

Running title: PI3K signalling as a therapeutic target in radioresistant OAC

PI3K inhibition as a novel therapeutic strategy for neoadjuvant chemoradiotherapy resistant oesophageal adenocarcinoma

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Full paper submission

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Abstract

Objectives: Neoadjuvant chemo-radiotherapy (neo-CRT) prior to surgery is the standard of care for oesophageal adenocarcinoma (OAC) patients. Unfortunately, most patients fail to respond to treatment. MiR-187 was previously shown to be downregulated in neo-CRT non-responders, whilst *in vitro* miR-187 overexpression enhanced radio-sensitivity and upregulated *PTEN*. This study evaluates the role of miR-187 and downstream PI3K signalling in radiation response in OAC.

Methods: The effect of miR-187 overexpression on downstream PI3K signalling was evaluated in OAC cell lines by qPCR and western blotting. *PTEN* expression was analysed in OAC pre-treatment biopsies of neo-CRT responders and non-responders. Pharmacological inhibition of PI3K using GDC-0941 was evaluated in combination with radiotherapy in 2D and 3D OAC models *in vitro* and as a single agent *in vivo*. Radiation response *in vitro* was assessed via clonogenic assay.

Results: *PTEN* expression was significantly decreased in neo-CRT non-responders. MiR-187 overexpression significantly upregulated *PTEN* expression and inhibited downstream PI3K signalling *in vitro*. GDC-0941 significantly reduced viability and enhanced radiation response *in vitro* and led to tumour growth inhibition as a single agent *in vivo*.

Conclusions: Targeting of PI3K signalling is a promising therapeutic strategy for OAC patients who have repressed miR-187 expression and do not respond to conventional neo-CRT.

Advances in knowledge: This is the first study evaluating the effect of PI3K inhibition on radio-sensitivity in OAC, with a particular focus on patients that do not respond to neo-CRT. We have shown for the 1st time that targeting of PI3K signalling is a promising alternative therapeutic strategy for OAC patients who do not respond to conventional neo-CRT.

Introduction

Oesophageal cancer has extremely poor prognosis and is the seventh most common and sixth most lethal cancer worldwide (1). Oesophageal adenocarcinoma (OAC) is the most prevalent histological subtype in western countries, with rapidly rising incidence rates (2)(3). Neoadjuvant chemoradiotherapy (neo-CRT) is becoming the standard of care for patients with locally advanced OAC (4). Patients achieving a pathological complete response (pCR) upon neo-CRT have been shown to have five-year survival rates up to 50% (5). Unfortunately, less than one third of OAC patients treated with neo-CRT achieve pCR (6). Therefore, identification of predictive therapy response biomarkers and novel therapeutic strategies is essential to improve patient prognosis.

MicroRNAs (miRNAs) are gene expression regulators, identified as predictors and modulators of treatment response in OAC (7). We have previously demonstrated that miR-31, miR-330-5p and miR-187 are significantly downregulated in pre-treatment tumour biopsies from OAC patients who are poor responders to neo-CRT (8-10). MiR-187 overexpression significantly enhanced sensitivity to radiotherapy and cisplatin *in vitro* and altered gene expression patterns, including *PTEN* (10). *PTEN* is a negative regulator of the phosphatidylinositol 3 kinase (PI3K)-protein kinase B (AKT) signalling pathway, whose hyperactivation is implicated cancer development and progression (11). Activation of PI3K-AKT signalling has been demonstrated in OAC (12), although the potential clinical relevance of PI3K inhibitors remains poorly understood.

GDC-0941 is a potent pan-PI3K inhibitor (13). Pre-clinical studies and phase I clinical trials have demonstrated that GDC-0941 promotes anti-tumorigenic effects and is suitable for treatment of solid tumours (13-16). GDC-0941 was also shown to enhance radio-sensitivity in

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glioblastoma multiforme *in vitro* and in thyroid carcinoma *in vivo* (17, 18). However, anti-tumorigenic properties of GDC-0941 have not previously been assessed in OAC.

In this study, we investigated the role of miR-187 and the PI3K axis in modulating response to neo-CRT. We showed that *PTEN* expression is downregulated in OAC patients who are poor responders to neo-CRT, and that miR-187 overexpression increases *PTEN* expression and downregulates p-AKT levels in OAC cells. We then targeted PI3K signalling in OAC cell lines using GDC-0941, which led to decreased cell survival, increased radio-sensitivity *in vitro*, and inhibition of *in vivo* tumour growth for the first time in OAC. Taken together, we are demonstrating for the 1st time that targeting PI3K in OAC is a promising therapeutic strategy in OAC and could improve outcome of patients who have repressed miR-187 expression and do not respond to conventional neo-CRT.

Methods and Materials

Cell culture, transfections, drug treatments, and irradiation treatment

OAC OE33, OE19 and SK-GT-4 cell lines were purchased from ECACC (UK). Cells were incubated at 37°C, 95% humidified air and 5% CO₂. Spheroids were generated by seeding 2.5x10⁴ cells per well in ultra-low adherence round-bottomed 96-well plates (Corning). Media was replaced every 2 days. Spheroids were imaged using the GelCount instrument (Oxford Optronix), and spheroid size determined as previously reported (19, 20).

Ambion pre-miR miRNA precursor molecules (Thermo Fisher Scientific, UK) were used for transient miR-187 overexpression. GDC-0941 stock solutions (Abcam, UK; Selleckchem, UK) were prepared in DMSO (dimethyl sulfoxide). X-ray irradiation was carried out using a RS-2000 biological irradiator (Rad Source Technologies, Georgia, USA) at a 1.87 Gy/min dose rate, calibrated to National Physics Laboratory (NPL) standards (21).

Patient treatment, tissue collection, and histology

Following ethical approval (Joint St James's Hospital/AMNCH ethical review board, Reference ID 2011/27/01) and written informed consent, diagnostic biopsy tumour specimens were taken from patients with a diagnosis of operable OAC, prior to neo-CRT. All patients received a complete course of neo-CRT, consisting of two courses of 5-fluorouracil (5-FU) and cisplatin, plus 40.05 Gy in 15 daily fractions (2.67 Gy/fraction) over 3 weeks as previously described (22). Diagnostic endoscopic biopsies were obtained prior to neo-CRT. Immediately adjacent tissue was taken for histologic confirmation, which was performed using routine Haematoxylin and Eosin staining. Specimens were immediately placed in RNAlater (Ambion) and refrigerated for 24 h, before removal of RNA later and storage at -80°C.

All specimens were assessed by an experienced pathologist. Tumour response to treatment was assigned 1 of 5 tumour regression grades (TRG) as previously described (23). Good responders were classified as achieving a TRG of 1 or 2, whilst poor responders were classified as having a TRG of 3, 4 or 5, as previously described (9). For the purposes of this study patients with a TRG 3 were excluded.

In vivo studies

Xenograft models were generated through the subcutaneous implant of 5×10^6 OE33 cells into the right-hand side of the middorsal region of the back of 16 x 52-58 day old female CB17.Cg-Prkdc^{scid}Lyst^{bg-J} SCID- Bg mice (sourced from Charles River UK). Animals were anaesthetised using 2% isoflurane, and a small area of fur shaved around the implant site prior to implant. The cell suspension was prepared in a 1:1 ratio of serum free RPMI medium and Cultrex BME (24), prior to implant of 0.1 mL of the 5×10^7 cells/ mL suspension. Tumour measurements were taken three times weekly with callipers and animals randomly assigned to control and treatment groups when tumour volume reached approximately 300-350mm³ (14, 25, 26). Three animals developed tumours at a later date and were not included in the analysis of tumour growth. Twice daily treatments of GDC-0941 or vehicle control (0.1 mL/ 10g) were administered via oral gavage at a concentration of 50 mg/kg for 8 days, as previously reported (14, 25, 26). Health status and weight of each animal were monitored throughout the experiment. Animals were sacrificed via a schedule 1 method (dislocation of the neck), and tumours were excised and snap frozen. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 and UKCCCR Guidelines 2010 using protocols approved by the University of Hull Animal Welfare and Ethical Review Body (AWERB) under Home Office Project Licence 60/4549. The study was powered using effect sizes and tumour

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growth variability seen in previous experiments (14, 25, 26) using G*Power software (27).

Tumour growth over the entire course of treatment was compared between groups.

RT-qPCR

Xenograft tumour tissue samples and patient tumour biopsies RNA was isolated using an All-in-One purification kit (Norgen Biotek). Aurum Total RNA Mini Kit (Biorad, UK) was used for RNA extraction from cell lines.

For miRNA expression analysis, 10 ng total RNA was first reverse transcribed to cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, UK). TaqMan microRNA assays and TaqMan 2X Universal PCR Master Mix (Applied Biosystems, UK) were used, with RNU48 used as the endogenous control. For mRNA analysis, 1 µg total RNA was reversed transcribed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, UK). QuantiNova SYBR Green PCR Kit and QuantiTECT primer assays (Qiagen, UK) were used, with *B2M* or *18S* used as the endogenous control. RT-qPCR was performed using the Step One Plus Real Time PCR System (Applied Bioscience, USA) and data was analysed using the $2^{-\Delta\Delta Ct}$ method (28). Primer details available in Supplementary Table 1.

Clonogenic assay

Cells were plated at a density of 500-5000 cells/well in 6-well plastic plates. Colonies were fixed and stained by crystal violet staining solution (0.1% w/v crystal violet, 70% v/v methanol, 30% v/v dH₂O) after 7-10 days post treatment. Colonies were counted using a Gel Count system (Oxford Optronics, UK).

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Cell viability assay

Short term cell viability was assessed using the Cell Titer 96 AQueous One Solution MTS cell proliferation assay (Promega, UK), as previously described (20).

Immunoblotting

Cells lysates were prepared in UTB as previously reported (29). Tumour samples were prepared for lysis using a BioPulverizer and Cryo-cup grinder (BioSpec, USA) and lysed in RIPA buffer (Cell Signalling Technology, USA) supplemented with protease (Mini, EDTA-free Protease Inhibitor Cocktail, Roche, UK) and phosphatase (PhosSTOP, Roche, UK) inhibitors. Western blotting was performed as previously described (29). Densitometric analysis of band intensity of blots was carried out using Image J (NIH, USA).

