Exploring mechanisms of EGFR inhibitor resistance in chordoma

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ABSTRACT

- Target discovery studies have identified EGFR as a promising therapeutic target in chordoma, motivating Phase II clinical trials with afatinib (NCT03083678) and cetuximab (NCT05041127).
- Recently-reported results from the afatinib trial include ORR = 9% with median DoR = 27.9 months (A. Lipplaa et al., *J Clin Oncol*, 2024), underscoring the need to identify therapeutic biomarkers. EGFR is not mutated in chordoma, leaving the mechanisms associated with sensitivity or resistance to EGFR inhibition unclear.
- We treated a panel of 14 chordoma cell lines with afatinib, an EGFR inhibitor (EGFRi) with potency against the wild-type receptor, and observed striking differential sensitivity. Sensitive cell lines have EC50 values < 50 nM, whereas resistant cell lines have EC50 values > 1 μM.
- Analysis of differentially-expressed genes in resistant versus sensitive cell lines revealed an association of interferon (IFN) signaling with afatinib resistance. Tumor cell-intrinsic IFN signaling has been associated with EGFRi resistance in lung cancer (K. Gong et al., *Nat Cancer*, 2020).
- In chordoma cells, modulation of IFN signaling does not reverse EGFRi sensitivity or resistance, suggesting the resistance driver may exist in an upstream IFN regulatory pathway. Studies are ongoing to identify the putative resistance driver.
- High IFN in chordoma may be linked to genomic instability and unresolved DNA damage, which leads to the activation of cytosolic nucleotide sensors. EGFR may play a role in DNA damage repair in chordoma.

RESULTS

1. Chordoma preclinical models exhibit differential sensitivities to afatinib treatment

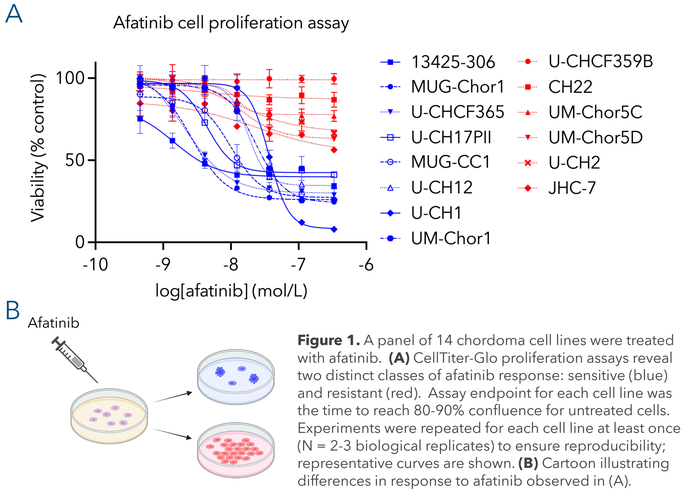

Figure 1. A panel of 14 chordoma cell lines were treated with afatinib. **(A)** CellTiter-Glo proliferation assays reveal two distinct classes of afatinib response: sensitive (blue) and resistant (red). Assay endpoint for each cell line was the time to reach 80-90% confluence for untreated cells. Experiments were repeated for each cell line at least once (N = 2-3 biological replicates) to ensure reproducibility; representative curves are shown. **(B)** Cartoon illustrating differences in response to afatinib observed in (A).

Cell line	Abs EC50 (nM)	Response
13425-306	3	Sensitive
U-CHCF365	5	Sensitive
MUG-Chor1	8	Sensitive
U-CH17PII	11	Sensitive
MUG-CC1	14	Sensitive
U-CH12	27	Sensitive
UM-Chor1	23	Sensitive
U-CH1	33	Sensitive
U-CHCF359B	> 1000	Resistant
CH22	> 1000	Resistant
UM-Chor5C	> 1000	Resistant
UM-Chor5D	> 1000	Resistant
U-CH2	> 1000	Resistant
JHC-7	> 1000	Resistant

Table 1. Absolute EC50 values (in nanomolar) plotted for chordoma cell lines. The Abs EC50 is defined as the afatinib concentration that inhibits proliferation by 50% compared to control cells.

