Clinical Cancer Research

# Targeting *HER2* Aberrations in Non–Small Cell Lung Cancer with Osimertinib

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## Abstract

**Purpose:** *HER2* (or *ERBB2*) aberrations, including both amplification and mutations, have been classified as oncogenic drivers that contribute to 2% to 6% of lung adenocarcinomas. *HER2* amplification is also an important mechanism for acquired resistance to EGFR tyrosine kinase inhibitors (TKI). 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 patients with non–small cell lung cancer (NSCLC), we performed a comprehensive preclinical study to evaluate the efficacy of a third-generation TKI, osimertinib (AZD9291).

**Experimental Design:** Three genetically modified mouse models (GEMM) 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 $HER2^{wt}$ 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, whereas JQ1 single treatment did not show efficacy.

**Conclusions:** Overall, our data indicated robust antitumor 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 patients with NSCLC. *Clin Cancer Res; 24(11); 2594–604. ©2018 AACR.*

*See related commentary by Cappuzzo and Landi, p. 2470*

## 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 (TKI) have been developed and are currently being used in lung cancer treatment (5).

HER2 is another receptor tyrosine kinase in the 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 is comprised of two groups: TKIs and antibody-based drugs. TKIs such as afatinib demonstrated efficacy from *in vitro* cell line assays (11, 12), and our previous preclinical study indicated that the combination with afatinib and rapamycin showed efficacy against lung tumors driven by *HER2* exon 20 insertions (13). However, the clinical benefit of afatinib in patients with HER2-positive lung cancer remains unclear, and more clinical trials are needed. Previous data indicated that 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. Although *HER2* amplification and mutation (mainly in-frame exon 20 insertions) are found in 1% to 3% and 2% to 4% of lung adenocarcinomas,

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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: $HER2^{wt}$ as an oncogene driver and 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. Although osimertinib showed robust efficacy as a monotherapy for $HER2^{wt}$, its combination with the 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 highlight the need to tailor treatment strategies for different *HER2* aberrations.

respectively, (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 that irreversibly and specifically targets both sensitizing and 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 mutations of EGFR but spares the wild-type 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 wild-type 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 that could be inhibited by osimertinib, including HER2, HER4, ACK1, ALK, and BLK (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* antitumor 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). Although 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 (26). Multiple BET inhibitors (BETi) have shown robust antitumor effects in different cancer types (26–28). Moreover, emerging evidence suggests that BETi could synergize with TKIs to boost antitumor responsiveness in a variety of cancer types (29–31). In this study, we also aimed to explore whether BETi could overcome TKI resistance in *HER2* aberrations.

Here, we designed a comprehensive preclinical study including individual *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 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 antitumor response to osimertinib treatment in NSCLC.

## Materials and Methods

### GEMM generation

The procedure to generate the tet-op-hHER2 mouse cohort was described before (13, 32). In short, a transgene DNA construct consisting of seven repeats of tetracycline operator, the wild-type human *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-hHER2 mice were crossed to Clara cell secretory protein (CCSP)–rtTA mice to obtain a tet-op-hHER2/CCSP-rtTA (HW) colony. The HW colony was fed with continuous doxycycline (doxy) diet from at least 6 weeks of age. CCSP-rtTA/tet-op-hEGFR Del-Luc and tet-op-$hHER2^{YVMA}$/CCSP-rtTA cohorts were generated as previously described (13, 33). All mouse breeding and treatment experiments were performed with the approval of the Dana-Farber Cancer Institute Animal Care and Use Committee.

### Magnetic resonance imaging and tumor volume quantification

Lung tumors were monitored by magnetic resonance imaging (MRI), and 3D Slicer was used to quantify the lung tumors as described before (34–36).

### Cell lines

NCI-H1781 cells were obtained from ATCC and maintained in RPMI-1640 medium containing 10% FBS, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. Ba/F3-$HER2^{wt}$ and Ba/F3-$HER2^{YVMA}$ cells were generated and maintained as described (37).

### CCK-8 assay

Two thousand Ba/F3 cells stably expressing $HER2^{wt}$ or $HER2^{YVMA}$ were plated into 96-well plates. Then either erlotinib or osimertinib was added the following day with indicated concentrations. After 3 days, the CCK-8 (Dojindo Molecular Technologies) was added to each well, and OD450 was measured after 1 to 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. Twenty to 40 μg of lysates were loaded on a NuPAGE 4% to 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; refs. 38–40).