Statistical analysis

Statistical analysis was carried out using the GraphPad PRISM (GraphPad software Inc, California, USA) and the data presented represent the mean +/- the standard error of the mean (SEM). Statistical analysis was carried out using an unpaired Student's T-test or a 2-way ANOVA. Significance was assumed if $p < 0.05$.

Detailed materials and methods are described in the Supplementary Materials.

Results

PTEN expression is associated with CRT response in OAC patients

We have previously shown that miR-187 is downregulated in poor responder patients in OAC and can impact on gene expression for survival pathways (10). Here, we confirmed that miR-187 overexpression resulted in significant upregulation of *PTEN* mRNA expression in a OAC cell line panel compared to non-transfected controls (Figure 1A, Supplementary Figure 1A), linking miR-187 levels with *PTEN* expression. As a search of the miR-code database (30) did not reveal a miR-187 binding site in the *PTEN* promotor, *PTEN* regulatory co-factors were identified using the UCSC genome browser, and those with miR-187 seed sites identified using the miR-code database (Supplementary Table 3). For the most promising negative regulators *EP300* or *DNMT1*, expression was not affected by miR-187 overexpression, while *KDMB5* expression was significantly increased in the OE33 and SK-GT-4 cell lines (Supplementary Figure 2), indicating that other factors must be responsible for miR-187-mediated *PTEN* modulation. The effect of miR-187 overexpression on downstream PI3K-AKT signalling was then assessed through analysis of phosphorylated AKT levels (Serine 473). MiR-187 overexpression resulted in significant reduction of pAKT levels compared to the non-transfected controls (Figure 1B, Supplementary Figure 3). Finally, we observed *PTEN* expression was significantly downregulated in the pre-treatment tumour biopsies of poor responders to neo-CRT compared to the responder group (Figure 1C). These data suggest that PTEN/PI3K-AKT signalling correlates with miR-187 expression *in vitro* and is associated with radiation response efficacy in OAC patients.

PI3K inhibition efficiently reduces OAC cell survival *in vitro* and *in vivo* tumour growth

We hypothesised that pharmacological inhibition of PI3K may provide an alternative novel therapeutic strategy for OAC in patients with repressed miR-187 expression who do not respond to neo-CRT. Clinically relevant PI3K inhibitor GDC-0941 was selected for further investigation due to the high efficacy shown in pre-clinical studies and phase I clinical trials for solid tumours (13-15, 25, 26, 31). GDC-0941 treatment resulted in PI3K inhibition as indicated via a reduction (lower doses) or abrogation (higher doses) in phospho-AKT in the OAC cell line panel (Figure 2A). Treatment with GDC-0941 significantly reduced both short-term viability (Figure 2B) and long-term clonogenic survival (Figure 2C) for the OAC cell line panel. These data confirm that GDC-0941 effectively reduces cell survival as a single agent *in vitro*.

Pre-clinical efficacy was then assessed *in vivo* using OE33 tumour xenograft models. Significant reduction in tumour growth was observed in mice treated with GDC-0941 (50 mg/kg) compared to those treated with the vehicle control (Figure 3A). pAKT levels were reduced in the GDC-0941 treated group compared to those treated with the vehicle control, indicating that PI3K signalling was successfully inhibited (Figure 3B). This is the first pre-clinical study to evaluate its efficacy for the treatment of OAC.

PI3K inhibition enhances radiosensitivity in OAC *in vitro*

Treatment with GDC-0941 significantly reduced long term clonogenic survival of OAC cell lines both as a single agent and in combination with irradiation as compared to the vehicle control, through an additive effect (Figure 4A, Supplementary Figure 4). Finally, treatment of all three OAC cell lines with GDC-0941 significantly reduced OAC spheroid volume, which was exacerbated in combination with ionising radiation (Figure 4B-D). These data suggest that

PI3K inhibition through treatment with GDC-0941 significantly reduces the long-term clonogenic survival of OAC cell lines as a single agent and in combination with physiologically relevant levels of ionising radiation.

Discussion

In the present study, miR-187 overexpression was shown to induce upregulation of the tumour suppressor gene *PTEN* which resulted in subsequent inhibition of downstream PI3K-AKT signalling in OAC cell lines. *PTEN* expression levels were also found to be significantly downregulated in the pre-treatment biopsy specimens of OAC patients displaying a poor response to neo-CRT (Figure 1). Our findings are supported by the findings of Saeed and colleagues, where AKT expression levels were increased in the pre-treatment tumour biopsies of OAC patients identified as partial or non-responders to neo-CRT compared to those with a pCR (32). AKT expression was also shown to significantly correlate to the degree of pathologic response (32). As *PTEN* is a well-established negative regulator of the PI3K-AKT signalling pathway, our current and previous findings, alongside those published by Saeed and colleagues (32), indicate that miR-187, *PTEN*, and downstream PI3K-AKT signalling are implicated in modulating neo-CRT response in OAC. It is well established that the PI3K-AKT signalling pathway becomes upregulated in response to radiotherapy and is implicated in radiation resistance (33). This suggests that decreased *PTEN* and/or miR-187 expression could be used as predictive biomarkers of radioresistance in OAC though the analysis of pre-treatment biopsies. Finally, manipulation of PI3K may therefore provide alternative therapeutic strategies for tackling radiation resistance in OAC.

We hypothesised that a negative *PTEN* transcriptional regulator harbouring a miR-187 binding site is responsible for miR-187 modulation of *PTEN* in OAC, as *PTEN* does not appear to be a direct target for miR-187. Unfortunately, candidate regulators *EP300*, *KDM5B* and *DMNT1* were also not affected by miR-187 overexpression (Supplementary Figure 2), indicating that other intermediate factors might be involved. Of particular interest from our *in silico* analysis (Supplementary Table 3), both *NFIC* (Nuclear Factor I C) and *PHF8* (PHD Finger Protein 8) were identified as potential miR-187 targets with an impact on *PTEN* expression through a yet unknown regulation mechanism, and have been shown to be overexpressed in oesophageal squamous cell carcinoma (34, 35), which will be evaluated in future studies.

Inhibition of PI3K signalling using the clinically relevant agent GDC-0941 significantly decreased cellular survival and enhanced radiation response in 2D and 3D models *in vitro*, as well as leading to tumour growth inhibition as a single agent in OAC xenograft models (Figures 2-4). Previous studies had shown that OAC and OSCC cell lines are sensitive to the previous generation PI3K inhibitors Wortmannin and LY294002, leading to inhibition of tumour growth chemoresistance (36-39). Unlike the previous generation PI3K inhibitors, the PI3K inhibitor GDC-0941 used in this study is a highly selective inhibitor that targets all four isoforms of class I PI3Ks, the effects of which have not previously been assessed in oesophageal cancer (13). Importantly, this is the first study to demonstrate the efficacy of GDC-0941 for the treatment of OAC, either as a single agent or in combination with radiotherapy. These findings support those of previous pre-clinical studies and clinical trials indicating that GDC-0941 is suitable for the treatment of solid tumours (13, 15, 16). PI3K inhibition has been shown to modulate radio-sensitivity in a range of malignancies including lung, prostate and thyroid carcinoma (40, 41), although this is the first study evaluating the effect of PI3K inhibition on radio-

sensitivity in OAC. Our study indicates, for the first time, that PI3K inhibition may enhance radiotherapy response in OAC. However, as PI3K is involved in normal physiological signalling pathways, potential associated normal toxicities and the previously described narrow therapeutic window has impaired the clinical success for these inhibitors as single agents (42). Therefore, careful consideration needs to be taken when designing dosing schedules for future clinical trials, if we are to see clinical benefit if using PI3K inhibitors for OAC patients.

Conclusions

We have shown that PTEN and PI3K signalling are implicated in modulating tumour response to neo-CRT in OAC patients. We have also demonstrated that the clinically relevant PI3K inhibitor GDC-0941 significantly decreased cellular survival and enhanced the efficacy of radiation treatment in OAC cell lines *in vitro*, as well as leading to inhibiting tumour growth as a single agent in *in vivo* xenograft models. Importantly, the preliminary data presented here demonstrate that PI3K inhibitors such as GDC-0941 may provide an alternative therapeutic strategy for the treatment of OAC, either as a single agent or in combination with neo-CRT.

Figure Legends

Figure 1 - PTEN expression is associated with CRT response in OAC patients.

A) OE33, OE19 and SK-GT-4 cells were transfected with pre-miR-187 precursor molecules and exposed to 2 Gy irradiation. PTEN fold change was analyzed by qPCR and expression calculated relative to the non-transfected control (NONT). Histogram represents an average of n=3 experimental repeats. B) OE33 cells were transfected with pre-miR-187 precursor molecules and tAKT/pAKT protein levels assessed by western blot. Western blot and densitometry shown is representative of n=3 repeats. C) PTEN expression was assessed in the pre-treatment diagnostic biopsy specimens of OAC patients (n=31) by qPCR. Patients were classified as neo-CRT responders (TRG 1 and 2, n=15) and non-responders (TRG 4 and 5, n=16). Error bars represent mean \pm SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Figure 2 –PI3K inhibition significantly reduces OAC proliferation and survival *in vitro*.

A) Cells were treated with GDC-0941 or vehicle control for 18 h and tAKT/ pAKT protein levels assessed by western blot. Western blot is representative of n=3 repeats. B) Cells were treated with GDC-0941 at a top concentration of 20 μ M and an 8-point dose response curve generated. The MTS reagent was added to the plates 48 h post treatment and absorbance measured at 490 nm 4 h later. Cell viability was calculated relative to the corresponding vehicle control for each dose (n=3). C) Cells were treated with GDC-0941 or vehicle control and the effect on long term survival assessed using the clonogenic assay. Data represent the average of n=3 experimental repeats. Error bars represent mean \pm SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Figure 3 – GDC-0941 treatment significantly decreases OAC tumour growth *in vivo*.

A) SCID-Beige female mice bearing OE33 tumour xenografts were treated twice daily with GDC-0941 (50 mg/kg) or vehicle control via oral gavage for 8 days. Data represents the average mean tumour volume of n=3 (treated) or n=3 (vehicle) animals per treatment group. B) Animals were sacrificed 1 hour after the final treatment dose and tAKT/ pAKT protein levels assessed by western blot. Western blot is representative of n=3 repeats. Error bars represent mean \pm SEM. *p < 0.05

Figure 4 - PI3K inhibition enhances radiotherapy treatment in 2D and 3D OAC models.

