Targeting HER2 Aberrations in Non-Small Cell Lung Cancer with Osimertinib

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Translational Relevance

Lung cancer is the leading cause of cancer mortality and therapies directly targeting HER2 aberrations in lung cancer remain an unmet clinical need. Here we generated three mouse models that recapitulated the clinical setting: HER2\textsuperscript{wt} as an oncogene driver, co-overexpression of HER2 with EGFR mutation and the activating HER2 mutation. Treatment studies using the third-generation tyrosine kinase inhibitor osimertinib demonstrated that not all HER2 aberrations should be treated equally. While osimertinib showed robust efficacy as a monotherapy for HER2\textsuperscript{wt}, its combination with BET inhibitor JQ1 was most efficacious for HER2 mutation. Therefore, our results not only provide a strong rationale for clinical evaluation of osimertinib against HER2-driven lung cancers, but also highlights the need to tailor treatment strategies for different HER2 aberrations.
Abstract:

**Purpose:** HER2 (or ERBB2) aberrations, including both amplification and mutations, have been classified as oncogenic drivers that contribute to 2-6 percent of lung adenocarcinomas. HER2 amplification is also an important mechanism for acquired resistance to EGFR tyrosine kinase inhibitors (TKIs). However, due to limited preclinical studies and clinical trials, currently there is still no available standard of care for lung cancer patients with HER2 aberrations. To fulfill the clinical need for targeting HER2 in non-small cell lung cancer (NSCLC) patients, we performed a comprehensive pre-clinical study to evaluate the efficacy of a third-generation TKI, osimertinib (AZD9291).

**Experimental Design:** Three genetically modified mouse models (GEMMs) mimicking individual HER2 alterations in NSCLC were generated and osimertinib was tested for its efficacy against these HER2 aberrations in vivo.

**Results:** Osimertinib treatment showed robust efficacy in HER2wt overexpression and EGFR del19/HER2 models but not in HER2 exon 20 insertion tumors. Interestingly, we further identified that combined treatment with osimertinib and the BET inhibitor JQ1 significantly increased the response rate in HER2-mutant NSCLC while JQ1 single treatment did not show efficacy.

**Conclusions:** Overall, our data indicated robust anti-tumor efficacy of osimertinib against multiple HER2 aberrations in lung cancer, either as a single agent or in combination with JQ1. Our study provides a strong rationale for future clinical trials using osimertinib either alone or in combination with epigenetic drugs to target aberrant HER2 in NSCLC patients.
Introduction

Lung cancer is the leading cause of cancer related mortality and non-small cell lung cancer (NSCLC) makes up about 85% of all lung cancers (1). There are three subtypes of NSCLC: adenocarcinoma, squamous cell carcinoma and large cell carcinoma (1). The rapid progress of targeted therapies appears mainly in lung adenocarcinomas with specific oncogenic drivers, especially EGFR and ALK mutations (2-4). For EGFR mutations, three generations of tyrosine kinase inhibitors (TKIs) have been developed and are currently being used in lung cancer treatment (5).

HER2 is another receptor tyrosine kinase in ErbB/HER family and forms heterodimers with other family members such as EGFR to activate downstream signaling (6,7). Compared with the recent clinical progress for EGFR TKIs, HER2 targeting remains an urgent clinical need in NSCLC. HER2 amplification and overexpression drive oncogenesis in several cancer types, such as breast, ovarian and gastric tumors (8). In breast cancers, targeted therapies such as trastuzumab and lapatinib are effective in clinic treatment (9). However, HER2 aberrations in lung cancer showed resistance to these treatments likely through tissue-specific mechanisms (10).

The current HER2 targeted therapies comprises of two groups, the TKIs and antibody based drugs. TKIs such as afatinib demonstrated efficacy from in vitro cell line assays (11,12) and our previous preclinical study had indicated that the combination with afatinib and rapamycin showed efficacy against lung tumor driven by HER2 exon 20 insertions(13). However, the clinical benefit of afatinib in HER2 positive lung cancer
patients remains unclear and more clinical trials are needed. Previous data indicated trastuzumab failed to demonstrate clinical benefit as a single therapy (14). Recently, two trastuzumab-based trials showed some promise for either the ado-trastuzumab-emtansine conjugate or trastuzumab/paclitaxel combination for HER2 positive lung cancers (15,16). Thus, new therapies need to be developed for lung cancer patients with HER2 aberrations.

HER2 aberrations found in NSCLC include both amplification and mutations and both lead to HER2 activation. While HER2 amplification and mutation (mainly in-frame exon 20 insertions) are found in 1-3% and 2-4%, respectively, of lung adenocarcinomas (1,17,18), they are typically not associated with each other (17,18). Rather, they are proposed to be clinically distinct driver alterations that can be used to subdivide lung adenocarcinoma patients for targeted therapy (18,19).