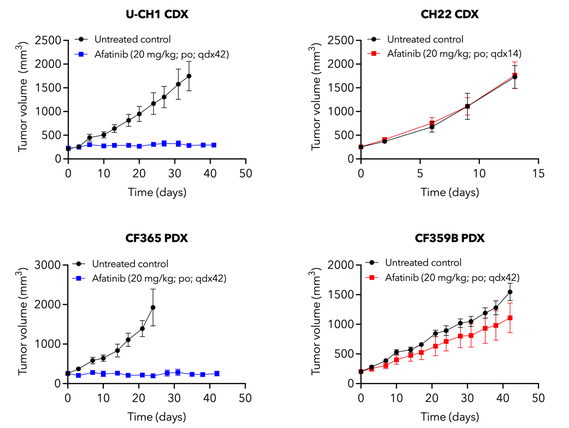

Figure 2. Afatinib responses are conserved in matched **(A)** Cell line-derived xenograft (CDX) and **(B)** Patient-derived xenograft (PDX) models (N = 5-7 mice/arm). (A) U-CH1 and CH22 are sensitive and resistant CDXs, respectively. (B) CF365 and CF359B are sensitive and resistant PDXs, respectively, corresponding to PDX-derived cell lines U-CHCF365 and U-CHCF359B. Cetuximab yields similar results in chordoma xenograft models (data not shown).

2. Differential gene expression analysis reveals an association between interferon signaling and afatinib resistance in chordoma