A) Cells were treated with GDC-0941 or vehicle control for 18 hours and exposed to 2 Gy irradiation. The effect of GDC-0941 as a single agent and in combination with 2 Gy irradiation was assessed using the clonogenic assay. The surviving fraction was calculated relative to the 0 Gy DMSO control. B-D) OE33, OE19, and SK-GT-4 spheroid models were generated as noted in Materials and Methods and treated with 1 μ M GDC-0941 or DMSO for 21 days in combination with a total of 5 x 2 Gy irradiation fractions or mock irradiated for the first 5 days. Spheroids were imaged using the GelCount instrument (Oxford Optronix), and spheroid size determined using ImageJ (NIH). Spheroid area is shown relative to day 0. Error bars represent mean \pm SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. E) Schematic of the effect of GDC-0941 treatment of the PI3K-AKT signalling pathway.

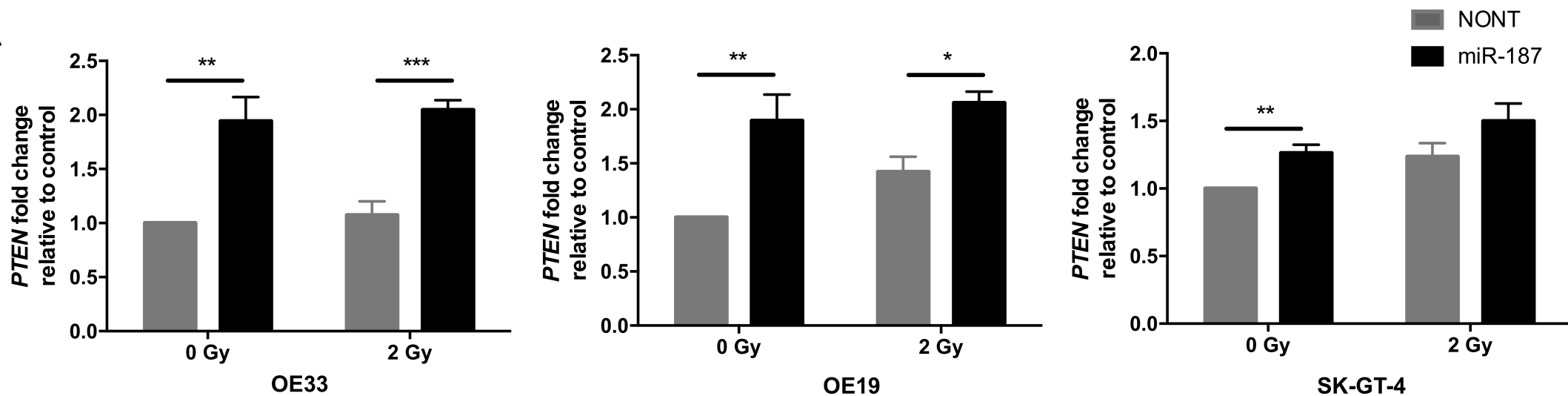
References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018;68(6):394-424.
2. Arnold M, Soerjomataram I, Ferlay J, Forman D. Global incidence of oesophageal cancer by histological subtype in 2012. *Gut.* 2015;64(3):381-7.
3. Arnold M, Laversanne M, Brown LM, Devesa SS, Bray F. Predicting the Future Burden of Esophageal Cancer by Histological Subtype: International Trends in Incidence up to 2030. *Am J Gastroenterol.* 2017;112(8):1247-55.
4. Rashid N, Elshaer M, Kosmin M, Riaz A. Current management of oesophageal cancer. *BJMP.* 2015;8(1):a804.
5. Reynolds JV, Muldoon C, Hollywood D, Ravi N, Rowley S, O'Byrne K, et al. Long-term outcomes following neoadjuvant chemoradiotherapy for esophageal cancer. *Ann Surg.* 2007;245(5):707-16.
6. van Hagen P, Hulshof MC, van Lanschot JJ, Steyerberg EW, van Berge Henegouwen MI, Wijnhoven BP, et al. Preoperative chemoradiotherapy for esophageal or junctional cancer. *N Engl J Med.* 2012;366(22):2074-84.
7. Skinner HD, Lee JH, Bhutani MS, Weston B, Hofstetter W, Komaki R, et al. A validated miRNA profile predicts response to therapy in esophageal adenocarcinoma. *Cancer.* 2014;120(23):3635-41.
8. Bibby BA, Reynolds JV, Maher SG. MicroRNA-330-5p as a Putative Modulator of Neoadjuvant Chemoradiotherapy Sensitivity in Oesophageal Adenocarcinoma. *PLoS One.* 2015;10(7):e0134180.
9. Lynam-Lennon N, Reynolds JV, Marignol L, Sheils OM, Pidgeon GP, Maher SG. MicroRNA-31 modulates tumour sensitivity to radiation in oesophageal adenocarcinoma. *J Mol Med (Berl).* 2012;90(12):1449-58.
10. Lynam-Lennon N, Bibby BA, Mongan AM, Marignol L, Paxton CN, Geiersbach K, et al. Low miR-187 expression promotes resistance to chemoradiation therapy in vitro and correlates with treatment failure in patients with esophageal adenocarcinoma. *Mol Med.* 2016;22.
11. Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, et al. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell.* 1998;95(1):29-39.
12. Kebenko M, Drenckhan A, Gros SJ, Jucker M, Grabinski N, Ewald F, et al. ErbB2 signaling activates the Hedgehog pathway via PI3K-Akt in human esophageal adenocarcinoma: identification of novel targets for concerted therapy concepts. *Cell Signal.* 2015;27(2):373-81.
13. Raynaud FI, Eccles SA, Patel S, Alix S, Box G, Chuckowree I, et al. Biological properties of potent inhibitors of class I phosphatidylinositide 3-kinases: from PI-103 through PI-540, PI-620 to the oral agent GDC-0941. *Mol Cancer Ther.* 2009;8(7):1725-38.
14. Burrows N, Babur M, Resch J, Ridsdale S, Mejin M, Rowling EJ, et al. GDC-0941 inhibits metastatic characteristics of thyroid carcinomas by targeting both the phosphoinositide-3 kinase (PI3K) and hypoxia-inducible factor-1alpha (HIF-1alpha) pathways. *J Clin Endocrinol Metab.* 2011;96(12):E1934-43.
15. Sarker D, Ang JE, Baird R, Kristeleit R, Shah K, Moreno V, et al. First-in-human phase I study of pictilisib (GDC-0941), a potent pan-class I phosphatidylinositol-3-kinase (PI3K) inhibitor, in patients with advanced solid tumors. *Clin Cancer Res.* 2015;21(1):77-86.
16. Schoffski P, Cresta S, Mayer IA, Wildiers H, Damian S, Gendreau S, et al. A phase Ib study of pictilisib (GDC-0941) in combination with paclitaxel, with and without bevacizumab or trastuzumab, and with letrozole in advanced breast cancer. *Breast Cancer Res.* 2018;20(1):109.
17. Shi F, Guo H, Zhang R, Liu H, Wu L, Wu Q, et al. The PI3K inhibitor GDC-0941 enhances radiosensitization and reduces chemoresistance to temozolomide in GBM cell lines. *Neuroscience.* 2017;346:298-308.
18. Burrows N, Williams J, Telfer BA, Resch J, Valentine HR, Fitzmaurice RJ, et al. Phosphatidylinositide 3-kinase (PI3K) and PI3K-related kinase (PIKK) activity contributes to radioresistance in thyroid carcinomas. *Oncotarget.* 2016;7(39):63106-23.

