Hyperactivation of mTORC1 drives resistance to the pan-HER tyrosine kinase inhibitor neratinib in *HER2*-mutant cancers

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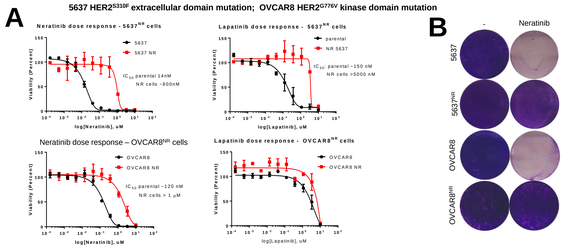
Clinical background

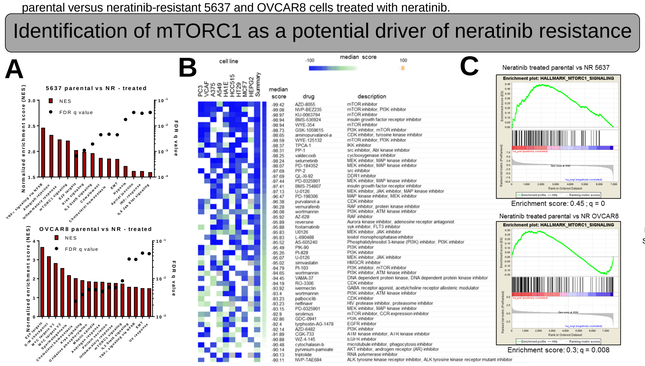
- Tumor genomic profiling has identified patients with cancers harboring activating *ERBB2* (HER2) mutations that are sensitive to HER2 targeted therapies.
- In the SUMMIT phase II 'basket' trial, a subset of patients with *ERBB2*-mutant cancers exhibited significant clinical benefit from treatment with the pan-HER irreversible tyrosine kinase inhibitor (TKI) neratinib.
- However, durable responses to neratinib are few, suggesting mechanisms of *de novo* and acquired drug resistance. Thus, we sought to identify actionable mechanisms of resistance to neratinib.

Neratinib-resistant HER2-mutant cells are cross-resistant to other HER2 TKIs



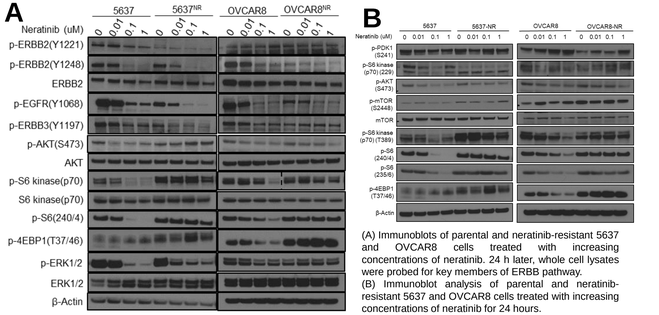
(A) 12-point dose response curves of parental and neratinib-resistant 5637 and OVCAR8 cells treated with neratinib or lapatinib. 6 days post-treatment, cells were counted on a Coulter counter. Neratinib-resistant cells were generated over a period of 6-8 months through gradual dose escalation. (B) Crystal violet stained images of parental versus neratinib-resistant 5637 and OVCAR8 cells treated with neratinib.