Osimertinib (AZD9291) is a third-generation TKI which irreversibly and specifically targets both sensitizing and the resistant T790M-mutated EGFRs (20). It has shown greater efficacy against EGFR T790M mutation than the standard platinum plus pemetrexed therapy and was thus recently fully approved by the FDA for metastatic EGFR T790M-positive NSCLC (21). Osimertinib covalently binds the cysteine-797 residue of both sensitizing and T790M mutation of EGFR but spares the wildtype form (20). This binding specificity leads to only mild side effects in a minority of patients as opposed to earlier generation TKIs that may cause severe toxicity due to their wildtype EGFR targeting (22).
When its targeting selectivity was explored, osimertinib was tested against a panel of 280 kinases and interestingly, this assay identified a limited number of kinases which could be inhibited by osimertinib, including HER2, HER4, ACK1, ALK, BLK et al (20). Considering the homology between HER2 and EGFR, we speculate that the covalent binding site for osimertinib may be C805 (analogous Cys797 to EGFR) of human HER2, which requires future investigation. Further cell line assays confirmed that HER2 could be targeted by osimertinib in vitro, implicating it as a potential HER2 targeting agent (20). However, it remains unknown whether osimertinib could demonstrate an in vivo anti-tumor efficacy against different HER2 aberrations in NSCLC.

Besides its role as an oncogenic driver, HER2 amplification is one of the major mechanisms of acquired resistance to first-generation TKIs in EGFR mutant lung cancers (12,23). Despite progress in EGFR-targeted therapy in lung cancers, intrinsic and acquired resistance remains a significant clinical challenge (12,23). EGFR T790M mutation is the most frequent event in acquired EGFR TKI resistance; HER2 amplification ranks second (12). While osimertinib could efficiently overcome T790M mediated EGFR TKI resistance, its efficacy remains to be explored against other resistance mechanisms, such as HER2 amplification.

Additionally, epigenetic therapy has become increasingly promising as a new treatment strategy in NSCLC (24). Recent studies have highlighted the abnormal epigenetic changes in many cancer types, and thus novel drugs targeting epigenetic modifiers have been developed (24,25). There are three subsets of epigenetic modifiers: writers, readers and erasers (24). Among the epigenetic readers, BET family members could recognize lysine acetylation of histones and are involved in chromatin remodeling
Multiple BET inhibitors have shown robust anti-tumor effects in different cancer types (26-28). Moreover, emerging evidence suggests that BET inhibitors could synergize with TKIs to boost anti-tumor responsiveness in a variety of cancer types (29-31). In this study, we also aimed to explore whether BET inhibitors could overcome TKI resistance in \textit{HER2} aberrations.

Here, we designed a comprehensive preclinical study including individual \textit{HER2} alterations to test their responsiveness to osimertinib treatment, hoping to shed light on future HER2-targeted lung cancer therapeutics. Given that a prior study has shown that the BET inhibitor (BETi) JQ1 can boost lapatinib efficacy in HER2 positive breast cancer (30), we also explored the question of whether JQ1 combination treatment could enhance the anti-tumor response to osimertinib treatment in NSCLC.

\textbf{Materials and Methods}

\textbf{GEMM generation}

The procedure to generate the tet-op-h\textit{HER2} mouse cohort was described before (13,32). In short, a transgene DNA construct consisting of seven repeats of tetracycline operator, the wild-type human \textit{HER2} gene, and the SV40 poly (A) was injected into FVB/N blastocysts. PCR targeting the transgene was used to screen positive progeny. Tet-op-h\textit{HER2} mice were crossed to Clara cell secretory protein (CCSP)-rtTA mice to obtain a tet-op-h\textit{HER2}/CCSP-rtTA (HW) colony. The HW colony was fed with continuous doxycycline diet from at least 6 weeks of age. CCSP-rtTA/Tet-op-hEGFR Del-Luc and tet-op h\textit{HER2}\textsuperscript{YVMA}/CCSP-rtTA cohorts were generated as previously described (13,33). All mouse breeding and treatment experiments were performed with the approval of DFCI Animal Care and Use Committee.

\textbf{Magnetic Resonance Imaging (MRI) and tumor volume quantification}
Lung tumors were monitored by MRI and 3D slicer was used to quantify the lung tumors as described before (34-36).

Cell lines
NCI-H1781 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI-1640 medium containing 10% FBS, 100 units/mL of penicillin and 100 ug/mL of streptomycin. Ba/F3-HER2\textsuperscript{wt} and Ba/F3-HER2\textsuperscript{YVMA} cells were generated and maintained as described (37).

CCK8 assay
2,000 Ba/F3 cells stably expressing HER2\textsuperscript{wt} or HER2\textsuperscript{YVMA} were plated into 96-well plates. Then either erlotinib or osimertinib was added the following day with indicated concentrations. After 3 days, the CCK8 (Dojindo Laboratories) was added to each well and OD450 was measured after 1-4 hours.