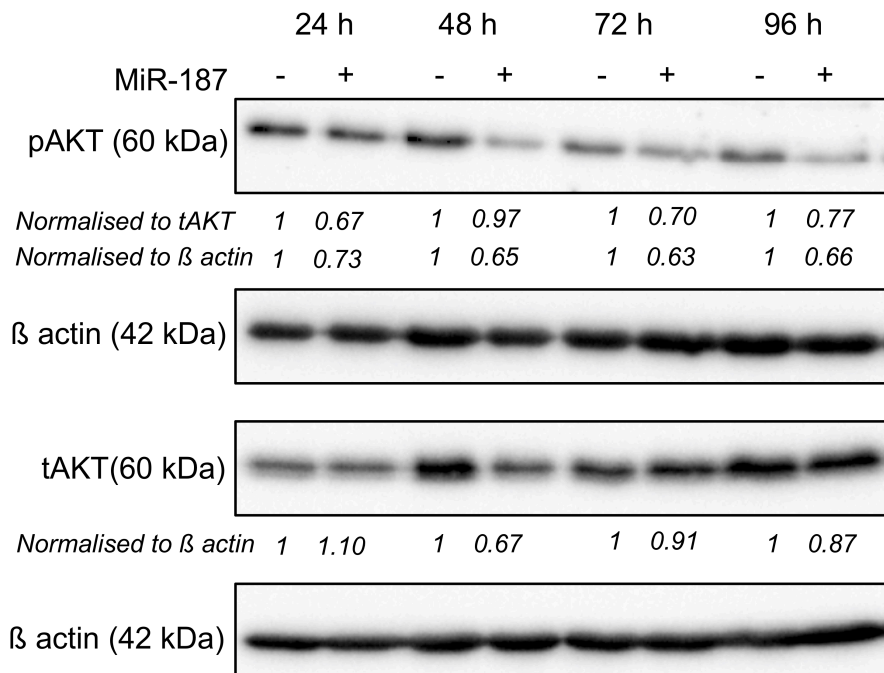
19. Pires IM, Olcina MM, Anbalagan S, Pollard JR, Reaper PM, Charlton PA, et al. Targeting radiation-resistant hypoxic tumour cells through ATR inhibition. *Br J Cancer*. 2012;107(2):291-9.
20. Beeby E, Magalhaes M, Pocas J, Collins T, Lemos MFL, Barros L, et al. Secondary metabolites (essential oils) from sand-dune plants induce cytotoxic effects in cancer cells. *J Ethnopharmacol*. 2020;258:112803.
21. Moore CS, Wood TJ, Cawthorne C, Hilton KL, Maher S, Saunderson JR, et al. A method to calibrate the RS 2000 x-ray biological irradiator for radiobiological flank irradiation of mice. *Biomedical Physics & Engineering Express*. 2016;2(3).
22. Maher SG, Gillham CM, Duggan SP, Smyth PC, Miller N, Muldoon C, et al. Gene expression analysis of diagnostic biopsies predicts pathological response to neoadjuvant chemoradiotherapy of esophageal cancer. *Ann Surg*. 2009;250(5):729-37.
23. Mandard AM, Dalibard F, Mandard JC, Marnay J, Henry-Amar M, Petiot JF, et al. Pathologic assessment of tumor regression after preoperative chemoradiotherapy of esophageal carcinoma. Clinicopathologic correlations. *Cancer*. 1994;73(11):2680-6.
24. Fridman R, Benton G, Aranoutova I, Kleinman HK, Bonfil RD. Increased initiation and growth of tumor cell lines, cancer stem cells and biopsy material in mice using basement membrane matrix protein (Cultrex or Matrigel) co-injection. *Nat Protoc*. 2012;7(6):1138-44.
25. Burrows N, Telfer B, Brabant G, Williams KJ. Inhibiting the phosphatidylinositide 3-kinase pathway blocks radiation-induced metastasis associated with Rho-GTPase and Hypoxia-inducible factor-1 activity. *Radiother Oncol*. 2013;108(3):548-53.
26. Cawthorne C, Burrows N, Gieling RG, Morrow CJ, Forster D, Gregory J, et al. [18F]-FLT positron emission tomography can be used to image the response of sensitive tumors to PI3-kinase inhibition with the novel agent GDC-0941. *Mol Cancer Ther*. 2013;12(5):819-28.
27. Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods*. 2007;39(2):175-91.
28. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods*. 2001;25(4):402-8.
29. Poujade FA, Mannion A, Brittain N, Theodosi A, Beeby E, Leszczynska KB, et al. WSB-1 regulates the metastatic potential of hormone receptor negative breast cancer. *Br J Cancer*. 2018;118(9):1229-37.
30. Jeggari A, Marks DS, Larsson E. miRcode: a map of putative microRNA target sites in the long non-coding transcriptome. *Bioinformatics*. 2012;28(15):2062-3.
31. O'Brien C, Wallin JJ, Sampath D, GuhaThakurta D, Savage H, Punnoose EA, et al. Predictive biomarkers of sensitivity to the phosphatidylinositol 3' kinase inhibitor GDC-0941 in breast cancer preclinical models. *Clin Cancer Res*. 2010;16(14):3670-83.
32. Saeed N, Shridhar R, Hoffe S, Almhanna K, Meredith KL. AKT expression is associated with degree of pathologic response in adenocarcinoma of the esophagus treated with neoadjuvant therapy. *J Gastrointest Oncol*. 2016;7(2):158-65.
33. Burris HA, 3rd. Overcoming acquired resistance to anticancer therapy: focus on the PI3K/AKT/mTOR pathway. *Cancer Chemother Pharmacol*. 2013;71(4):829-42.
34. Wang H, Shi X, Wu S. miR-550a-3/NFIC plays a driving role in esophageal squamous cell cancer cells proliferation and metastasis partly through EMT process. *Mol Cell Biochem*. 2020;472(1-2):115-23.
35. Sun X, Qiu JJ, Zhu S, Cao B, Sun L, Li S, et al. Oncogenic features of PHF8 histone demethylase in esophageal squamous cell carcinoma. *PLoS One*. 2013;8(10):e77353.
36. Li B, Cheung PY, Wang X, Tsao SW, Ling MT, Wong YC, et al. Id-1 activation of PI3K/Akt/NFkappaB signaling pathway and its significance in promoting survival of esophageal cancer cells. *Carcinogenesis*. 2007;28(11):2313-20.
37. Li B, Li J, Xu WW, Guan XY, Qin YR, Zhang LY, et al. Suppression of esophageal tumor growth and chemoresistance by directly targeting the PI3K/AKT pathway. *Oncotarget*. 2014;5(22):11576-87.

38. Li B, Tsao SW, Li YY, Wang X, Ling MT, Wong YC, et al. Id-1 promotes tumorigenicity and metastasis of human esophageal cancer cells through activation of PI3K/AKT signaling pathway. *Int J Cancer*. 2009;125(11):2576-85.
39. Pal J, Fulciniti M, Nanjappa P, Buon L, Tai YT, Tassone P, et al. Targeting PI3K and RAD51 in Barrett's adenocarcinoma: impact on DNA damage checkpoints, expression profile and tumor growth. *Cancer Genomics Proteomics*. 2012;9(2):55-66.
40. Schuurbiers OC, Kaanders JH, van der Heijden HF, Dekhuijzen RP, Oyen WJ, Bussink J. The PI3K/AKT-pathway and radiation resistance mechanisms in non-small cell lung cancer. *J Thorac Oncol*. 2009;4(6):761-7.
41. Chang L, Graham PH, Hao J, Ni J, Bucci J, Cozzi PJ, et al. PI3K/Akt/mTOR pathway inhibitors enhance radiosensitivity in radioresistant prostate cancer cells through inducing apoptosis, reducing autophagy, suppressing NHEJ and HR repair pathways. *Cell Death Dis*. 2014;5:e1437.
42. Bauer TM, Patel MR, Infante JR. Targeting PI3 kinase in cancer. *Pharmacol Ther*. 2015;146:53-60.

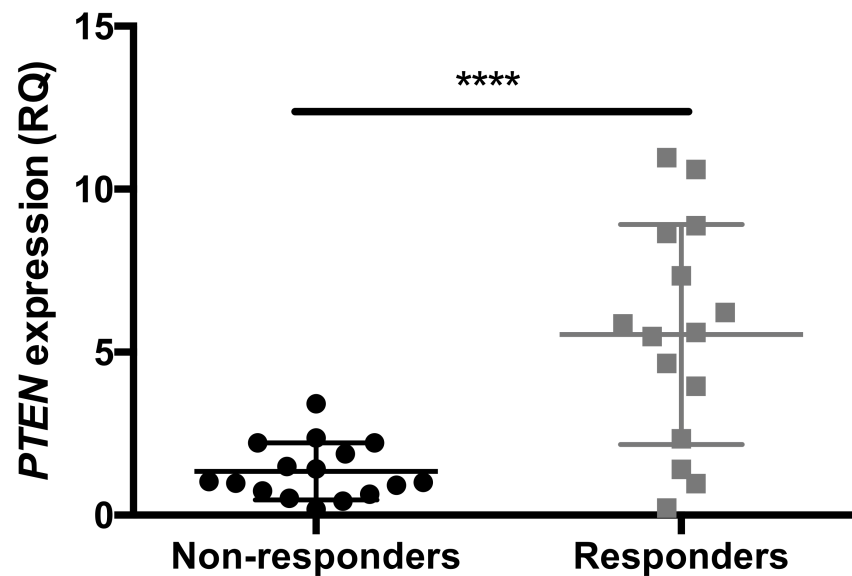
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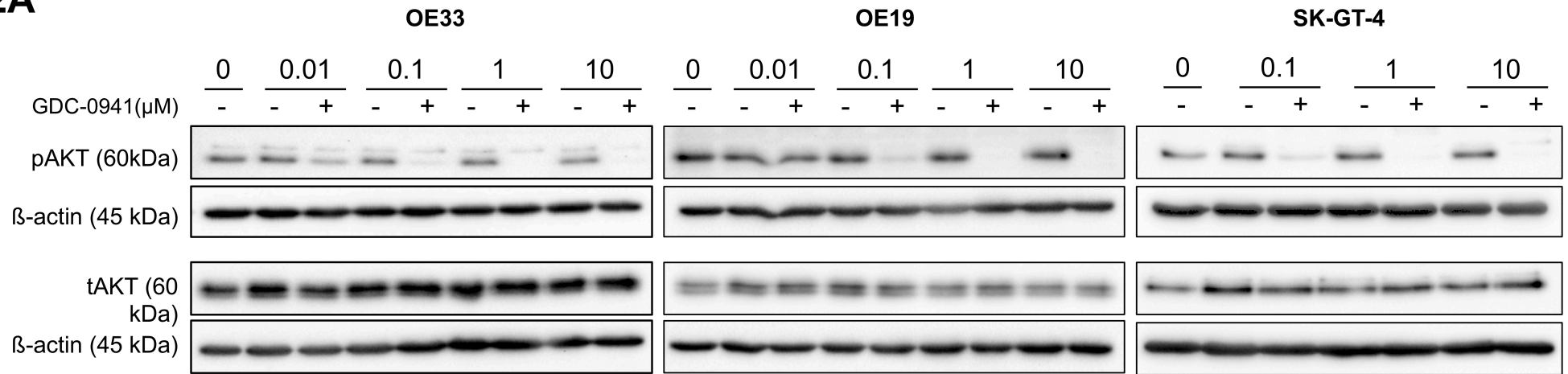
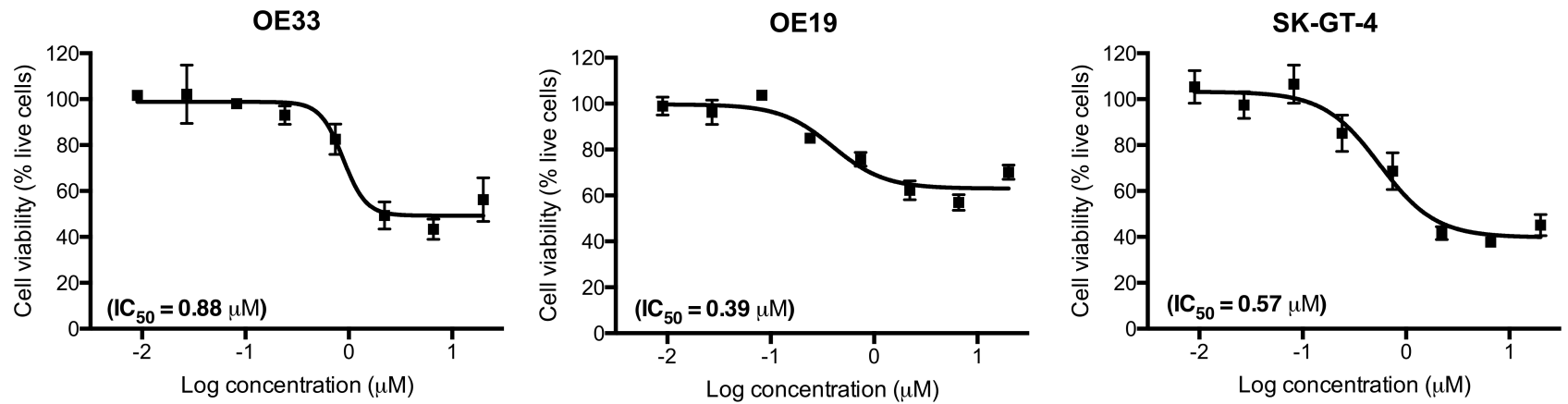
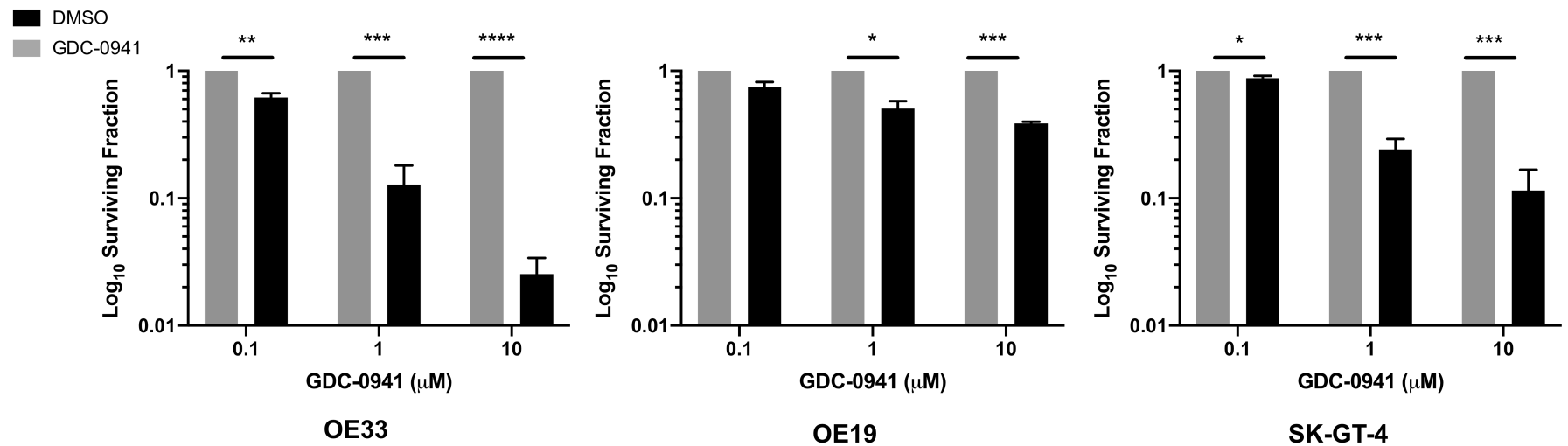