Identification of mTORC1 as a potential driver of neratinib resistance



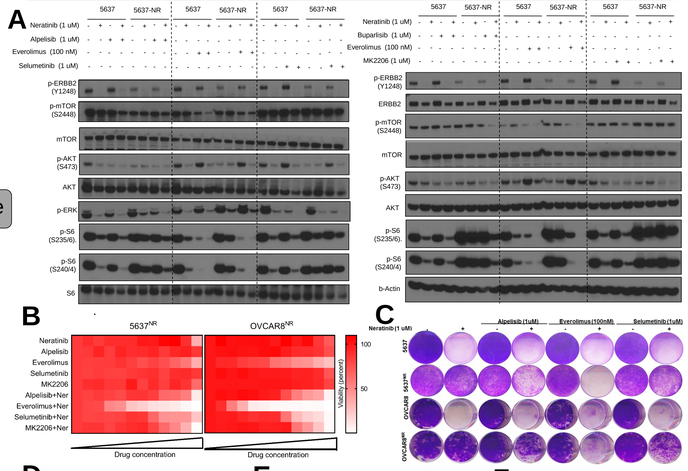
(A) RNA-seq based gene set enrichment analysis of pathways significantly upregulated in neratinib treated parental vs. neratinib-resistant cells. (B) Connectivity map analysis to identify drugs that could potentially reverse expression of resistance associated genes. (C) Enrichment plots for mTOR pathway related genes in neratinib treated parental vs. neratinib-resistant cells.

Neratinib resistant *HER2*-mutant cells sustain S6 phosphorylation in the presence of neratinib



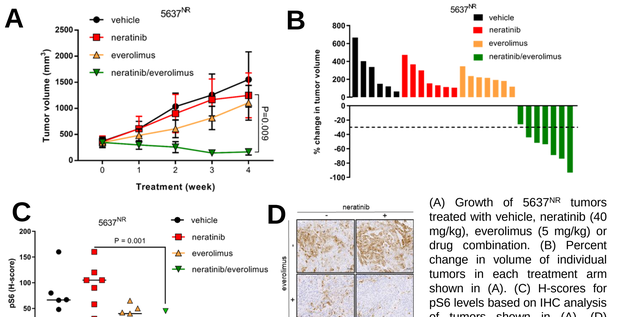
(A) Immunoblots of parental and neratinib-resistant 5637 and OVCAR8 cells treated with increasing concentrations of neratinib. 24 h later, whole cell lysates were probed for key members of ERBB pathway. (B) Immunoblot analysis of parental and neratinib-resistant 5637 and OVCAR8 cells treated with increasing concentrations of neratinib for 24 hours.

Combined neratinib and mTORC1 inhibition alone suppresses S6 phosphorylation in neratinib-resistant cells



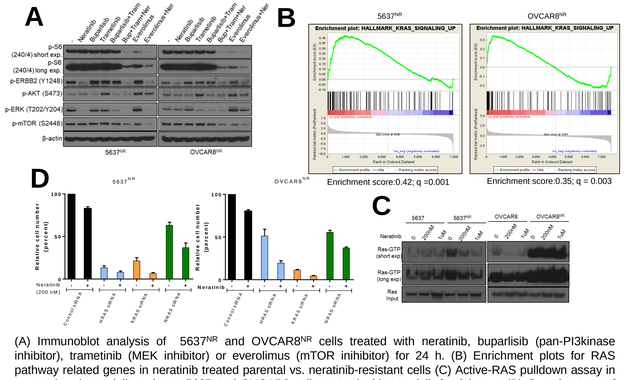
((A) Immunoblot analysis of parental and neratinib-resistant 5637 cells treated with indicated drugs combinations for 24 hours; alpelsib (PI3K-alpha specific inhibitor), everolimus (mTORC1 inhibitor), selumetinib (MEK1/2 inhibitor), Buparlisib (pan-PI3K inhibitor), MK2206 (AKT inhibitor). (B) Heatmaps representing 12-point dose response assays of 5637^{NR} and OVCAR8^{NR} cells treated with indicated single agent or drug combination. (C) Representative images of cells seeded in a 12 well plate, treated with indicated drug combination every 72 hours. (D) Immunoblot analysis of neratinib treated 5637^{NR} and OVCAR8^{NR} cells transfected with indicated siRNA. (E) Growth assay of neratinib treated cells transfected with indicated siRNAs. (F) Growth assay of TSC2 knockdown 5637, OVCAR8, MCF7 HER2^{L755S} and HER2^{V777L} cells treated every 3 days with indicated concentrations of neratinib.