Western blotting
Ba/F3 or H1781 cells with erlotinib or osimertinib treatment were lysed with RIPA buffer with Halt™ Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) and Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific). Frozen lung tumor nodules were homogenized in the same lysis buffer. 20-40 µg of lysates were loaded on a NuPAGE 4-12% Bis-Tris protein gel. After transfer to PVDF membrane, western blots were probed with Phospho-HER2/ErbB2 (Tyr1221/1222) (6B12), HER2/ErbB2 (29D8), Phospho-Akt (Ser473) (D9E) XP®, Akt (#9272), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP®, p44/42 MAPK (Erk1/2) (137F5), c-Myc (#9402), p21 Waf1/Cip1 (2947T) and β-actin (#4967) antibodies (all from Cell Signaling Technology). Then the blots were developed with ECL-Plus kits (GE healthcare) (38-40).

IHC and H&E staining
Mice were euthanized and lung tissues were collected and fixed with 10% formalin. Immunohistochemistry staining was performed as described (13,35), using the following antibodies: HER2 (Cell Signaling Technology, #2165), Phospho-HER2 (Tyr1221/1222) (Cell Signaling Technology, #2243), TTF1 ( Epitomics; 5883-1), SOX2 (EMD Millipore;
AB5603), p63 (Abcam, ab53039), EGFR (Cell Signaling Technology, #4267), Phospho-EGFR (Tyr1068) (Cell Signaling Technology, #3777).

**Treatment study**

HW, DH and SH26 mice were fed with doxycycline diets and lung tumors were monitored by MRI (34). Tumor-bearing mice were treated with erlotinib (selleckchem, #S7786), osimertinib (AstraZeneca), afatinib (selleckchem, #S1011) or JQ1 and tracked by MRI every two weeks. Erlotinib (in 0.5% HPMC) was dosed at 50 mg/kg, afatinib (in 0.5% HPMC) was dosed at 20 mg/kg and osimertinib (in 0.5% HPMC) was dosed at 25 mg/kg daily all by oral gavage. JQ1, provided by Dr. Jun Qi and Dr. James Bradner (Dana Farber Cancer Institute, Boston, MA), was prepared in 10% DMSO (Thermo Fisher Scientific) and further diluted 1:10 with 10% 2-hydroxylpropyl β-cyclodextrin (Sigma). JQ1 was dosed to mice intraperitoneally (I.P.) at 50 mg/kg daily.

**Fluorescent in situ hybridization (FISH)**

The peripheral blood sample from the HW mouse was prepared for FISH by direct preparation (no culture or stimulation) with standard cytogenetic methods, and an air-dried slide with nuclei was prepared and hybridized according to instructions provided with the commercial probes. A custom dual-color FISH probe was used to evaluate the presence and copy number of human HER2 in the blood sample using a commercial genomic probe for human HER2 (Abbott Molecular, labeled in orange/red, R) and as an internal control, a commercial mouse probe for D15Mit224, a single-copy region on chromosome 15 (ID Labs, labeled in green, G).

One hundred nuclei were scored, 50 cells each by two observers. All scorable nuclei had at least one orange and one green signal; the green (internal control) probe gave the expected 2 signal pattern in 92/100 cells, reflecting an excellent hybridization efficiency.

**Copy number variation analysis using TaqMan assays**

Genomic DNA was prepared from ear tissues from HW mice using Quick-DNA Miniprep Kit (Zymo Research) and used as templates in following real-time qPCR assays.
Standard real-time qPCR was performed following the vendor's instruction for copy number assays. Taqman copy number assay targeting human HER2 (Thermo Fisher Scientific; assay ID Hs01074948_cn) was used together with TaqMan copy number reference assay (Thermo Fisher Scientific; mouse, Tert, #4458368).

**Statistical analysis**

Data were analyzed using mean ± standard error of the mean (SEM). Student’s t-test was used for comparisons between two groups using GraphPad Prism software. P values < 0.05 were considered statistically significant (*); P values < 0.01 are marked **, and P values < 0.001 are marked ***.

**Results**

**Generation and characterization of doxycycline induced lung-specific hHER2\(^{wt}\) GEMM**

Previous data has shown the *in vitro* efficacy of osimertinib against wildtype HER2 with cell line assays (20). To confirm this *in vitro* efficacy and test whether osimertinib can target \(HER2^{wt}\), we overexpressed wild-type human HER2 in Ba/F3 cells (37). Treatment of Ba/F3-hHER2\(^{wt}\) cells with either osimertinib or first-generation TKI erlotinib for 6 hours showed that while erlotinib did not inhibit HER2 phosphorylation at up to 500 nM, osimertinib demonstrated potent activity in a dose-dependent manner from a concentration of 100 nM, confirming that osimertinib indeed targets human wildtype HER2 (Fig. 1A).
We further treated Ba/F3-hHER2<sup>wt</sup> cells with either osimertinib or erlotinib for 72 hours to calculate growth inhibition (GI50) and found that osimertinib achieved a significantly lower GI50 (10.4 nM) compared to erlotinib (438 nM) (Fig. 1 B). Taken together, these results confirmed the efficacy of osimertinib against human HER2 and efficiently inhibited HER2 phosphorylation at a low dose in vitro.