1B



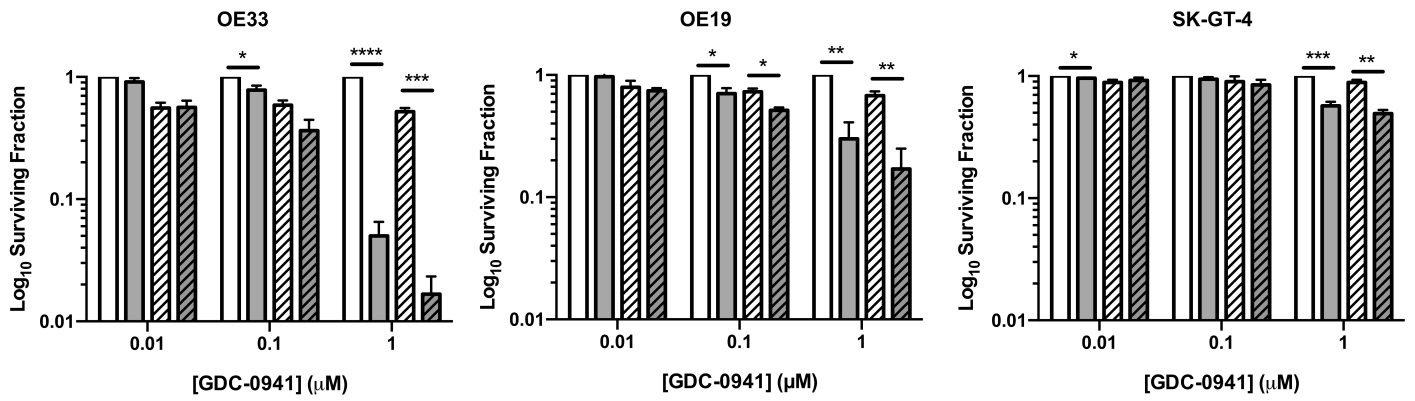
1C



2A**2B****2C**

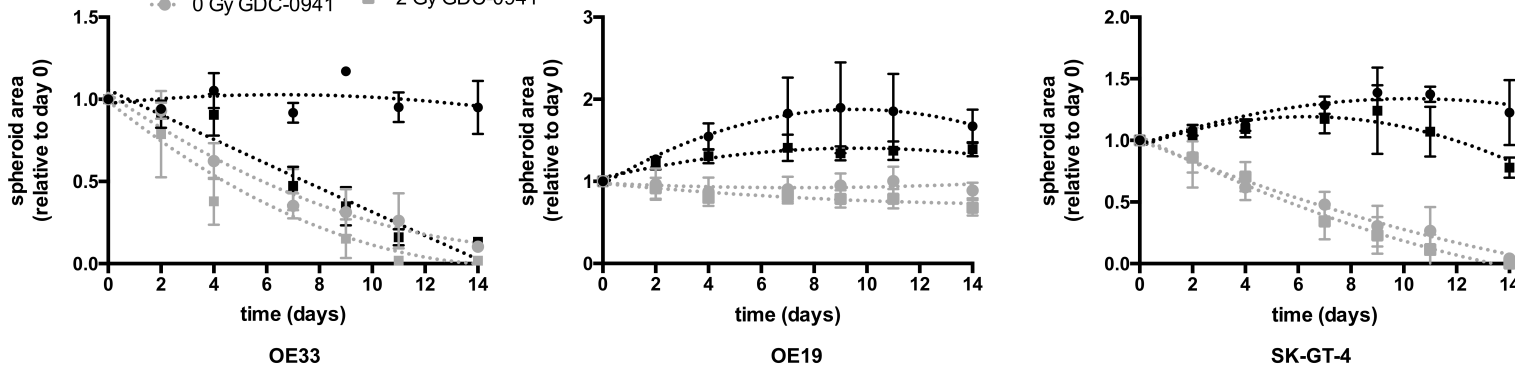
4A

DMSO 0Gy DMSO 2Gy
 GDC-0941 0Gy GDC-0941 2Gy



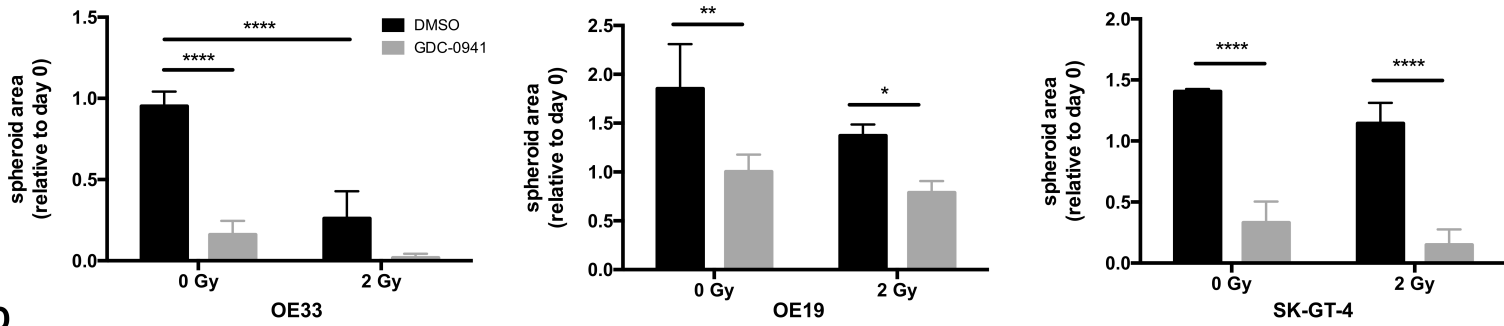
4B

0 Gy DMSO 2 Gy DMSO
 0 Gy GDC-0941 2 Gy GDC-0941



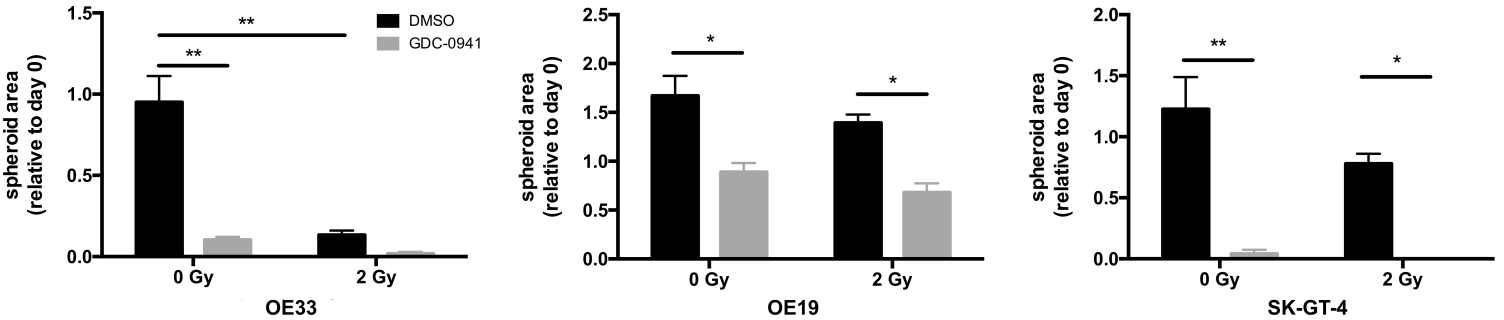
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Day 11