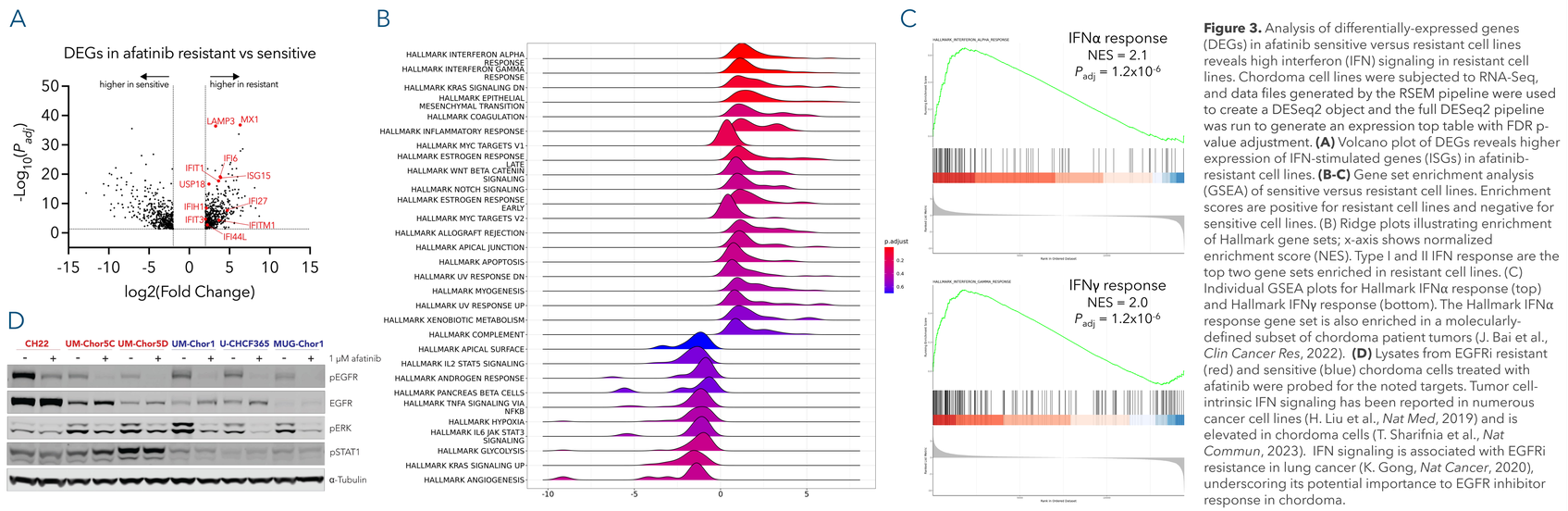

Figure 3. Analysis of differentially-expressed genes (DEGs) in afatinib sensitive versus resistant cell lines reveals high interferon (IFN) signaling in resistant cell lines. Chordoma cell lines were subjected to RNA-Seq, and data files generated by the RSEM pipeline were used to create a DESeq2 object and the full DESeq2 pipeline was run to generate an expression top table with FDR p-value adjustment. **(A)** Volcano plot of DEGs reveals higher expression of IFN-stimulated genes (ISGs) in afatinib-resistant cell lines. **(B-C)** Gene set enrichment analysis (GSEA) of sensitive versus resistant cell lines. Enrichment scores are positive for resistant cell lines and negative for sensitive cell lines. (B) Ridge plots illustrating enrichment of Hallmark gene sets; x-axis shows normalized enrichment score (NES). Type I and II IFN response are the top two gene sets enriched in resistant cell lines. (C) Individual GSEA plots for Hallmark IFNα response (top) and Hallmark IFNγ response (bottom). The Hallmark IFNα response gene set is also enriched in a molecularly-defined subset of chordoma patient tumors (J. Bai et al., *Clin Cancer Res*, 2022). **(D)** Lysates from EGFRi resistant (red) and sensitive (blue) chordoma cells treated with afatinib were probed for the noted targets. Tumor cell-intrinsic IFN signaling has been reported in numerous cancer cell lines (H. Liu et al., *Nat Med*, 2019) and is elevated in chordoma cells (T. Sharifnia et al., *Nat Commun*, 2023). IFN signaling is associated with EGFRi resistance in lung cancer (K. Gong, *Nat Cancer*, 2020), underscoring its potential importance to EGFR inhibitor response in chordoma.

3. High IFN in chordoma may be linked to DNA damage

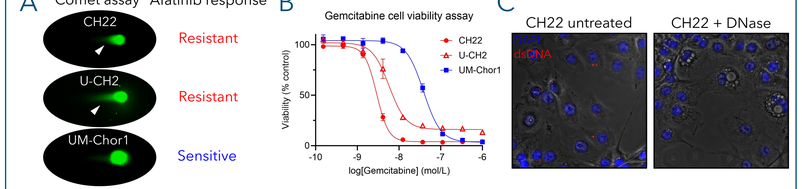

Figure 6. EGFRi resistant chordoma cell lines exhibit endogenous DNA damage and higher sensitivity to therapy-induced replication stress. **(A)** Representative comet assays reveal endogenous DNA damage in afatinib-resistant CH22 and U-CH2 cells but not sensitive UM-Chor1 cells. **(B)** Gemcitabine cell proliferation assays show increased sensitivity to gemcitabine in EGFRi-resistant cells with underlying DNA damage. **(C)** CH22 chordoma cells harbor cytoplasmic dsDNA puncta.

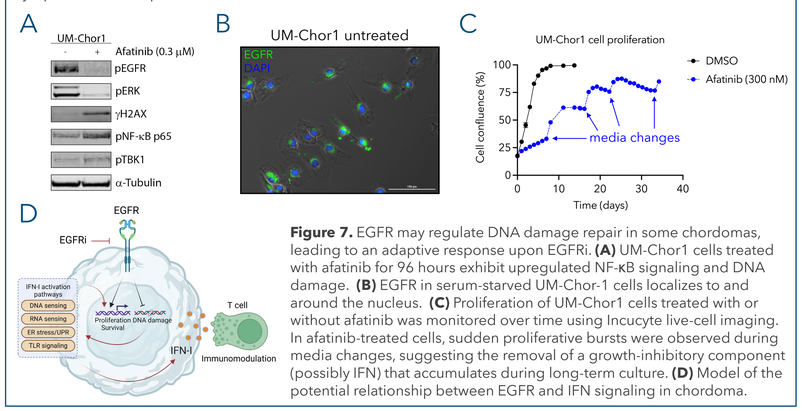

Figure 7. EGFR may regulate DNA damage repair in some chordomas, leading to an adaptive response upon EGFRi. **(A)** UM-Chor1 cells treated with afatinib for 96 hours exhibit upregulated NF-κB signaling and DNA damage. **(B)** EGFR in serum-starved UM-Chor-1 cells localizes to and around the nucleus. **(C)** Proliferation of UM-Chor1 cells treated with or without afatinib was monitored over time using Incucyte live-cell imaging. In afatinib-treated cells, sudden proliferative bursts were observed during media changes, suggesting the removal of a growth-inhibitory component (possibly IFN) that accumulates during long-term culture. **(D)** Model of the potential relationship between EGFR and IFN signaling in chordoma.