To study the in vivo role of osimertinib against hHER2, we first generated a tetO-hHER2 transgenic mouse founder by injecting into FVB/N blastocysts a 4.75-kb DNA segment containing seven direct repeats of the tetracycline operator sequence followed by wild-type human HER2 ORF (open reading frame) and SV40 polyA(32) (Fig. 1 C; top). The tetO-HER2 mouse founders were bred with CCSP-rtTA mice to generate the inducible lung-specific bitransgenic hHER2<sup>wt</sup>/CCSP-rtTA (HW) cohorts, which harbor both activator and responder transgenes (Fig. 1 C; bottom). To confirm that the hHER2<sup>wt</sup> was integrated into mouse genome, we performed fluorescence in situ hybridization (FISH) using blood sample collected from HW mouse. FISH analysis showed that there were two copies of human HER2 in HW mouse genome (supplementary Fig. S1A and S1B). Real-time qPCR assays also confirmed two copies of hHER2, using mouse TERT gene as a control (supplementary Fig. S1B). We further aimed to check hHER2 expression at protein level with doxy induction. Lungs of the doxycycline (doxy)-induced HW mice exhibited hHER2 expression after 5 weeks with doxy induction, and HER2 phosphorylation (pHER2) increased over time (Fig. 1 D). To validate whether expression of HER2 was dependent on doxycycline, the doxy food was switched into normal diet for 3 days. After 3-day doxy removal, hHER2 expression was almost undetectable, confirming hHER2 expression was doxy-dependent (Fig. 1 D).
To validate whether hHER2 expression can initiate lung cancer development, HW mice were fed with a continuous doxycycline diet and monitored by lung magnetic resonance imaging (MRI). Tumors began to form after 6 weeks and developed into high grade tumors after 16 weeks (Fig. 1E). Immunohistochemistry (IHC) of HW tumors revealed high levels of HER2 and pHER2 (Fig. 1F, upper panel). We also examined markers and found these HW tumors showed adenocarcinoma features with positive staining for TTF1 and low expression of SOX2 and p63(13,35,41) (Fig. 1F, lower panel). These histology features indicated the HW model mimicked the clinical setting as most lung cancers with HER2 aberrations are indeed adenocarcinomas. Having clarified that hHER2 could drive de novo tumorigenesis of lung adenocarcinomas, we further explored whether HER2 was also required for tumor maintenance. The doxycycline diet was replaced with normal food after tumor formation, and tumors disappeared two weeks after doxycycline diet removal (Fig. 1G). This confirmed HER2 was both an oncogenic driver in lung cancer and was required for tumor maintenance. Continuous MRI monitoring showed tumors developed faster 10 weeks after induction (supplementary Fig. S2 A), and mice had a median survival of 19.4 weeks after doxycycline induction (supplementary Fig. S2 B).

**In vivo anti-tumor efficacy of osimertinib in HER2wt GEMM.**

The HW mice were fed with doxycycline food and lung tumors were monitored by MRI. Osimertinib was then administered orally at 25 mg/kg daily, an equivalent dose to clinical 80mg QD in tumor-bearing HW mice (42). Osimertinib treatment was efficacious after four weeks; in contrast, erlotinib and afatinib demonstrated limited anti-tumor response (Fig. 2A-B). Both vehicle and erlotinib treated mice showed progressive
disease by the 4-week time point (PD: more than 20% increase in tumor volume compared to baseline) and afatinib treated mice remained in stable disease (SD: between 30% decrease and 20% increase in tumor volume change). In contrast, all mice (n=7) treated with osimertinib showed significant tumor regression with mice achieving up to an 80% decrease in tumor volume compared to baseline (Fig. 2 B).

To investigate whether osimertinib targeted HER2 signaling in vivo, we next performed a pharmacodynamic study. The tumor-bearing HW mice were dosed with HPMC, osimertinib or erlotinib for 3 days and then tumor nodules were collected. The tissue lysates were used for HER2 signaling analysis with western blot. Osimertinib effectively abolished HER2 phosphorylation and inhibited major downstream signaling targets such as pAKT and pERK (Fig. 2 C). This pharmacodynamic data indicated the on-target efficacy of osimertinib against wildtype HER2 in vivo.

**Long-term survival benefit with osimertinib treatment in HER2wt mice**

To further test whether osimertinib could maintain a durable anti-tumor response in HW mice, we performed long-term treatment with osimertinib or erlotinib in HW mice and monitored the tumor volume by lung MRI every two weeks. Osimertinib showed continuous anti-tumor efficacy for 16 weeks (Fig. 3 A). The HW mice treated with osimertinib showed significantly longer progression-free survival (PFS) and overall survival (OS) compared to those treated with erlotinib (Fig. 3 B-C). This data demonstrated a long-term survival benefit for HW tumors with osimertinib treatment, which was consistent with the short-term efficacy and pharmacodynamic results. Taken
together, osimertinib treatment showed an on-target, efficacious and durable anti-tumor effect against HER2 in HW tumor model.