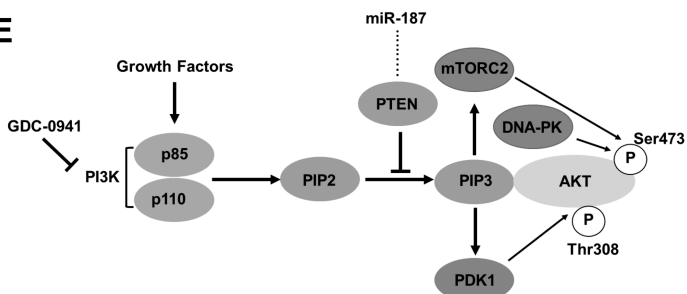


4D

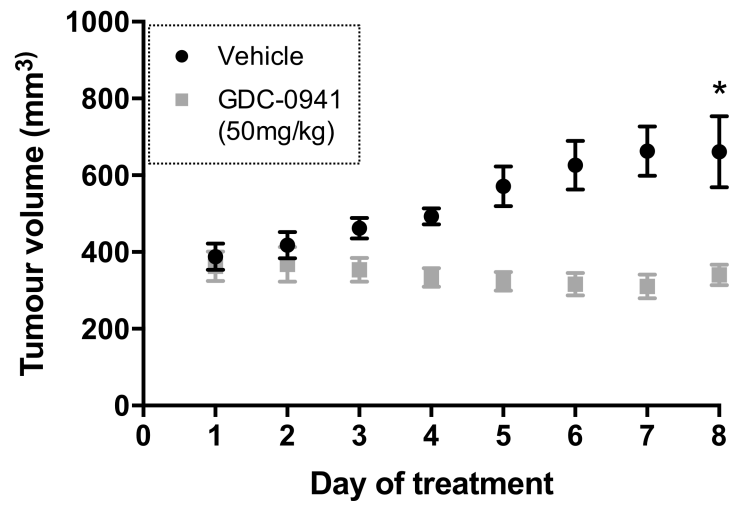
Day 14



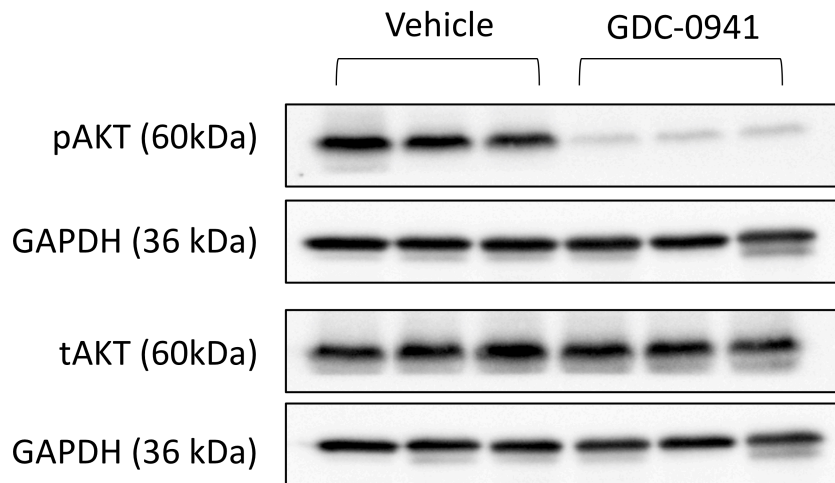
4E



3A



3B



Supplementary Information

Supplementary methods

Cell culture and spheroid generation and treatment

The human OAC cell lines OE33, OE19 and SK-GT-4 were purchased from the European Collection of Authenticated Cell Cultures, UK (ECACC). Cell lines were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium with L-glutamine (Gibco, Life Technologies, UK) supplemented with 10% (v/v) heat inactivated foetal bovine serum (FBS; Gibco, Life Technologies, UK). Cells were incubated at 37°C, with 95% humidified air and 5% CO₂. All cell lines tested negative for mycoplasma infection.

Spheroids were generated by seeding cells at a density of 2.5×10^4 cells per well in ultra-low adherence round-bottomed 96-well plates (Corning). After aggregation, spheroids were treated according to specific experimental conditions, with media replaced every 2 days. Spheroids were imaged using the GelCount instrument (Oxford Optronix), and spheroid size was determined as previously reported^{1,2}

Transfection with Pre-miR miRNA pre-cursor molecules

Ambion pre-miR miRNA precursor molecules (Thermo Fisher Scientific, UK) were used to achieve transient miR-187 overexpression. Cells were seeded into 6 cm dishes at a density of 0.8×10^6 cells/dish prior to reverse transfection with 5 nM Pre-miR-187 precursor molecules in combination with Lipofectamine RNAiMAX (Invitrogen, UK). Non-transfected control cells were concurrently seeded and treated using an identical protocol whereby the miRNA precursor molecules were substituted for the vehicle control (nuclease free water).

Patient treatment and histology

Following ethical approval (Joint St James's Hospital/AMNCH ethical review board, Reference ID 2011/27/01) and written informed consent, diagnostic biopsy tumour specimens were taken from patients with a diagnosis of operable OAC, prior to neoadjuvant therapy. All patients received a complete course of neoadjuvant CRT. Chemotherapy consisted of 2 courses of 5-fluorouracil (5-FU)

and cisplatin, as previously described ³. Patients received 40.05 Gy in 15 daily fractions (2.67 Gy/fraction) over 3 weeks as previously described ³. Surgical resection was performed approximately one month following completion of the CRT regimen. All resected oesophagectomy specimens were assessed by an experienced pathologist. Tumour response to treatment was assigned 1 of 5 tumour regression grades (TRG) as previously described ⁴. Good responders were classified as patients achieving a TRG of 1 or 2, whilst poor responders were classified as patients having a TRG of 3, 4 or 5, as previously described ⁵. For the purposes of this study patients with a TRG 3 were excluded.

Tissue collection

Diagnostic endoscopic biopsies were obtained by a qualified endoscopist prior to neoadjuvant therapy. Immediately adjacent tissue was taken for histologic confirmation, which was performed using routine Haematoxylin and Eosin staining. Specimens were immediately placed in RNAlater (Ambion) and refrigerated for 24 h, before removal of RNA later and storage at -80°C.

Clonogenic assay

Cell seeding densities were optimised for each treatment condition to ensure that at least 100 colonies, each consisting of at least 50 cells were counted. Cells were seeded directly into 6 well plates at the optimised seeding densities (500-5000 cells/well). Once adhered, cells were treated with GDC-0941 or the DMSO 18 hours and then irradiated/ mock irradiated. Clonogenic plates were then placed in the incubator 7-10 days, fixed, and stained through the application of crystal violet staining solution (0.1% w/v crystal violet, 70% v/v methanol, 30% v/v dH₂O). Colonies were counted using a Gel Count system (Oxford Optronics, UK). Plating efficiencies were calculated as follows: average colony number / number of cells seeded. The surviving fraction (SF) for each treatment group was then determined as follows: (average colony number/plating efficiency of control) X seeding density ⁶.

Cell viability assay

Short term cell viability was assessed using the Cell Titer 96 AQueous One Solution MTS cell proliferation assay (Promega, UK) as previously described ². Cells were seeded into 96 well plates at a seeding density of 3000 cells/well and incubated over night to allow cells to adhere. Complete medium was replaced with treatment conditions and plates were returned to the incubator for a further 48 h,

and MTS reagent added as per manufacturer's instructions. Media only absorbance was subtracted from mean absorbance values and cell viability for each treatment condition was calculated relative to DMSO vehicle control, normalised to 100% viability.

Western blotting

Cells were lysed in UTB (9M Urea, 75 mM Tris- HCL pH 7.5, 0.1M β - Mercaptoethanol) as previously reported ⁷. Tumour samples were prepared for lysis using a BioPulverizer and Cryo-cup grinder (BioSpec, USA) and lysed in RIPA buffer (Cell Signalling Technology, USA) supplemented with protease inhibitors (Mini, EDTA-free Protease Inhibitor Cocktail, Roche, UK) and phosphatase inhibitors (PhosSTOP, Roche, UK). Western blotting was performed as previously reported ⁷. Antibodies used include AKT (pan), phospho AKT Serine 473, PARP, β -actin or GAPDH, details noted in Supplementary Table 2. Detection was carried out using the ChemiDoc XRS+ (BioRad, UK). Densitometric analysis of band intensity of blots was carried out using Image J (NIH, USA).

Supplementary Table 1 – Details of qPCR primers used in this study

Target	RNA type	Method	Manufacturer	Reference
miR-187-3p	MicroRNA	TaqMan	Applied Biosystems	001193
<i>RNU48</i>	MicroRNA	TaqMan	Applied Biosystems	001006
<i>PTEN</i>	mRNA	SYBR	Qiagen	QT00086933
<i>KDM5B</i>	mRNA	SYBR	Qiagen	QT00060648
<i>DNMT1</i>	mRNA	SYBR	Qiagen	QT00034335
<i>EP300</i>	mRNA	SYBR	Qiagen	QT00094500
<i>B2M</i>	mRNA	SYBR	Qiagen	QT00088935

