tumour progression post-irradiation. Supporting the concept of enhanced growth factor dependence in treatment-resistant HNSCC, afatinib is reported to have superior activity compared with methotrexate following progression on platinum therapy (25). If our findings are confirmed in future reports, there may be merit in testing whether combined IGF:EGF blockade delays progression of LSCCs that recur following radiotherapy, or reduces risk of recurrence after salvage surgery.

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	Laryngeal Cancer Treatment Group, n (%)			
	LTR (n=23)	RT Failure (n=18)	Primary Laryngectomy (n=22)	P-Value (Chi-squared)
Sex				
Male	23(100)	16(88.9)	17(77.3)	0.053
Female	0(0)	2(11.1)	5(22.7)	
≥65	15(65.2)	9(50)	12(54.5)	0.59
<65	8(34.8)	9(50)	10(45.5)	
ASA				
1/2	21(91.3)	15(83.3)	22(100)	0.15
3/4	2(8.7)	3(16.7)	0(0)	
Smoking Status				
Never	8(34.8)	3(16.7)	2(9.1)	0.26
Current	9(39.1)	8(44.4)	13(59.1)	
Ex-smoker	6(26.1)	7(38.9)	7(31.8)	
Drinking Status				
Nil	12(52.2)	13(72.2)	10(45.5)	0.53
1-21 units/week	5(21.7)	1(5.6)	4(18.2)	
22-40 units/week	4(17.4)	2(11.1)	3(13.6)	
>40 units/week	2(8.7)	2(11.1)	5(22.7)	
T Classification				
T1/2	17(73.9)	14(77.8)	0(0)	0.001
T3/4	6(26.1)	4(22.2)	22(100)	
N Classification				
N0/1	22(95.7)	17(94.4)	14(63.6)	0.005
N2/3	1(4.3)	1(5.6)	8(36.4)	
M Classification				
M0	23(100)	18(100)	22(100)	n/a
M1	0(0)	0(0)	0(0)	
Radiotherapy				
55 Gy 20 Fr	19(82.6)	12(66.7)	n/a	
60-66Gy 30-33 Fr	4(17.4)	2(11.1)	n/a	0.80
Unknown	0(0)	4(22.2)	n/a	

Table 1: Patient Demographics and Tumour Characteristics by Primary Treatment

Abbreviations: ASA, American Society of Anaesthesiologists Performance Status; n/a, not applicable; RT, radiotherapy; Gy, Gray; Fr, fractions; LTR, long term remission.

Figure legends

Figure 1: Expression of IGF-1R and EGFR in LSCC. A. Flow diagram to show included tumour samples from which IHC scores were obtained. LTR, long term remission; RT, radiotherapy; 1°TL, primary total laryngectomy; STL, salvage total laryngectomy. Three IGF-1R-stained and 2 EGFR-stained samples were excluded because IHC was uninterpretable**. B**. LSCCs stained for EGFR and IGF-1R and scored for signal intensity: a) EGFR membrane 3, cytoplasm 1; b) EGFR membrane 3, cytoplasm 3; c) IGF-1R membrane 1, cytoplasm 1; d) IGF-1R membrane 3, cytoplasm3. Scale bar 20 µm. Analysis of associations between clinical parameters and membrane or cytoplasmic scores gave no additional information over that provided by total IGF-1R and EGFR. Therefore, subsequent analysis focuses on total EGFR and IGF-1R. **C**. Tumour IGF-1R and EGFR signal show weak positive correlation: r = 0.26 ± 0.038-0.46 (95% confidence intervals), p=0.022. **D**. Immunoreactive scores for: left, IGF-1R; right, EGFR in tumour biopsies from patients experiencing long-term remission (LTR) vs those undergoing primary laryngectomy (1^{o}L).

Figure 2: Increase in IGF-1R content of LSCCs that recur following radiotherapy. A. No correlation between content of IGF-1R (left) or EGFR (right) and time to recurrence in n=18 patients with LSCC experiencing recurrence post-radiotherapy. **B, C.** Comparison of B, IGF-1R and C, EGFR content in: left, diagnostic biopsies of LSCC patients treated with radiotherapy and achieving long-term remission (LTR) or post-irradiation relapse (radiotherapy failure, RF); right, diagnostic biopsies of LTR group vs biopsies of LSCCs that recurred post-irradiation. Graphs show mean IGF-1R score ± 95% confidence intervals. Compared with biopsies of patients in the LTR group, LSCCs that recurred post-irradiation contained significantly more IGF-1R (p=0.01 by Mann-Whitney U test) but not EGF. **D, E**. Graphs show IGF-1R (D) and EGFR (E) scores in the plasma membranes and cytoplasm, and the total score (sum of membrane and cytoplasm score) in paired primary and recurrent tumour biopsies from the same patients. When analysed by paired t-test, IGF-1R scores were found to be significantly higher in the

tumour biopsies taken post-radiotherapy recurrence. EGFR showed the same trend but differences were not significant.