**In vivo anti-tumor efficacy of osimertinib in EGFR<sup>del19</sup>/HER2<sup>wt</sup> GEMMs**

hHER2<sup>wt</sup> overexpression was proven to be an oncogenic driver in lung cancer based on the HW mice model. Besides, it was also identified as an important mechanism underlying acquired resistance to TKIs such as erlotinib in EGFR mutant lung cancer patients(12). Having clarified the anti-tumor efficacy of osimertinib against hHER2 as a driver oncogene in the HW model, we further tested osimertinib against co-expression of hHER2 in EGFR-mutant lung cancers.

There was no available GEMMs which could mimic the clinical setting of HER2 amplification-mediated acquired resistance in EGFR mutant lung cancers. We first treated CCSP-rtTA/Tet-op-hEGFR Del19-Luc (Del19) mice (33) with osimertinib or erlotinib. As expected, both drugs demonstrated robust anti-tumor efficacy after 4 weeks of treatment (supplementary Fig. S3). Next, we crossed Del19 mice with HW mice to produce tritransgenic tet-op EGFR-del19/hHER2<sup>wt</sup>/CCSP-rtTA (Del19HW; DH) mice (Fig. 4 A). In this model, lung tumors were co-driven by both EGFR<sup>del19</sup> and HER2<sup>wt</sup>. Osimertinib efficiently inhibited DH tumors after two weeks (Fig. 4 B). Erlotinib reduced tumors after two weeks as well, but tumors relapsed quickly after initial response (Fig. 4 B). To check the expression level of both oncogenes after treatment, pEGFR, HER2 and pHER2 were compared in the DH mice either before treatment or after relapse with erlotinib treatment with IHC. Two representative DH mouse lungs were examined: one mouse before erlotinib treatment and another treated with erlotinib for 12 weeks. While
the pEGFR level was high, HER2 expression remained low before treatment (Fig. 4 C; top panel). After relapse, pEGFR was nearly undetectable while HER2 and pHER2 levels increased significantly (Fig. 4 C; bottom panel). Furthermore, we monitored the long-term efficacy for osimertinib treatment in DH mice and the PFS was significantly improved with osimertinib treatment (Fig. 4 D). Taken together, these data indicated that osimertinib can also target HER2 together with an activating EGFR mutation.

**Anti-tumor efficacy of combined osimertinib and JQ1 treatment against HER2 exon 20 insertions**

Previous *in vitro* data showed modest efficacy of osimertinib against HER2 exon 20 insertion (20). Having clarified the robust efficacy of osimertinib against HER2 amplification, we further tested its effect against HER2 exon 20 insertion mutations. Ba/F3 cells stably expressing A775_G776insYVMA HER2 (Ba/F3-HER2<sub>YVMA</sub>), the most frequent exon 20 insertion, were treated with osimertinib or erlotinib for 6 hours. Osimertinib inhibited pHER2 at 500 nM, while erlotinib did not (Fig. 5 A). We also performed a 3-day proliferation assay and found osimertinib suppressed Ba/F3-HER2<sub>YVMA</sub> at a much lower concentration (GI50=44 nM) compared to erlotinib (Fig. 5 B). However, HER2<sub>YVMA</sub> (GI50=44 nM) was less sensitive than HER2<sub>wt</sub> (GI50=10.4 nM). Because Ba/F3 is a murine pro-B cell line, we next detected the inhibitory role of osimertinib in human lung cancer lines with HER2 mutations.

H1781 cells, a human lung cancer cell line harboring another exon 20 insertion (13), HER2<sub>G776insV_G/C</sub>, were treated with osimertinib for various times, from 6 hours to 5 days. Osimertinib significantly inhibited pHER2 at 500 nM at all time points. Of note, total
HER2 levels significantly increased with osimertinib treatment in a dose-dependent manner after one day (Fig. 5 C, lane 1-3). Downstream phosphorylation of AKT and ERK was also suppressed by osimertinib treatment (Fig. 5 C, lane 1-3). Interestingly, MYC was also inhibited by osimertinib at all time points, and p21 was reduced by osimertinib after 5 days (Fig. 5 C, lane 1-3). Considering both MYC and p21 are downstream targets of BET inhibitor JQ1 (27,28), we investigated whether JQ1 and osimertinib combination could further decrease HER2 signaling. Compared with osimertinib single treatment, H1781 cells treated with osimertinib and JQ1 combination demonstrated marked reduction in phosphorylation of HER2, AKT and ERK (Fig. 5 C, lane 4-6). Moreover, combination treatment downregulated total HER2 and MYC levels while upregulating p21 (Fig. 5 C, lane 4-6). We also treated H1781 cells with osimertinib with and without JQ1 for 5 days. JQ1 further inhibited cell proliferation when combined with different doses of osimertinib, suggesting that JQ1 and osimertinib combination could suppress HER2 exon 20 insertion in H1781 cells (Fig. 5 D).