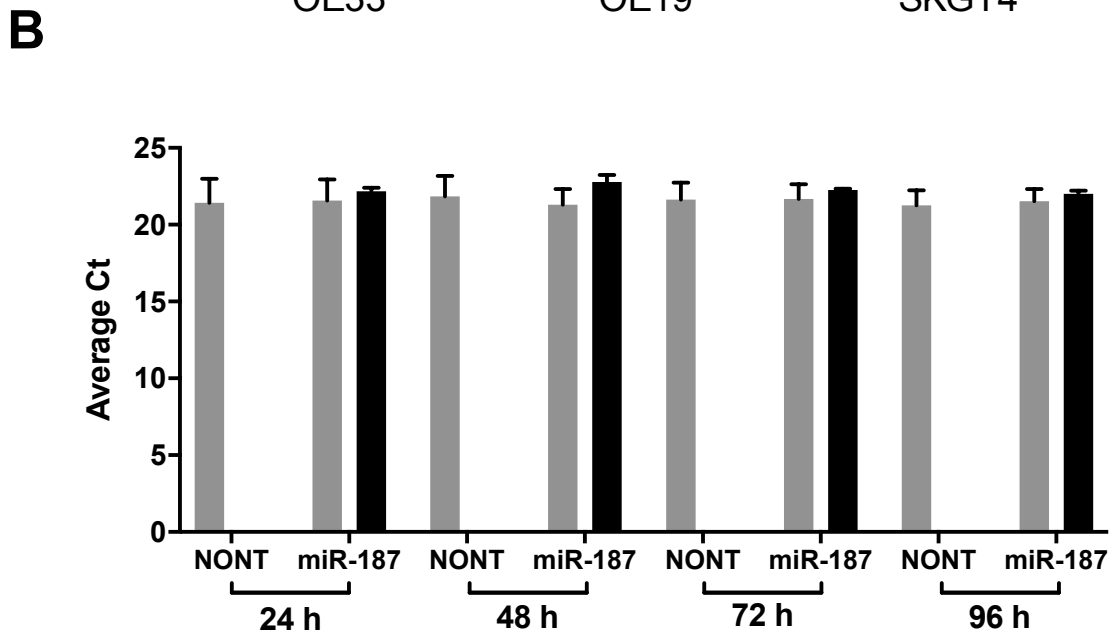
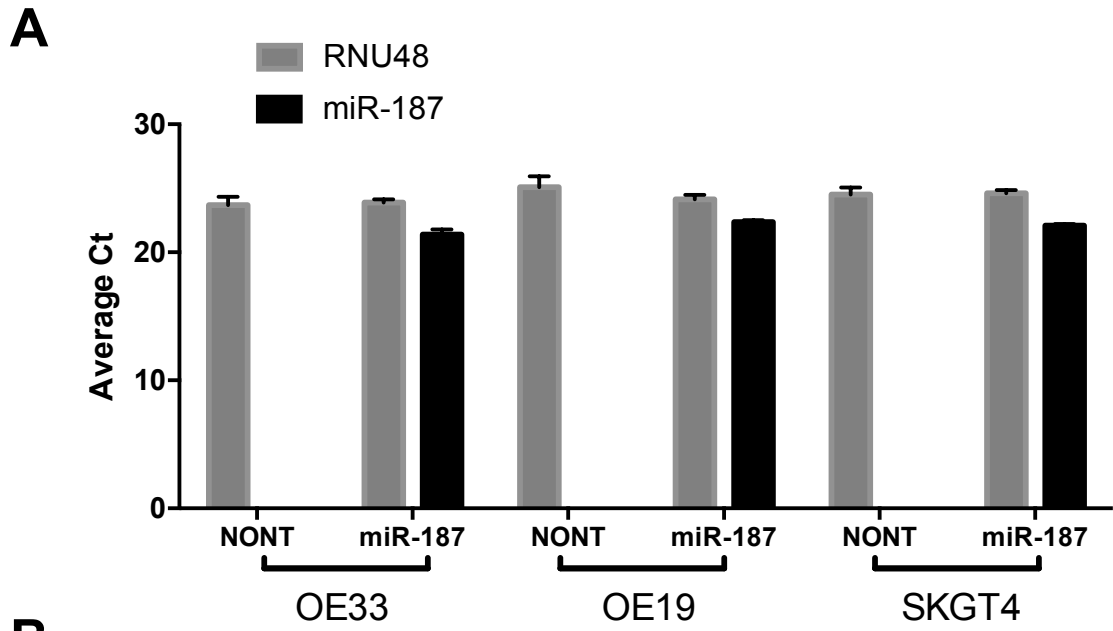
Supplementary Table 2 – Details Antibodies used

Target	Manufacturer	Reference	Dilution	Origin Species
AKT (pan)	Cell signalling	4691	1:1000	Rabbit mAb
Phospho AKT	Cell signalling	4060	1:2000	Rabbit mAb
LC3b	Cell signalling	2775	1:1000	Rabbit pAb
PARP	Cell signalling	9542	1:1000	Rabbit pAb
PTEN	Cell signalling	9188	1:1000	Rabbit mAb
β-actin	Santa Cruz	SC-69879	1:10,000	Mouse mAb
GAPDH	Ambion	AM4300	1:10,000	Mouse mAb
Mouse HRP (2°)	Dako	P0449	1:2000	Rabbit pAb
Rabbit HRP (2°)	Dako	P0448	1:2000	Goat pAb

mAb, Monoclonal antibody; *pAb*, Polyclonal antibody

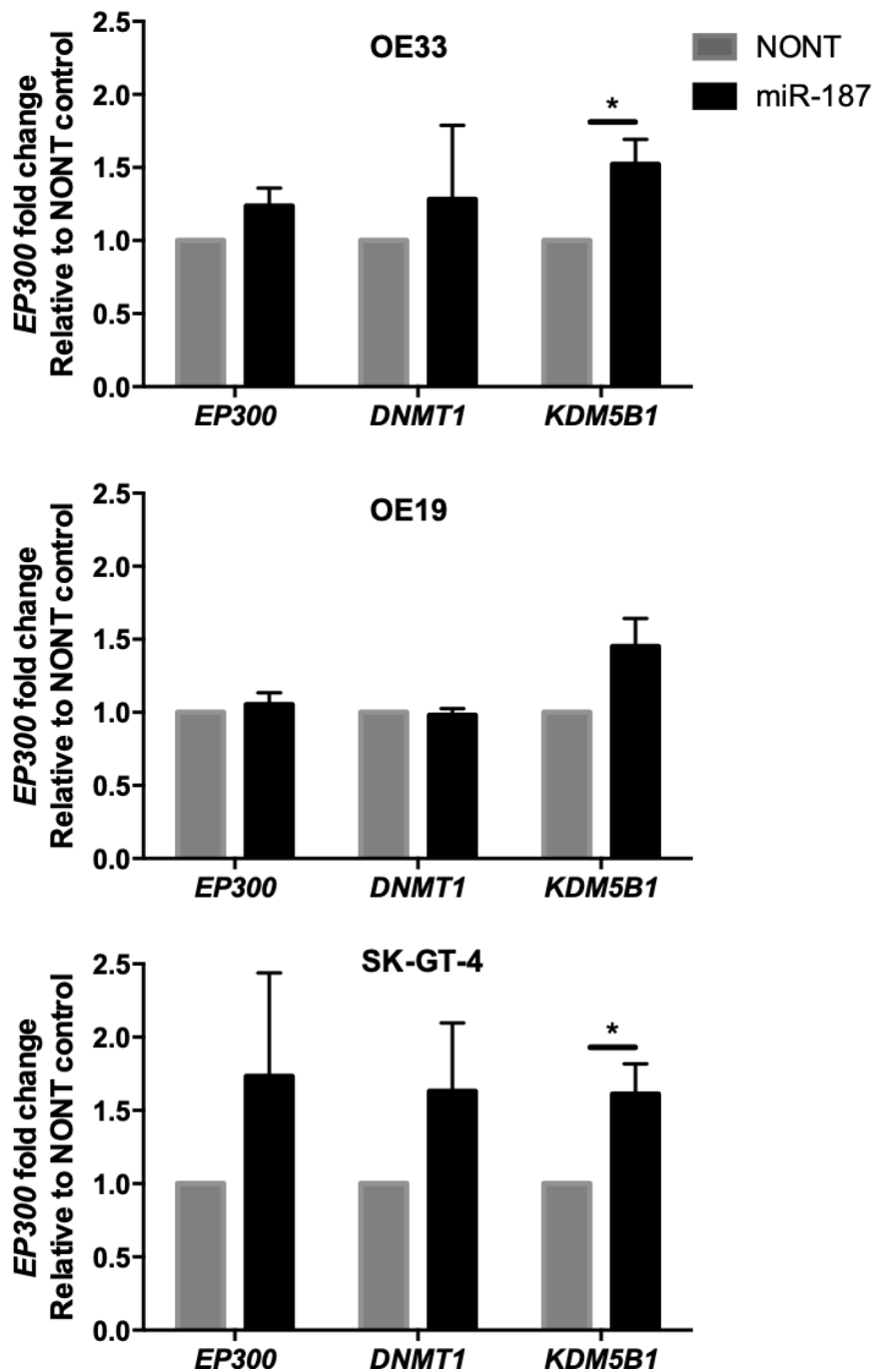
Supplementary Table 3. Genes encoding PTEN transcription factors with miR-187 binding site

<i>PTEN</i> transcription factor (TF)	MiR-187 seedposition on TF	Conservation of miR-187 binding site on TF in primates (%)	Known TF function in transcriptional regulation of <i>PTEN</i>	Reference
<i>MAZ</i>	chr16:29819949	89	Mechanism of PTEN regulation unknown	N/A
<i>NFIC</i>	chr19:3435127	89	Mechanism of PTEN regulation unknown	N/A
<i>E2F1</i>	chr20:32264211	78	One study demonstrated PTEN activation via binding to 5' UTR	⁸
<i>EP300</i>	chr22:41572511	78	Negatively regulates PTEN	⁹
<i>BLC3</i>	chr19:45259490	67	Mechanism of PTEN regulation unknown	N/A
<i>MYCBP2</i>	chr13:77835389	67	Mechanism of PTEN regulation unknown	N/A
<i>NFIC</i>	chr19:3433521	67	Mechanism of PTEN regulation unknown	N/A
<i>PHF8</i>	chrX:54069203	67	Mechanism of PTEN regulation unknown	N/A
<i>PPARG</i>	chr4:23816000	67	Positive regulator	¹⁰
<i>KDM5B</i>	chr1:202698500	56	Histone demethylase which transcriptionally represses PTEN	¹¹
<i>MAZ</i>	chr16:29821826	56	Mechanism of PTEN regulation unknown	N/A
<i>SRFBP1</i>	chr5:121356312	56	Mechanism of PTEN regulation unknown	N/A
<i>PPARG</i>	chr4:23822068	56	Positive regulator	¹⁰
<i>FOXP2</i>	chr7:113726400	44	Mechanism of PTEN regulation unknown	N/A
<i>MAFK</i>	chr7:1580362	44	Mechanism of PTEN regulation unknown	N/A
<i>WRNIP1</i>	chr6:2766547	44	Mechanism of PTEN regulation unknown	N/A
<i>DNMT1</i>	chr19:10248642	44	Negative regulator	¹⁰