Figure 3: EGFR-positive LSCC cells are radiosensitised by inhibition of EGF but not IGF signaling. A. SQ20B and BICR18 cells were treated with 50 nM IGF-1 or 20 nM EGF for 20 min and then lysed for analysis by western blot. Representative result from three independent sets of cell lysates. **B**. SQ20B (left) and BICR18 (right) cells were pretreated with solvent (control), afatinib or xentuzumab for 60 min and in the final 20 min with IGF-1 or EGF as in A. **C.** Cells were treated with solvent (control) or IGF-1 plus EGF, alone or with the combination of 100 nM afatinib and 100 nM xentuzumab, prior to lysis for western blotting. **D.** Graphs show mean ± SEM clonogenic survival expressed as % survival in control cells, to assess effects of afatinib and xentuzumab alone and in combination on left: SQ20B, right: BICR18 cells. Afatinib caused significant inhibition of SQ20B cell survival, with no additional inhibitory effect when combined with xentuzumab. **E, F.** SQ20B (E) and BICR18 cells (F) were treated with afatinib and/or xentuzumab and the following day were irradiated. Graphs show relative survival (mean ± SEM) under each condition, expressed as % survival in unirradiated dishes. Effects on radiosensitivity are shown in legends as SF_{50} (radiation dose required to suppress survival to 50% of control dishes) and dose-enhancement ratio (DER) at 2 Gy, calculated as the ratio of relative survival at 2 Gy of controls/relative survival at 2 Gy of treated cells. SQ20B cells were radiosensitised by afatinib but not xentuzumab. Neither drug had any significant effect on the radiosensitivity of BICR18 cells.

Figure 4: Repeatedly-irradiated LSCC cells upregulate IGF and EGF receptors and show increased IGF:EGF dependence for cell survival. A. Parental cells and repeatedly-irradiated sublines were treated with IGF-1 or EGF and analysed by western blot as in Figure 3A. **B.** Mean ± SEM of parental and repeatedly-irradiated cells, including data on the survival of relatively early (passage 14, P14) and later passage (P23, P24) to exclude passage number as a reason for any change in relative clonogenic survival. The repeatedly-irradiated cells of both sublines showed significant reduction in survival compared with parental cells. In subsequent assays SQ20B_55 cells were seeded at 10,000 cells/10 cm dish (increased from 2000) to allow sufficient survival to assess effects of IR. **C.** Graph shows

radiation survival (mean ± SEM as % survival of unirradiated cells) in SQ20B and SQ20B_55 cells, representative result from 4 independent assays. Legend shows SF_{50} values (with 95% confidence intervals) and DERs at 2 Gy, mean data from all 4 assays. SQ20B_55 cells were more radioresistant than parental cells (*p=0.01, **p=0.002). **D.** Radiation survival of BICR18 and BICR_55 cells, performed and analysed as C. Graph: representative result, showing that radioresistance was enhanced in BICR_55 cells only at 8 Gy (*p=0.007). Legend: pooled data from 4 independent assays indicate that there was little change in SF_{50} and DER. **E, F**. Radiation survival assays performed as in C, D in: E, SQ20B_55; F, BICR_55 cells pre-treated with 100 nM afatinib, 100 nM xentuzumab or the combination. There were minor changes in SF_{50} and DER values but no significant radiosensitisation in any condition. **G, H.** Survival of: G, SQ20B and SQ20B_55; H, BICR18 and BICR_55 treated with 100 nM afatinib, 100 nM xentuzumab or the combination in the absence of IR. The survival of BICR_55 cells was reduced by afatinib plus xentuzumab (***p<0.001), while SQ20B_55 cells were significantly more sensitive to afatinib and the combination of afatinib and xentuzumab than parental cells (*p<0.05, ***p<0.01, ***p<0.001).