**Osimertinib and JQ1 combination treatment against HER2^{YVMA} in vivo**

Tet-op hER2^{YVMA}/CCSP-rtTA (SH26) mice were generated and used for preclinical study in our previous research as described (13). To test osimertinib and JQ1 combination in vivo we treated tumor-bearing SH26 mice with either single agent or the two in combination. After two weeks, neither osimertinib nor JQ1 alone showed efficacy, but combination treatment led to significant tumor regression (Fig. 6 A). After 4 weeks, only 3 out of 8 mice showed tumor regression with osimertinib treatment (Fig. 6 B). In contrast, JQ1 and osimertinib combination showed a better anti-tumor benefit than single treatments (Fig. 6 B). Moreover, the long-term treatment study indicated the PFS...
was greatly improved with JQ1 and osimertinib combination, compared with single-agent treatments in SH26 mice (Fig. 6 C). These data provided the first in vivo evidence that although HER2 mutant tumors were resistant to osimertinib and JQ1 as single agents, they became vulnerable when treated with their combination.

Discussion

Both HER2 and EGFR (or HER1) belong to the ErbB / HER tyrosine kinase family which are activated by ligand binding and receptor dimerization (6). Despite the rapid progress of EGFR targeted therapy (2,25), HER2 targeting remains an urgent clinical challenge in lung cancer. In this study, we clarified the unique response signature of lung cancer HER2 alterations to the FDA-approved third-generation TKI, osimertinib, using three GEMMs. Both HER2 overexpression events, either as an oncogenic driver itself or as a concurrent event with EGFR mutation, were effectively targeted by osimertinib while HER2 exon 20 insertions were resistant to osimertinib single-treatment in vivo. Our findings demonstrate for the first time that the BET inhibitor JQ1 could synergize with osimertinib to inhibit tumor growth of HER2-mutated lung cancers. Our study implicates the need to subdivide the lung cancer patients carrying aberrant HER2 for osimertinib.

Irreversible dual EGFR/HER2 inhibitors (HKIs) such as afatinib, neratinib and dacomitinib have recently been tested in lung cancer patients with HER2 aberrations and demonstrated partial response in a few patients with HER2 exon 20 insertions (19,43,44). But due to the limited patient number and low response rate within the small population, the overall response for each HER2 alteration subtype remains unclear. Moreover, considering that HER2 amplification has only recently been considered as an
oncogenic driver in NSCLC (17,18), previous clinical studies were predominantly focused on HER2 mutations, especially the exon 20 insertions. Another restriction to the pre-clinical study in HER2-altered lung cancer is the shortage of available lung cancer lines. Compared with other driver mutations such as KRAS and EGFR mutations, human cell line models with HER2 amplification and exon 20 insertions are very limited. The H1781 is one of the most commonly used cell lines for HER2-mutation-driven lung cancer research. Considering the shortage of lung cancer cell lines with HER2 aberrations, generating mouse models that mimic individual clinical presentations is of great translational significance. Our study provides an invaluable tool to study different HER2 aberrations in lung cancer under a tissue-specific activation system.

The role of HER2 amplification as a lung cancer driver was identified from a recent cancer genomic study (17). To our knowledge, we provided the first in vivo evidence that HER2 overexpression drives de novo tumorigenesis of lung adenocarcinomas. Moreover, we also generated a unique DH model by crossing HW mice with EGFR-del19 mice and this DH strain closely mimicked HER2 overexpression in some EGFR-mutant tumors with acquired resistance to first-generation TKIs. These two novel HER2 strains, provided the first available GEMM tools to test therapeutics against HER2wt in NSCLCs. HER2 was proposed as a potential resistance mechanism to osimertinib in EGFR T790M tumors (45), but the potency of osimertinib against HW tumors demonstrated in our study may not support this hypothesis. It was also noteworthy that although osimertinib demonstrated a robust and durable anti-tumor response in HW mice model, acquired resistance to osimertinib developed after long-term treatment, which ultimately led to the death of HW mice. The mechanism underlying this resistance
needs further investigation, especially when osimertinib is used in clinic for patients with HER2 amplification. It would be interesting to address whether BRD4 inhibitors can overcome the resistance to osimertinib in HW mouse model.

The potent efficacy of osimertinib against HER2\textsuperscript{wt} also provides a rationale to test it in other cancers with HER2 amplification or overexpression, such as breast cancer. HER2 positive breast cancer makes up about 20% of this cancer type and the HKI lapatinib is widely used in combination therapy for this subset of patients (9). However, it may cause severe side effects such as diarrhea in a small proportion of patients (9). Considering the great efficacy of osimertinib as a single agent with minimum side effects, it may also benefit HER2 positive breast cancer treatment.

