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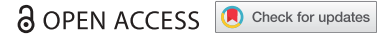
Novel mechanism of resistance to targeted therapies in lung cancer

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ABSTRACT
We have identified a non-canonical role of0 Notch3 in response to epidermal growth factor receptor (*EGFR*) tyrosine kinase inhibitor (TKI) therapy, whereby Notch3 associates with β-catenin, resulting in increased catenin beta-1 (CTNNB1, best known as β-catenin) stability and increased survival of drug persister cells (DPCs). Furthermore, combined treatment of an EGFR TKI with a β-catenin inhibitor demonstrated improved therapeutic outcomes in xenograft models.

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Lung cancer accounts for about 25 percent of cancer deaths each year in the United States, and is the leading cause of cancer-related deaths world-wide.[1] Approximately, 15 percent of non-small cell lung cancer (NSCLC) harbors "driver" mutations in epidermal growth factor receptor (EGFR), which dramatically respond to treatment with EGFR tyrosine kinase inhibitors (TKIs).[2] However, in these cases, resistance to EGFR TKI therapy inevitably develops, resulting in tumor recurrence. This resistance is partly due to pre-existing cellular subpopulations that harbor resistance mutations, such as *EGFR* T790M mutation, associated with resistance to erlotinib[3]. However, in most cases, resistance develops stochastically over time from a subpopulation of the original tumor that "persists" after TKI exposure. Previous work from our lab has demonstrated that treatment with erlotinib rapidly induces drug-persistent cells (DPCs) that have a stem cell-like phenotype, such as aldehyde dehydrogenase (ALDH) positivity.[4] That work also suggested that NOTCH3 (and not NOTCH1) plays an important role in mediating the development of DPCs, but is hard to selectively target.

Notch receptors are a family of evolutionarily conserved genes that play a critical role in differentiation, proliferation and cell death. Canonical Notch signaling culminates in the cleavage of the Notch intracellular domain (NICD), which translocates into the nucleus and interacts with the CSL (CBF1, Supressor of Hairless, Lag-1) transcription factor complex to transcribe target genes. In cancers, Notch has been shown to have both oncogenic and tumor-suppressive roles, depending on context.[5] In our recent work, we find that drug persistence by NOTCH3 is mediated by catenin beta-1 (CTNNB1, best known as β-catenin) induction by non-canonical NOTCH3 activation, in which it binds to and stabilizes β-catenin following EGFR TKI therapy, increasing the activity of β-catenin and resulting in the development of DPCs.[6]

In our recent work, we utilized a microarray analysis to determine differentially expressed genes between NOTCH3 and NOTCH1 knockdowns to identify genes that regulate drug persister cells. Surprisingly, canonical Notch transcripts such as Hes or Hey family members[7] were not identified; instead, we found an increase in transcriptional targets associated with β-catenin signaling, such as plasminogen activator inhibitor-1 (PAI1), specifically in a NOTCH3 dependent manner.[8] We then overexpressed dominant negative mastermind-like (DN-MAML1), an inhibitor of canonical Notch signalling,[7] and demonstrated that the induction of DPCs did not depend on canonical Notch activity. Protein co-immunoprecipitation and immunofluorescence studies showed that NOTCH3 physically bound to β-catenin in the cytoplasm, where the association led to stability of β-catenin and increased nuclear β-catenin accumulation and downstream target activation. Therefore, our results demonstrate a novel role of NOTCH3 in the induction of stem-like DPCs through the stabilization of and subsequent increase in β-catenin activity (Figure 1).

Examination of patient samples via immunohistochemistry before and after EGFR TKI therapy showed that both NOTCH3 and β-catenin exhibited increased expression after treatment, highlighting this important pathway in the development of resistance and recurrence that is connected to DPCs in patients.