Figure 1

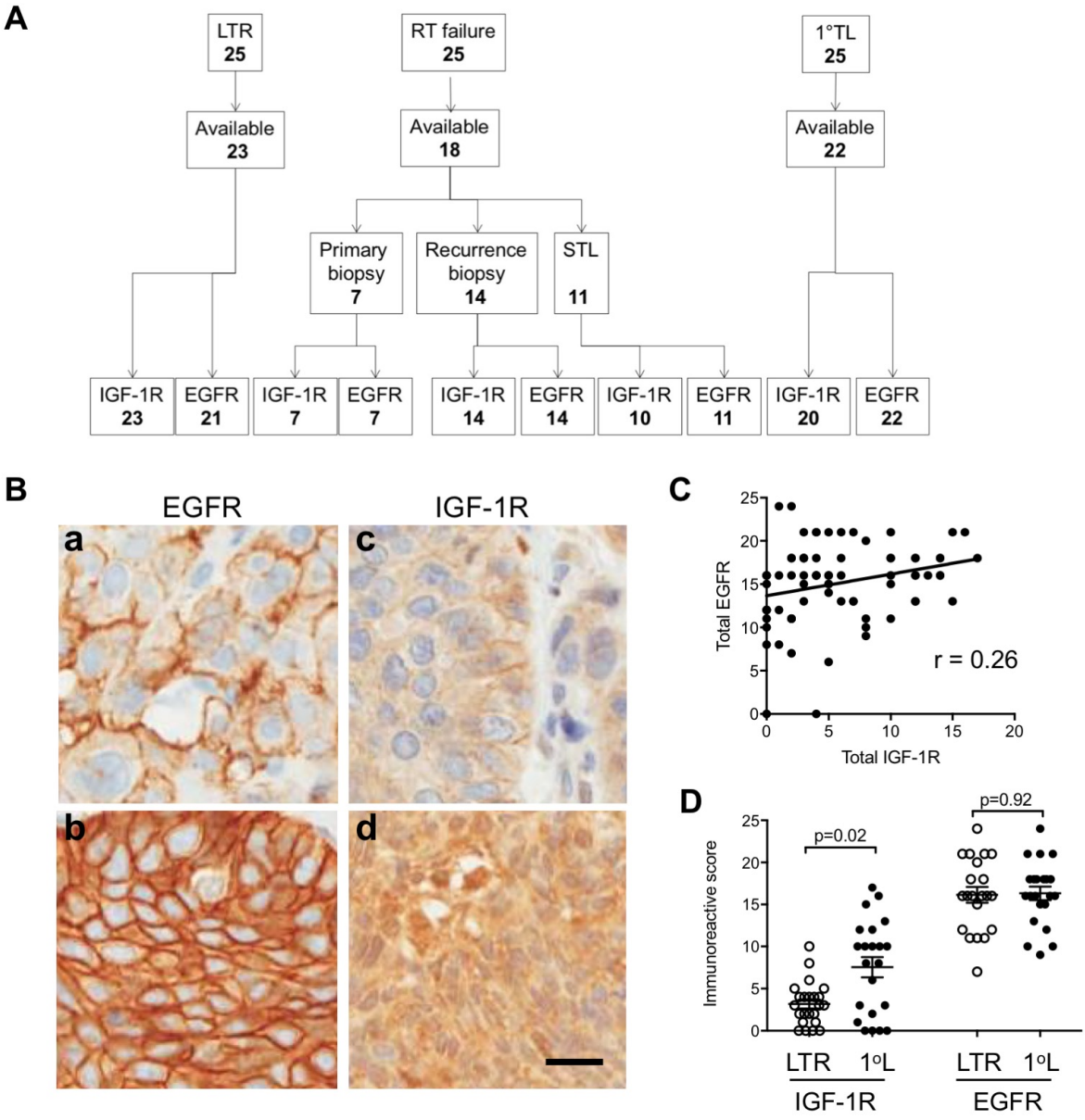

A
LTR 25
RT failure 25
1°TL 25
Available 23
Available 18
Available 22
Primary biopsy 7
Recurrence biopsy 14
STL 11
IGF-1R 23
EGFR 21
IGF-1R 7
EGFR 7
IGF-1R 14
EGFR 14
IGF-1R 10
EGFR 11
IGF-1R 20
EGFR 22
B
EGFR
IGF-1R
a
b
c
d
C
Total EGFR
Total IGF-1R
r = 0.26
D
Immunoreactive score
p=0.02
p=0.92
LTR
1°L
IGF-1R
EGFR

