Overcoming acquired resistance in NSCLC with targeted beam irradiation in combination with targeted agents

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Abstract: 765

Introduction

- Lung cancer is the largest cancer killer with poor 5-year survival rate. Non-small cell lung cancer (NSCLC) patients undergo primary, adjuvant or neoadjuvant radiotherapy treatment for NSCLC with image-guided radiotherapy (IGRT) being widely used to treat cancer patients providing benefit with more accurate treatment plans and reduced side effects.
- NSCLC patients that have activating mutations in the EGFR gene are treated with epidermal growth factor receptor (EGFR) inhibitors e.g. Erlotinib (Tarceva®) and Gefitinib (Iressa®). However, resistance emerges in the majority of patients due to secondary gatekeeper mutations (T790M) or amplification of genes such c-MET and Her2.
- The HCC827 NSCLC adenocarcinoma cell line, which harbours an activating EGFR mutation (del E746-A750), was used to generate EGFR inhibitor (EGFRi) resistant models.
- Here we demonstrate the application of the image-guided small animal radiation research platform (SARRP, Xstrahl Ltd) to treat subcutaneous xenograft tumours with irradiation, using planned protocols similar to those utilised in the clinic, with little or no adverse effects on mice and to report on combination treatment strategies to overcome EGFR inhibitor resistance.

Methods

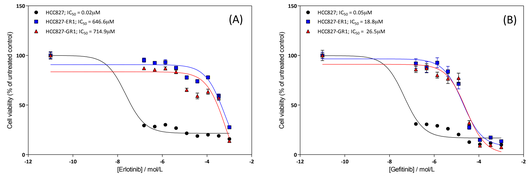
- **Generation of resistance variants of HCC827;** EGFRi resistant variants of HCC827 (ER1 and GR1) were generated *in vitro* following sub-culture with escalating doses of Erlotinib or Gefitinib respectively. Further models of HCC827 resistance were generated following single-cell cloning of the resistant HCC827 variants. HCC827 and the resistant variants were characterised for c-MET genomic amplification by qPCR (RNAse P reference) and Axl gene expression via RT-PCR (HPRT reference). STR profiling was also carried out (LGC Promochem). Resistance was also generated *in vivo* by allowing tumours to outgrow under dosing pressure. Xenograft tissue was excised, disaggregated and purified *in vitro;* this cell line is denoted PCS030.
- ***In vitro IR* assay**: Cells were grown in T25 flasks and treated with irradiation and counted after 6 days. For IC_{50} evaluation cells were seeded in 384-well plates and viability assessed by CellTitre Blue™ (Promega).
- ***In vivo* studies**: Cells were implanted subcutaneously in nude mice (ValidatedXeno™ in HsdOla:MF1-*Foxn1^{nu}*). Erlotinib was dosed at 25mg/kg po QD and Crizotinib was dosed at 50mg/kg po QD. Tumour measurements and body weights were taken 3 times weekly and dosing initiated in the 2 models when the tumours reached a mean volume of ~200mm^3.
- ***In vivo* Irradiation**: Mice were anaesthetised and transported to the SARRP where CBCT images were acquired. Using the MuriSlice software the isocenter of the tumour was identified and aligned with the central axis of the beam. Fractionated irradiation was administered with the SARRP (225 kV peak X-ray beams; dose rate of 2.5 Gy/min) using collimators of various dimensions and a double beam (gantry position at 0° and 180°) under the guidance of the CBCT. A tolerability was performed initially to evaluate 3Gy/day x 5 days for 2 weeks.

Results: Generation of resistance

EGFRi resistant variants of HCC827 (ER1 and GR1) exhibited a >500 fold shift in IC_{50} compared with the parental line and exhibited cross-resistance with the alternate EGFRi (Figure 1). STR profiling confirmed no changes to the wild-type HCC827 DNA profile.

Figure 1: *In vitro* characterisation of Erlotinib and Gefitinib response in EGFRi resistance variants HCC827-ER1 and HCC827-GR1

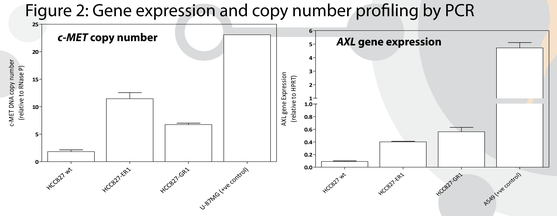
A: Erlotinib IC_{50} values. B: Gefitinib IC_{50} values.



Treatment naive and post-treatment resistance phase tumour material was also characterised for mutations in EGFR by direct sequencing of exons 19 (del E746-A750) and 20 (T790M; gatekeeper).

- Exon 19 deletion was confirmed in all samples.
- No exon 20 T790M gatekeeper mutations were detected in any of the test samples.
- Treatment naïve and post-treatment resistance phase HCC827 wild-type/resistant variants were characterised for *c-MET* genomic amplification by qPCR and *AXL* gene expression via RT-PCR (figure 2) .

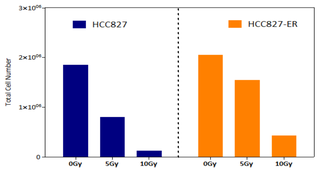
Figure 2: Gene expression and copy number profiling by PCR



Results: *In Vitro* sensitivity to irradiation

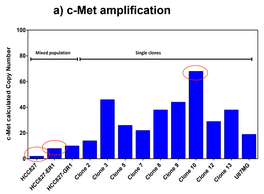
Irradiation of HCC827 cells resulted in a dose-dependent cytotoxicity (figure 3). Similarly the Erlotinib resistant cell line, HCC827-ER1, showed a dose response to IR; however, the response was attenuated when compared to that of the parental cell line suggesting that the acquired resistance to Erlotinib bestowed some resistance to irradiation.