### Immunohistochemistry and H&E staining

Mice were euthanized, and lung tissues were collected and fixed with 10% formalin. Immunohistochemistry (IHC) 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), and Phospho-EGFR (Tyr1068; Cell Signaling Technology, #3777).

### Treatment study

HW, DH, and SH26 mice were fed with doxy diets, and lung tumors were monitored by MRI (34). Tumor-bearing mice were treated with erlotinib (Selleck Chemicals, #S7786), osimertinib (AstraZeneca), afatinib (Selleck Chemicals, #S1011), or JQ1 and tracked by MRI every 2 weeks. Erlotinib (in 0.5% HPMC) was dosed at 50 mg/kg, afatinib (in 0.5% HPMC) at 20 mg/kg, and osimertinib (in 0.5% HPMC) 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-Aldrich). JQ1 was dosed to mice intraperitoneally (i.p.) at 50 mg/kg daily.

### Fluorescent *in situ* hybridization

The peripheral blood sample from the HW mouse was prepared for fluorescent *in situ* hybridization (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 two-signal pattern in 92 of 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 the 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 ± SEM. Student *t* test was used for comparisons between two groups using GraphPad Prism software. *P* values reported were considered statistically significant ($^{*}$, $P < 0.05$; $^{**}$, $P < 0.01$; $^{***}$, $P < 0.001$).

## Results

### Generation and characterization of doxy-induced lung-specific hHER2$^{wt}$ GEMM

Previous data have shown the *in vitro* efficacy of osimertinib against wild-type 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 the first-generation TKI erlotinib for 6 hours showed that although erlotinib did not inhibit HER2 phosphorylation (pHER2) at up to 500 nmol/L, osimertinib demonstrated potent activity in a dose-dependent manner from a concentration of 100 nmol/L, confirming that osimertinib indeed targets human wild-type HER2 (Fig. 1A).

We further treated Ba/F3-hHER2$^{wt}$ cells with either osimertinib or erlotinib for 72 hours to calculate growth inhibition ($GI_{50}$) and found that osimertinib achieved a significantly lower $GI_{50}$ (10.4 nmol/L) compared with erlotinib (438 nmol/L; Fig. 1B). Taken together, these results confirmed the efficacy of osimertinib against human HER2 and efficiently inhibited pHER2 at a low dose *in vitro*.

To study the *in vivo* role of osimertinib against h*HER2*, 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* open reading frame (ORF) and SV40 polyA (ref. 32; Fig. 1C, top). The tetO-HER2 mouse founders were bred with CCSP-rtTA mice to generate the inducible lung-specific bitransgenic hHER2$^{wt}$/CCSP-rtTA (HW) cohorts, which harbor both activator and responder transgenes (Fig. 1C, bottom). To confirm that the hHER2$^{wt}$ was integrated into mouse genome, we performed FISH using a blood sample collected from an 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 the protein level with doxy induction. Lungs of the doxy-induced HW mice exhibited hHER2 expression after 5 weeks with doxy induction, and pHER2 increased over time (Fig. 1D). To validate whether expression of HER2 was dependent on doxy, the doxy food was switched to normal diet for 3 days. After 3-day doxy removal, hHER2 expression was almost undetectable, confirming that hHER2 expression was doxy dependent (Fig. 1D).

To validate whether hHER2 expression can initiate lung cancer development, HW mice were fed with a continuous doxy diet and monitored by lung MRI. Tumors began to form after 6 weeks and developed into high-grade tumors after 16 weeks (Fig. 1E). IHC of HW tumors revealed high levels of HER2 and