Combined neratinib and mTORC1 suppression overcomes neratinib resistance



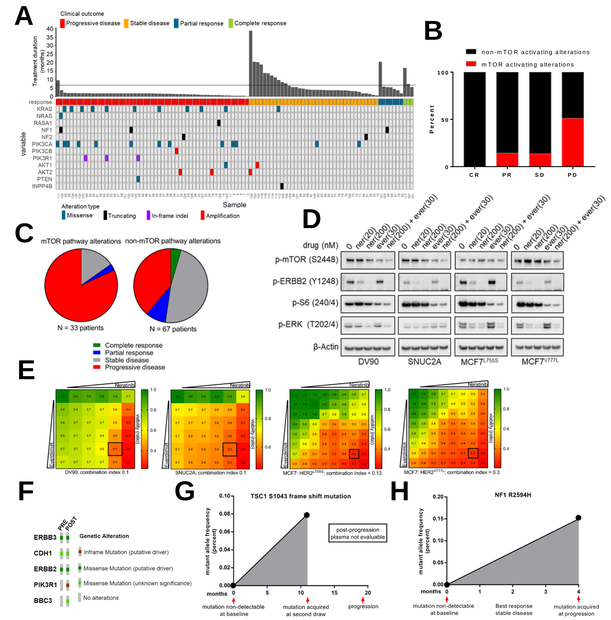
(A) Growth of 5637^{NR} tumors treated with vehicle, neratinib (40 mg/kg), everolimus (5 mg/kg) or drug combination. (B) Percent change in volume of individual tumors in each treatment arm shown in (A). (C) H-scores for pS6 levels based on IHC analysis of tumors shown in (A). (D) Representative images from (C),

mTORC1 activation could be partly attributed to RAS pathway upregulation



(A) Immunoblot analysis of 5637^{NR} and OVCAR8^{NR} cells treated with neratinib, buparlisib (pan-PI3kinase inhibitor), trametinib (MEK inhibitor) or everolimus (mTOR inihibitor) for 24 h. (B) Enrichment plots for RAS pathway related genes in neratinib treated parental vs. neratinib-resistant cells (C) Active-RAS pulldown assay in parental and neratinib resistant 5637 and OVCAR8 cells treated with neratinib for 6 hours. (D) Growth assay of neratinib treated cells transfected with indicated siRNA.

Clinical response to neratinib in phase 2 SUMMIT 'basket' trial



(A-C) Outcomes of patients enrolled in SUMMIT 'basket' trial based on mTOR pathway alteration status. Source: cBioPortal SUMMIT (Nature 2018). (D) Immunoblot analysis of DV90, SNUC2A, MCF7 HER2^{L755S} and HER2^{V777L} cells treated with indicated concentrations of neratinib, everolimus or combination for 24 hours. (E) Viability assay to test synergy between neratinib and everolimus. Cells were treated with increasing concentrations of single agents or drug combinations. Staining intensities were quantified colorimetrically and combination indices were determined using Chou-Talalay test. (F-H) mTOR pathway mutations acquired in cancers progressing on neratinib.

Conclusions

- Neratinib-resistant *HER2*-mutant cells remained cross-resistant to other HER2 targeting agents. RNAseq revealed significant enrichment of mTORC1 pathway in neratinib-resistant cells.
- Addition of the TORC1 inhibitor everolimus to neratinib as well as knockdown of RHEB or RPTOR overcame resistance to neratinib.
- mTORC1 activation in neratinib resistance cells was achieved, at-least in part through RAS upregulation. Knockdown of RAS suppressed S6 phosphorylation and restored sensitivity to neratinib.
- Patients with cancers harboring mTOR activating alterations did not exhibit clinical benefit from neratinib compared to those without mTOR activating alterations.
- Addition of TORC1 inhibitors may improve the activity of irreversible HER2 TKIs against cancers with HER2 activating mutations.