Compared with HER2\textsuperscript{wt}, osimertinib alone had limited efficacy against HER2 exon 20 insertions in vivo and we explored whether combination treatment may overcome this resistance to osimertinib. Previous studies have shown that BET inhibitor JQ1 could synergize with multiple TKIs in different cancer types (29-31). In a subset of AML, JQ1 synergized with FLT3 TKI ponatinib to attenuate c-Myc, Bcl-2, CDK4/6 and increase p21, BIM and cPARP, thus inducing significant AML apoptosis (29). In HER2-driven breast cancer, HKI lapatinib was found to induce expression of multiple kinases and reprogram the kinome, which could contribute to drug resistance (30). However, JQ1 could suppress the kinase induction and kinome adaptation, thus making lapatinib response more durable (30). Our previous studies showed JQ1 could both target Kras tumors and play an immunoregulatory role in NSCLC(46,47). Here we showed that JQ1 could also synergize with osimertinib against HER2 exon20 insertions by attenuating HER2 re-expression and myc-mediated downstream signaling. We also demonstrated
that the combination treatment reversed osimertinib-induced downregulation of the senescence marker p21. BET inhibitors are an important group of epigenetic readers and currently there are multiple BET inhibitors in clinical trials for different cancer types, including lung cancer. It will be interesting to understand the mechanism of the BETi-TKI synergy at the epigenetic, transcriptional and metabolic levels in HER2-driven lung cancers.

HER2 exon 20 insertions share structure analogy with EGFR exon 20 insertions, which comprise 4-10% of all EGFR mutations in lung cancer (37,48). Most EGFR mutations, including exon 19 deletion and L858R mutation, are sensitizing mutations which are vulnerable to current TKIs. Other rare mutations, including exon 20 insertions, are generally resistant to current TKIs (23,37,48,49). Similar to HER2, exon 20 insertions in EGFR also render it resistant to osimertinib as in vitro assays have indicated (20). Thus, our identification that BRD4 inhibitor JQ1 could synergize with osimertinib to overcome the resistance revealed the importance to test the combination of TKIs with BRD4 inhibitors in the current TKI-resistant tumors with EGFR exon 20 insertions.

Taken together, our results provided a strong rationale to test osimertinib as a single agent in lung cancer patients with HER2 amplification and as combinational therapies against HER2 mutations. The HER2 GEMMs used here are also invaluable preclinical tools to evaluate other future drug regimens against individual HER2 aberrations in lung cancer. Besides lung cancer, it is also worthwhile to evaluate osimertinib efficacy in other HER2-driven tumor types such as breast cancer.
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Author Contributions

S.L., S.L., D.A.E.C and K.K.W. designed the study; S.L., S.L., J.H. conducted the experiments and acquired the data; X.W., T.C., M.M.Q, P.G. and Y.Z provided technical assistance; H.J. generated animals; S.L. and K.K.W. wrote the manuscript; all authors reviewed and contributed to the revision of the manuscript.
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Figure legends

**Fig. 1. Overexpression of hHER2 drives development of lung adenocarcinoma.**

(A) Ba/F3-HER2<sup>wt</sup> cells stably expressing wild-type human HER2 were treated with either osimertinib or erlotinib for 6 hours at indicated concentrations before HER2 phosphorylation was detected. (B) Ba/F3-HER2<sup>wt</sup> cells were plated into 96-well plates and treated with osimertinib and erlotinib for 72 hours, and growth inhibition rate (GI50) was calculated based on CCK8 assay. (C) Schematic of transgene used to generate tet-op-hHER2 cohort and breeding strategy into tet-op-hHER2/CCSP-rtTA (HW) mice. (D) HW mice were fed with either normal diet or with doxycycline food for 1 week, 5 weeks or 11 weeks, or 5 weeks of doxycycline then switched into normal diet for 3 days. HER2 expression and phosphorylation were detected in whole lung lysate samples from these mice. (E) Representative MRI images of HW mice fed with doxycycline food for 6, 12 and 16 weeks. (F) H&E staining and IHC analysis for HER2 and phosphorylated HER2, and adeno/squamous markers TTF1, SOX2 and p63. Scale bar: 100um. (G) representative MRI image of an HW mouse fed with doxycycline food for 14 weeks and then switched to normal food for 2 weeks.

**Fig. 2. Osimertinib induces tumor regression in HER2-wildtype GEMMs.**

(A) Representative MRI image of HW mice before and after treatment with vehicle, erlotinib, osimertinib or afatinib for 4 weeks. (B) Waterfall plots showed tumor volume change compared with before treatment, and each column represented one individual mouse. (C) HW mice were treated with vehicle, osimertinib or erlotinib for 3 days and tumor nodules were collected and lysates were used in western blot to detect HER2 phosphorylation and downstream signaling. Two representative tumor samples from each group are shown.

**Fig. 3. Osimertinib demonstrates long-term survival benefit in HER2-wildtype lung cancer.**

(A) Long-term monitoring of tumor volume change with erlotinib or osimertinib in HW mice. (B) PFS of HW mice with each treatment. (C) OS of HW mice with each treatment.
Fig. 4. Osimertinib induces regression in lung tumors co-driven by both EGFR<sup>del</sup><sup>19</sup> and HER2<sup>wt</sup>.