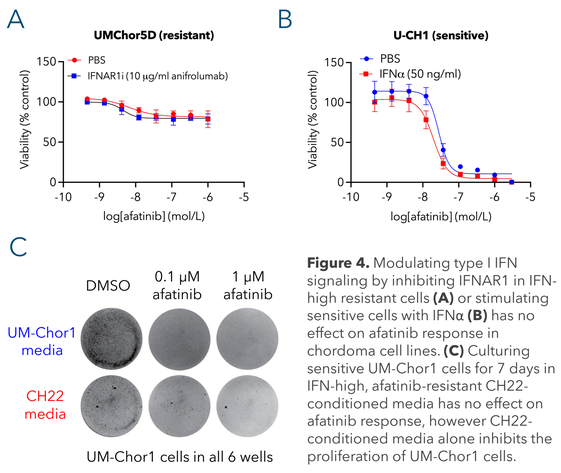

Figure 4. Modulating type I IFN signaling by inhibiting IFNAR1 in IFN-high resistant cells **(A)** or stimulating sensitive cells with IFNα **(B)** has no effect on afatinib response in chordoma cell lines. **(C)** Culturing sensitive UM-Chor1 cells for 7 days in IFN-high, afatinib-resistant CH22-conditioned media has no effect on afatinib response, however CH22-conditioned media alone inhibits the proliferation of UM-Chor1 cells.

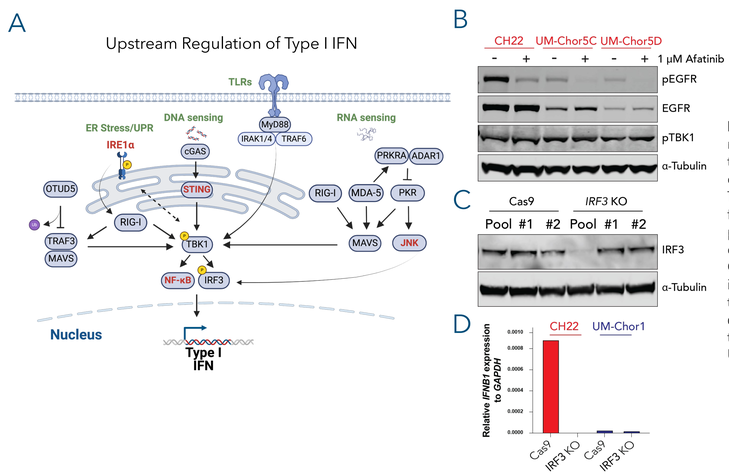

Figure 5. (A) Schematic overview of known EGFRi resistance drivers (colored in red) in pathways (green) that regulate type I IFN production. **(B)** EGFRi-resistant chordoma cells exhibit high levels of phosphorylated TBK1 at baseline and during EGFRi. Whole-cell lysates from chordoma cells treated with 1 μM afatinib were probed for the noted targets. **(C)** Cas9-mediated gene editing in chordoma cells. Whole-cell lysates from UM-Chor1 cells expressing Cas9 (transduced pools and isolated Cas9-expressing clones #1 and #2) were transduced with an IRF3 gRNA and probed for IRF3 and α-tubulin by western blotting. **(D)** RT-PCR of the IRF3 target IFNB1, a type I IFN, was measured in CH22 and UM-Chor1 Cas9 and IRF3 KO cells.

SUMMARY

- Chordoma cell lines exhibit striking differential sensitivity to afatinib, which is mirrored in xenograft mouse models.
- Combining *in vitro* afatinib sensitivity data with gene expression analysis identified an enrichment of interferon (IFN) signaling in resistant cell lines.
- IFN signaling per se does not promote resistance in chordoma cell lines. Instead, it may serve as a biomarker to guide patient selection for EGFR-targeted therapy.
- Studies are ongoing to identify the putative EGFRi resistance driver, and to characterize the causes and consequences of high IFN in chordoma.

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