Using xenograft tumor models, we were able to recapitulate the observed increases in NOTCH3 and β-catenin following EGFR TKI therapy. Furthermore, we observed a significant increase in survival and a decrease in tumor recurrence when treated with EGFR TKI in combination with ICG-001, a β-catenin inhibitor, compared to either drug alone.[9] These results indicate that combining EGFR TKI therapy with a β-catenin inhibitor may reduce overall morbidity and mortality in EGFR mutant lung cancers by inhibiting the generation of DPCs and reducing recurrence.
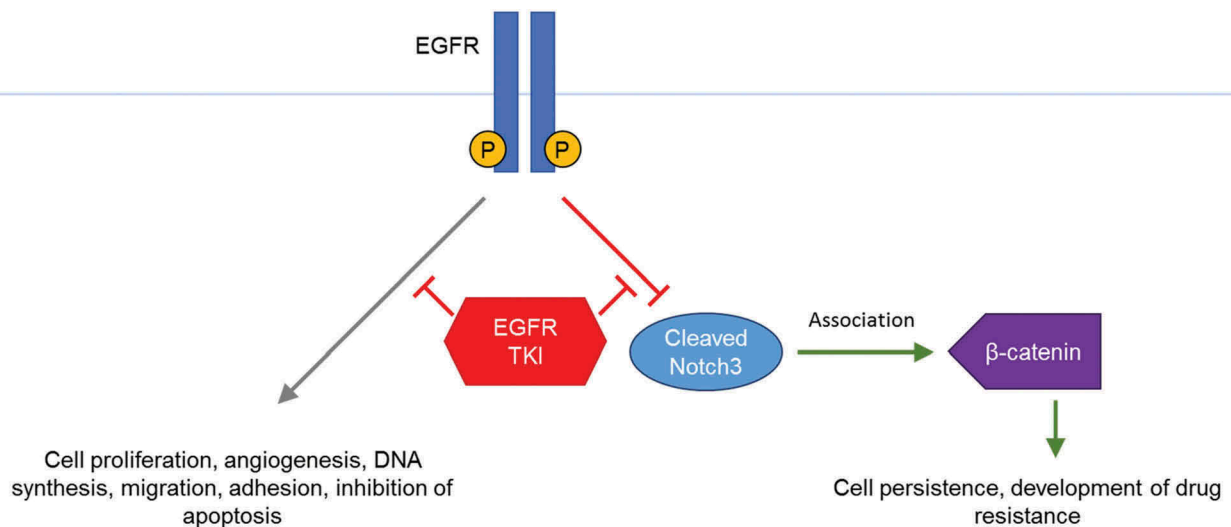

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Figure 1. Increased β-catenin activity and chemoresistance in lung cancer. Sustained epidermal growth factor receptor (EGFR) signaling in cancer cells leads to direct oncogenic effects and directly suppresses NOTCH3 activity. EGFR tyrosine kinase inhibitor (TKI) therapies inhibit EGFR mediated downstream signaling essential for cell proliferation, but also upregulate NOTCH3, stabilizing catenin beta-1 (CTNNB1, best known as β-catenin) and increasing downstream β-catenin signaling, leading to the development of drug persistent cells and enabling the acquisition of genetic changes necessary for the development of drug resistance. EGFR TKI therapy in conjunction with targeting β-catenin can prevent the development of persistent cells and represents a novel combination therapy that could be of therapeutic value to increase the depth and duration of response in patients with EGFR mutant non-small cell lung cancer (NSCLC).

Furthermore, transcriptional targets of β-catenin could be used as mechanism-related biomarkers to measure *in vivo* β-catenin activation. In our studies, we demonstrated that baseline and changes in levels of PAI1 were correlated with the development of resistance to EGFR TKIs. *In vitro* studies showed that levels of PAI1 secreted into the media increased after EGFR TKI therapy and diminished with combination therapy. Patient serum samples also had increased levels of PAI1 following EGFR TKI therapy, and high levels of PAI1 was associated with decreased progression-free survival in patients. Thus, it is possible that PAI1 could be used to assess response to therapy and be used to monitor the development of DPCs in real time in patients.

The non-canonical interaction between NOTCH3 and β-catenin after EGFR TKI therapy and the subsequent increase in β-catenin transcriptional activity is critical to the generation of DPCs and represents a novel actionable target that can improve patient outcomes by increasing the depth and duration of clinical responses to EGFR TKI therapy. Clinical trials of β-catenin inhibitors are already underway, and initial results indicate that they are well-tolerated.[10] Our study also suggests that PAI1 could be used to select patients most likely to benefit from this combination, monitor induction of β-catenin, and predict tumor recurrence.

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