Figure 2

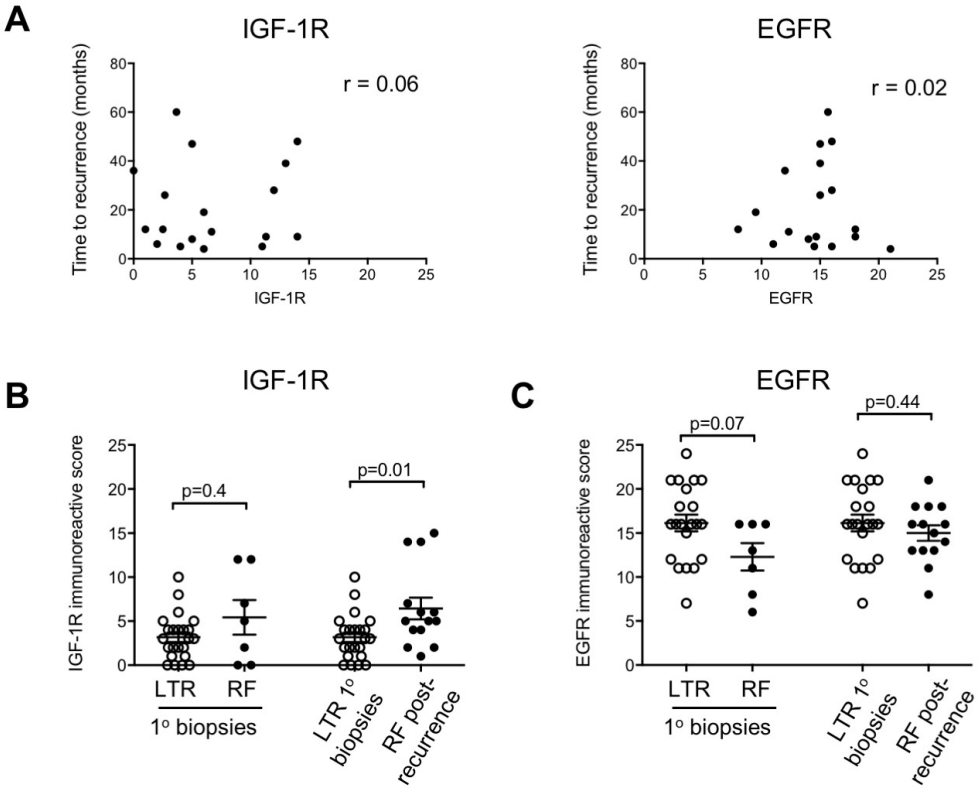
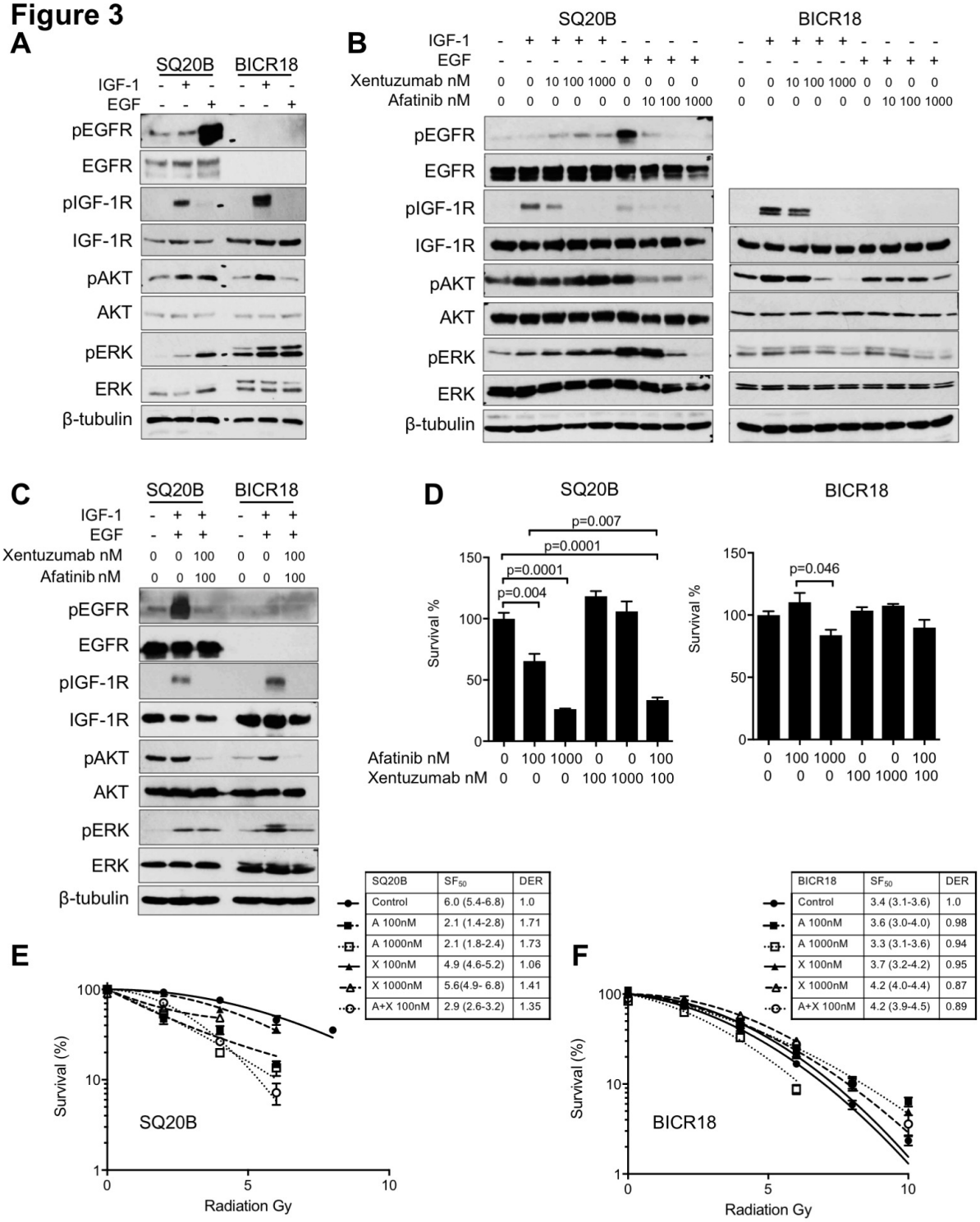