(A) Breeding scheme of DH mice. (B) Long-term tumor change for DH mice following treatment with vehicle, erlotinib or osimertinib. (C) Lungs from two representative mice, one without treatment and another treated with erlotinib for 12 weeks, were harvested. pEGFR, HER2 and pHER2 were examined by immunohistochemistry. Scale bar: 100um. (D) PFS of DH mice treated with vehicle, erlotinib or osimertinib.

Fig. 5. Combined osimertinib and JQ1 therapy suppresses NSCLC with HER2 exon 20 insertions in vitro.

(A) Ba/F3-HER2<sup>YVMA</sup> cells stably expressing human HER2 exon 20 insertion (YVMA) were treated with either osimertinib or erlotinib for 6 hours at indicated concentrations, then HER2 phosphorylation was detected. (B) Ba/F3-HER2<sup>YVMA</sup> cells were plated into 96-well plates and treated with osimertinib and erlotinib for three days. Growth inhibition rate (GI50) was calculated based on CCK8 assay. (C) H1781 cells were treated with osimertinib and/or JQ1 for indicated time, then HER2 signaling was evaluated. (D) H1781 cells were treated with osimertinib and/or JQ1 for 5 days, and growth rate was measured with CCK8.

Fig. 6. Osimertinib and JQ1 combination induces tumor regression and long-term survival benefit in HER2<sup>YVMA</sup> GEMMs

(A-B) SH26 mice were treated with osimertinib, JQ1 or their combination for 2 weeks (A) or for 4 (B) weeks, and tumor volume change was calculated compared with before treatment based on MRI quantification. (C) PFS of SH26 mice treated with vehicle, osimertinib, JQ1 or combination. P values < 0.05 were considered statistically significant (*); P values < 0.01 are marked **, and P values < 0.001 are marked ***.
**Fig 1**

A. Western blot analysis showing the expression of p-HER2, HER2, and β-actin. The samples were treated with DMSO, Osimertinib, and Erlotinib at various concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 1.0, and 0.1, 0.5 nM).

B. Graph showing cell viability (%) of Ba/F3-HER2 wt cells treated with different concentrations of Erlotinib and Osimertinib. The x-axis represents the concentration (nM) of the drugs, and the y-axis represents cell viability (%). The graph shows a decrease in cell viability with increasing drug concentration.

C. Diagram illustrating the Tet-off system with Tet-op-hHER2 transgene, Tet Operator, Human HER2, and Poly A. The system is activated by Doxy on and inhibited by Doxy off.

D. Western blot analysis showing the expression of p-HER2, HER2, and β-actin under Doxy treated conditions at 6 weeks, 12 weeks, and 16 weeks.

E. Images showing the progression of tumor growth over 6, 12, and 16 weeks under Doxy treated conditions.

F. Immunohistochemical stains for H&E, HER2, p-HER2, TTF1, p63, and SOX2.

G. Images showing the effect of Doxy on and Doxy off after 2 weeks.
Fig 2

A 0 week 4 weeks

Vehicle
Erlotinib
Osimertinib
Afatinib

B

Tumor Volume Change (%)

Vehicle
Erlotinib
Osimertinib
Afatinib

C

Vehicle Osi Erl

p-HER2 HER2 pAKT
AKT pERK ERK β-actin

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Fig 3

A

Tumor Volume Change (%)
Weeks after treatment

Erlotinib
Osimertinib

B

PFS
Weeks after treatment
Vehicle
Erlotinib
Osimertinib

C

OS
Weeks after treatment
Vehicle
Erlotinib
Osimertinib
Fig 4

A

\[
\text{tet-op-hHER2}(+/-) \quad \times \quad \text{Tet-op-hEGFR Del}(+/-) \\
\text{CCSP-rtTA}(+/+) \quad \text{CCSP-rtTA}(+/+) \\
\text{Del}(+/+)/\text{CCSP-rtTA}(+/+) \\
\text{DH}
\]

B

-\text{Tumor volume (percentage)}

Weeks after treatment

C

\[
\begin{array}{ccc}
\text{pEGFR} & \text{HER2} & \text{pHER2} \\
\text{Pre-treatment} & \text{12 weeks} \\
\end{array}
\]

D

-\text{PFS}

Weeks after treatment

\begin{align*}
\text{Vehicle} & \quad \text{Erlotinib} \\
\text{Osimertinib}
\end{align*}
Fig 5

A

B

C

D

Fig 5

A

B

C

D
**Fig 6**

A. *2 weeks*

B. *4 weeks*

C. Long-term survival

- **Vehicle**
- **JQ1**
- **Osimertinib**
- **Osimertinib + JQ1**

[Graphs showing tumor volume change and long-term survival data with statistical significance indicated by asterisks.]
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