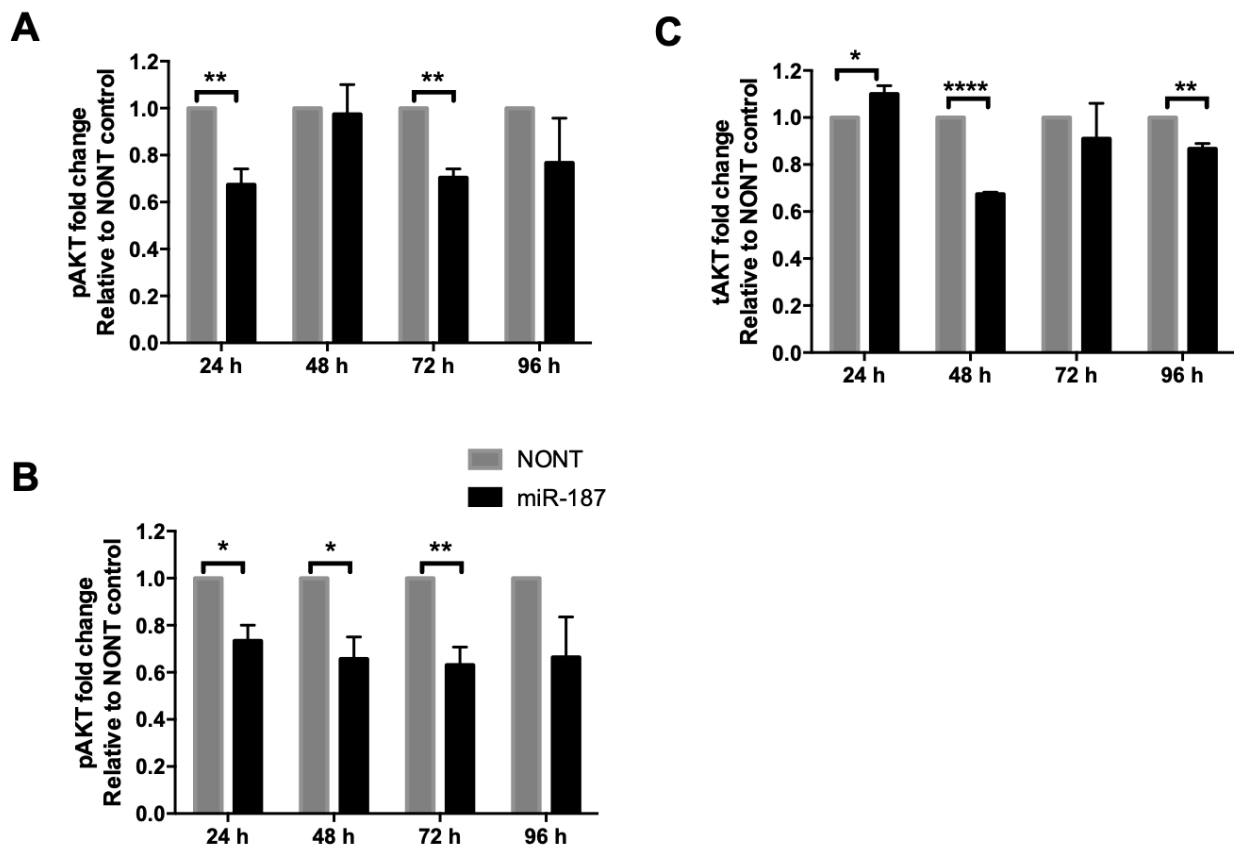
Supplementary Figure 1 – Validation of mir-187 overexpression

Cells were seeded into 6cm dishes and transiently transfected with 5 nM pre-miR-187. miR-187 expression levels were assessed 24 h post transfection for all cell lines (A) or for a series of timepoints post transfection for OE33 cell line (B) using TaqMan qPCR. MiR-187 expression was also assessed in mock transfected (NONT) control cells. The average Ct values for miR-187 and the endogenous control RNU48 are presented for each sample. Data represent the average of n=3 experimental repeats. Error bars represent mean \pm SEM.



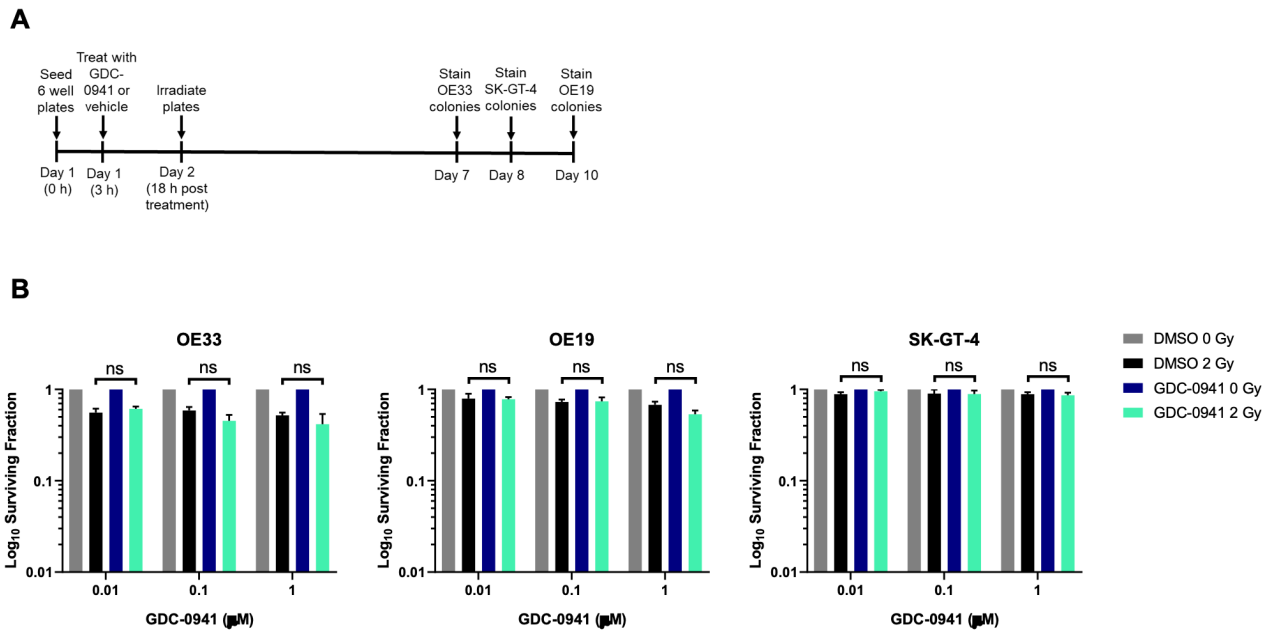
Supplementary Figure 2 – Evaluation of potential transcriptional regulators of PTEN downstream of miR-187

Cells were reverse transfected with 5 nM miR-187 precursor or mock transfected (NONT) and harvested 24 h post transfection. *EP300*, *DNMT1*, and *KDM5B* expression was analysed by qPCR, and transcript levels were normalised to mock transfected control cells (NONT). *B2M* was used as a housekeeping gene. Histogram represents an average of n=3 independent experimental repeats. Error bars represent mean \pm SEM. Statistical analysis was performed using the Student's t-test.



Supplementary Figure 3 – Densitometry quantification of pAKT and tAKT

Densitometry of western blots shown in Figure 2B. Band intensity was quantified using ImageJ. Histograms represent the average band intensity for pAKT normalised to tAKT (A) pAKT normalised to β -actin control (B) and tAKT normalised to β -actin control (C) All calculations are relative to the mock transfected control (NONT) for each time point. Histogram represents an average of n=3 independent experimental repeats. Error bars represent mean \pm SEM. Statistical analysis was performed using the Student's t-test: *p < 0.05, **p < 0.01, ****p < 0.0001.



Supplementary Figure 4 – GDC-0941 significantly reduced SK-GT-4 clonogenic survival as a single agent and has an additive effect in combination with physiologically relevant levels of irradiation

Cells were seeded into 6 well plates for the clonogenic assay and treated with GDC-0941 or the corresponding vehicle control once adhered. Plates were transported to the irradiator and exposed to 0 Gy or 2 Gy irradiation doses 18 h post treatment. Colonies were stained and counted. The surviving fraction was calculated relative to the mock transfected (Mock) 0 Gy control. Treatment with GDC-0941 or vehicle control (DMSO) was removed at the time of staining. B Treatment with GDC-0941 or vehicle control (DMSO) was removed 24 h post irradiation and replaced with complete medium. (A) Treatment scheduling. (B) Survival fractions for all conditions, relative to GDC-0941-treated samples. Error bars represent mean \pm SEM. Statistical analysis was performed using the Student's t-test.

Supplementary references

1. Pires IM, Olcina MM, Anbalagan S, et al. Targeting radiation-resistant hypoxic tumour cells through ATR inhibition. *Br J Cancer*. 2012;107(2):291-299.
2. Beeby E, Magalhaes M, Pocas J, et al. Secondary metabolites (essential oils) from sand-dune plants induce cytotoxic effects in cancer cells. *J Ethnopharmacol*. 2020;258:112803.
3. Maher SG, Gillham CM, Duggan SP, et al. Gene expression analysis of diagnostic biopsies predicts pathological response to neoadjuvant chemoradiotherapy of esophageal cancer. *Ann Surg*. 2009;250(5):729-737.
4. Mandard AM, Dalibard F, Mandard JC, et al. Pathologic assessment of tumor regression after preoperative chemoradiotherapy of esophageal carcinoma. Clinicopathologic correlations. *Cancer*. 1994;73(11):2680-2686.
5. Lynam-Lennon N, Reynolds JV, Marignol L, Sheils OM, Pidgeon GP, Maher SG. MicroRNA-31 modulates tumour sensitivity to radiation in oesophageal adenocarcinoma. *J Mol Med (Berl)*. 2012;90(12):1449-1458.
6. Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. *Nat Protoc*. 2006;1(5):2315-2319.
7. Poujade FA, Mannion A, Brittain N, et al. WSB-1 regulates the metastatic potential of hormone receptor negative breast cancer. *Br J Cancer*. 2018;118(9):1229-1237.
8. Boosani CS, Agrawal DK. PTEN modulators: a patent review. *Expert Opin Ther Pat*. 2013;23(5):569-580.
9. Correia NC, Girio A, Antunes I, Martins LR, Barata JT. The multiple layers of non-genetic regulation of PTEN tumour suppressor activity. *Eur J Cancer*. 2014;50(1):216-225.
10. Bermudez Brito M, Goulielmaki E, Papakonstanti EA. Focus on PTEN Regulation. *Front Oncol*. 2015;5:166.
11. Tang B, Qi G, Tang F, et al. JARID1B promotes metastasis and epithelial-mesenchymal transition via PTEN/AKT signaling in hepatocellular carcinoma cells. *Oncotarget*. 2015;6(14):12723-12739.