A
IGF-1R
r = 0.06
Time to recurrence (months)
IGF-1R
EGFR
r = 0.02
EGFR
B
IGF-1R
IGF-1R immunoreactive score
p=0.4
p=0.01
LTR
RF
1° biopsies
LTR 1° biopsies
RF post-recurrence
C
EGFR
EGFR immunoreactive score
p=0.07
p=0.44
D
IGF-1R
Membrane
Cytoplasm
Total
p=0.048
p=0.033
p=0.006
Pre
Post
E
EGFR
p=0.08
p=0.38
p=0.13

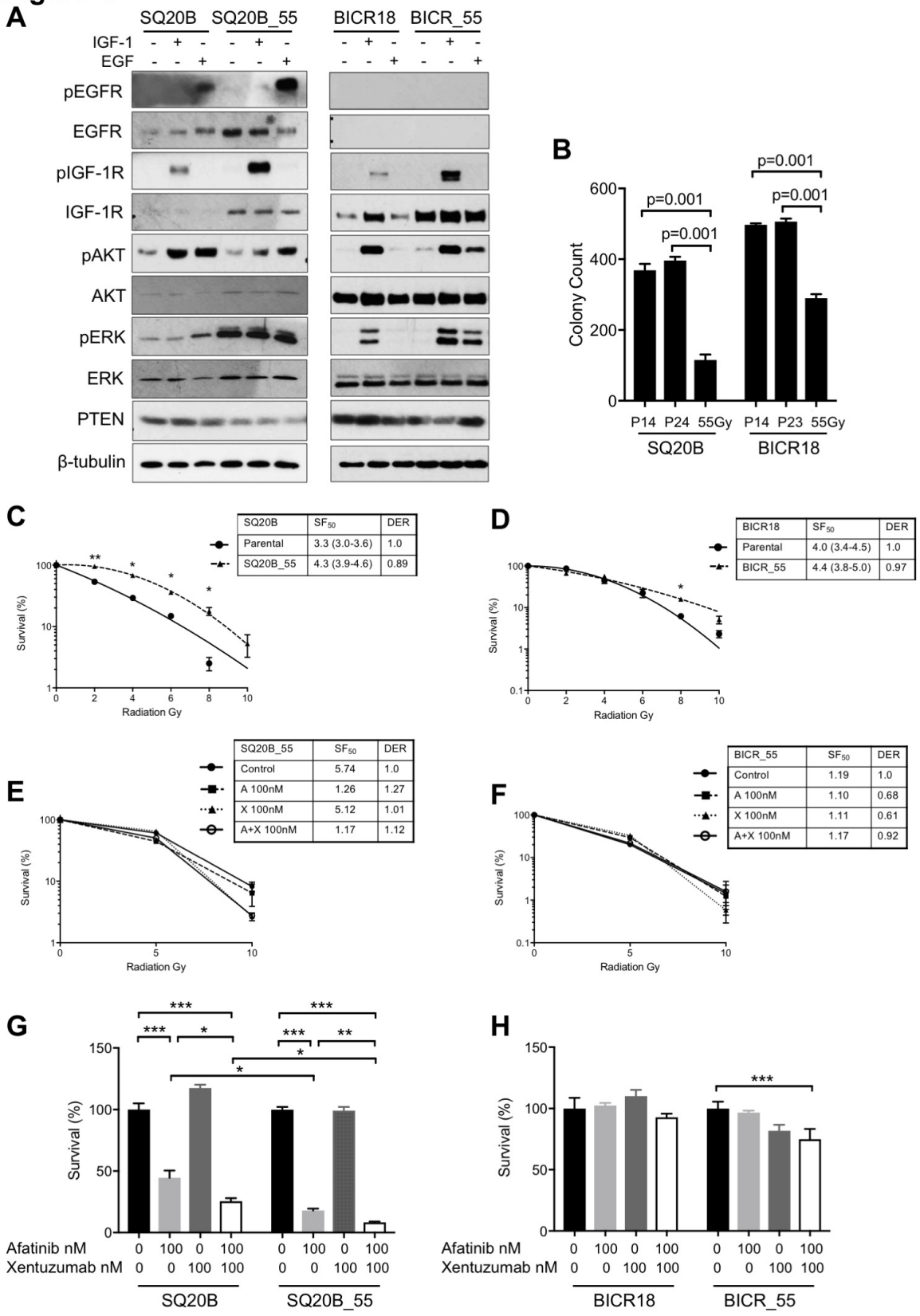
Figure 3



SQ20B	SF_{50}	DER
Control	6.0 (5.4-6.8)	1.0
A 100nM	2.1 (1.4-2.8)	1.71
A 1000nM	2.1 (1.8-2.4)	1.73
X 100nM	4.9 (4.6-5.2)	1.06
X 1000nM	5.6(4.9- 6.8)	1.41
A+X 100nM	2.9 (2.6-3.2)	1.35

BICR18	SF_{50}	DER
Control	3.4 (3.1-3.6)	1.0
A 100nM	3.6 (3.0-4.0)	0.98
A 1000nM	3.3 (3.1-3.6)	0.94
X 100nM	3.7 (3.2-4.2)	0.95
X 1000nM	4.2 (4.0-4.4)	0.87
A+X 100nM	4.2 (3.9-4.5)	0.89

Figure 4


A
SQ20B
SQ20B_55
BICR18