Figure 3: Dose dependent effect of irradiation on cell number of HCC827 and HCC827-ER1 cells



Results: Combination index *in vitro*

a) Single cell clones from EGFRi resistant variants of HCC827 were generated and characterised for c-Met genomic amplification by qPCR.
b) Three of the cell lines, exhibiting an increasing scale of c-Met amplification (HCC827, HCC827-ER1 and HCC827-GR1 clone 10), were assessed *in vitro* to determine their response to combination treatment.



Cells were treated with Crizotinib, Erlotinib or combinations of both for 72h and cell viability were assessed by using CellTiter Blue™ (Promega). Combination ratios of 1:1 and 1:100 (Erlotinib:Crizotinib) in the parental line and 1:1 and 100:1 were tested in resistant lines.

b) Combination (CI) analysis

Cell line	Compound / combination	Combination Index (CI) ED50	ED75	ED90	ED95	Weighted CI	Score
HCC827	Erlotinib:Crizotinib; 1:1	340.40	1.37	6.98	21.57	45.04	No effect
	Erlotinib:Crizotinib; 1:100	64.75	0.08	0.12	0.17	6.60	No effect
HCC827-ER1	Erlotinib:Crizotinib; 1:1	0.33	0.79	1.89	3.42	2.12	Antagonism (synergism near IC50)
	Erlotinib:Crizotinib; 100:1	0.21	0.14	0.10	0.07	0.11	Strong synergism
HCC827-GR1 (clone 10)	Erlotinib:Crizotinib; 1:1	0.57	0.46	0.37	0.32	0.39	Synergism
	Erlotinib:Crizotinib; 100:1	0.12	0.04	0.02	0.01	0.03	Very strong synergism

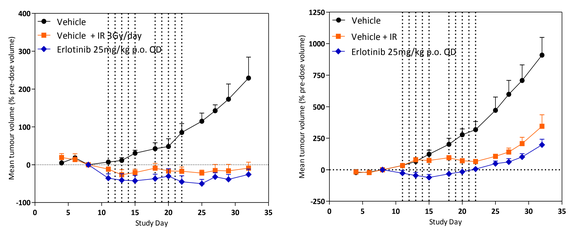
Synergistic effect was calculated based on combination index values according to the Chou and Talalay method (CalcuSyn). The increasing scale of synergistic response corresponds with c-Met amplification. Results could be used to guide further *in vivo* efficacy testing and dose optimisation.

Results: *In vivo* resistance

- Mice bearing subcutaneous HCC827 xenograft tumours showed high sensitivity to Erlotinib treatment (25mg/kg po QD, $p<0.001$ Two way ANOVA) resulting in tumour regression (figure 4A).
- Similarly treatment with 2 cycles of 3Gy/day for 5 days using the SARRP resulted in tumour regression ($p<0.001$).
- In the Erlotinib resistant variant, HCC827-ER1, the sensitivity to Erlotinib was reduced and growth rate higher (Figure 4B).
- The sensitivity to irradiation was also reduced.

Figure 4: The effect of 3Gy/day irradiation on HCC827 & HCC827-ER1 subcutaneous xenografts.

A. HCC827 tumour volume B. HCC827-ER1 tumour volume



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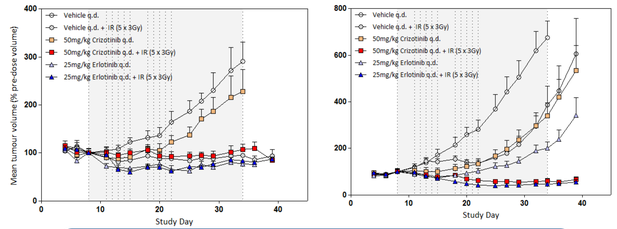
Results: Combination studies

- Due to c-met amplification in resistant model and implication of c-met in resistant mechanisms, Crizotinib was tested in the WT and resistant model alongside Erlotinib +/- IR.
- No effect with Crizotinib alone in WT model (figure 5a) whereas in HCC827-ER1 model (figure 5b) there was a significant reduction ($p<0.001$; ~60% tumour growth inhibition), which supported the role of c-Met amplification in the resistance mechanism.
- In the resistant model, the tumour regression induced by IR was lost; however, when combined with Crizotinib the efficacy was restored. Additionally, treatment with Erlotinib and IR also resulted in tumour regression

Figure 5: The effect of 3Gy/day irradiation on HCC827 & HCC827-ER1 xenografts in combination with Crizotinib and Erlotinib.

A. HCC827 tumour volume B. HCC827-ER1 tumour volume



Summary

- EGFRi resistant variants were generated *in vitro* and *in vivo* from the NSCLC cell line HCC827. Cross-resistance to EGFRi was observed along with an elevation in the *c-MET* copy number.
- Combination treatment (c-Met and EGFR inhibitors) overcomes resistance in c-Met driven EGFRi resistant models (*in vitro* & *in vivo*).
- Resistance to Erlotinib confers resistance to IR, but sensitivity to c-Met inhibition supports the hypothesis of c-Met driven resistance mechanisms.
- Combination treatment with IR restores tumour regression with both c-Met and EGFR inhibitors.

Conclusions

- Models of acquired resistance to EGFR inhibitors are invaluable in assessing novel agents targeting resistance pathways.
- These models open up opportunities for the assessment of new combination strategies which seek to prevent or overcome the emergence of resistance.
- Offer proof of concept for generation other resistant lines/models for current or new treatment strategies for MAbs or small molecules.
- Small animal irradiation platforms such as the SARRP allows the use of irradiation to interrogate IR combination strategies with anti-cancer agents in mice with reduced side effects and improved outcome.