BICR_55
IGF-1
EGF
pEGFR
EGFR
pIGF-1R
IGF-1R
pAKT
AKT
pERK
ERK
PTEN
β-tubulin
B
Colony Count
p=0.001
p=0.001
p=0.001
p=0.001
P14 P24 55Gy
P14 P23 55Gy
SQ20B
BICR18
C
SQ20B | SF50 | DER
Parental | 3.3 (3.0-3.6) | 1.0
SQ20B_55 | 4.3 (3.9-4.6) | 0.89
Survival (%)
Radiation Gy
D
BICR18 | SF50 | DER
Parental | 4.0 (3.4-4.5) | 1.0
BICR_55 | 4.4 (3.8-5.0) | 0.97
Survival (%)
Radiation Gy
E
SQ20B_55 | SF50 | DER
Control | 5.74 | 1.0
A 100nM | 1.26 | 1.27
X 100nM | 5.12 | 1.01
A+X 100nM | 1.17 | 1.12
Survival (%)
Radiation Gy
F
BICR_55 | SF50 | DER
Control | 1.19 | 1.0
A 100nM | 1.10 | 0.68
X 100nM | 1.11 | 0.61
A+X 100nM | 1.17 | 0.92
Survival (%)
Radiation Gy
G
Survival (%)
Afatinib nM
Xentuzumab nM
SQ20B
SQ20B_55
H
Survival (%)
Afatinib nM
Xentuzumab nM
BICR18
BICR_55