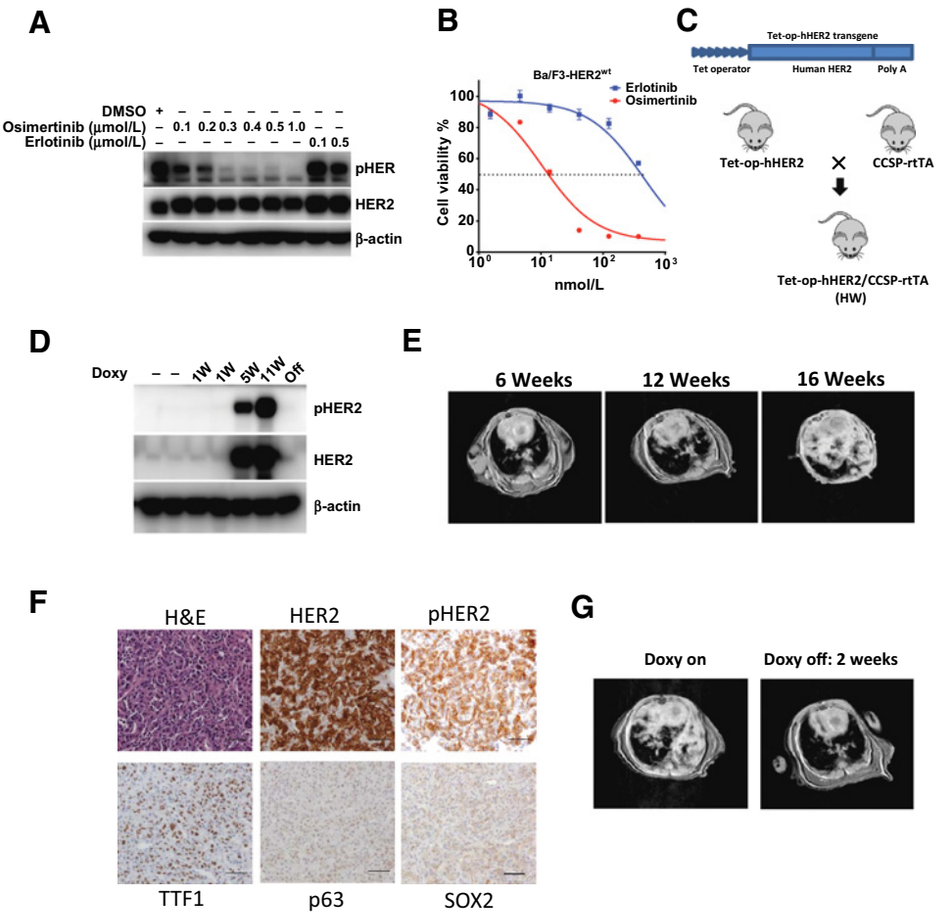


**Figure 1.**

Overexpression of hHER2 drives development of lung adenocarcinoma. **A,** Ba/F3-HER2$^{wt}$ cells stably expressing wild-type human HER2 were treated with either osimertinib or erlotinib for 6 hours at indicated concentrations before pHER2 was detected. **B,** Ba/F3-HER2$^{wt}$ cells were plated into 96-well plates and treated with osimertinib and erlotinib for 72 hours, and growth inhibition rate ($GI_{50}$) was calculated based on the CCK-8 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 doxy food for 1 week, 5 weeks, or 11 weeks or 5 weeks of doxy then switched to normal diet for 3 days. HER2 expression and phosphorylation were detected in whole-lung lysate samples from these mice. w, week(s). **E,** Representative MRI images of HW mice fed with doxy food for 6, 12, and 16 weeks. **F,** H&E staining and IHC analysis for HER2 and pHER2, and adeno/squamous markers TTF1, p63, and SOX2. Scale bar, 100 μm. **G,** Representative MRI image of an HW mouse fed with doxy food for 14 weeks and then switched to normal food for 2 weeks.

pHER2 (Fig. 1F, top). We also examined markers and found that these HW tumors showed adenocarcinoma features with positive staining for TTF1 and low expression of SOX2 and p63 (ref. 13, 35, 41; Fig. 1F, bottom). These histology features indicated that 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 doxy diet was replaced with normal food after tumor formation, and tumors disappeared 2 weeks after doxy diet removal (Fig. 1G). This confirmed that HER2 was 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. S2A), and mice had a median survival of 19.4 weeks after doxy induction (Supplementary Fig. S2B).

### *In vivo* antitumor efficacy of osimertinib in HER2$^{wt}$ GEMM

The HW mice were fed with doxy food, and lung tumors were monitored by MRI. Osimertinib was then administered orally at 25 mg/kg daily, an equivalent dose to clinical 80 mg daily in tumor-bearing HW mice (42). Osimertinib treatment was efficacious after 4 weeks; in contrast, erlotinib and afatinib demonstrated limited antitumor response (Fig. 2A and B). Both vehicle- and erlotinib-treated mice showed progressive disease (PD) by the 4-week time point (PD: more than 20% increase in tumor volume compared with 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 with baseline (Fig. 2B).

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 pHER2 and inhibited major downstream signaling targets such as pAKT and pERK (Fig. 2C). These pharmacodynamic data indicated the on-target efficacy of osimertinib against wild-type HER2 *in vivo*.

### Long-term survival benefit with osimertinib treatment in HER2$^{wt}$ mice

To further test whether osimertinib could maintain a durable antitumor 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 2 weeks. Osimertinib showed continuous antitumor efficacy for 16 weeks (Fig. 3A). The HW mice treated with osimertinib showed significantly longer progression-free survival (PFS) and overall

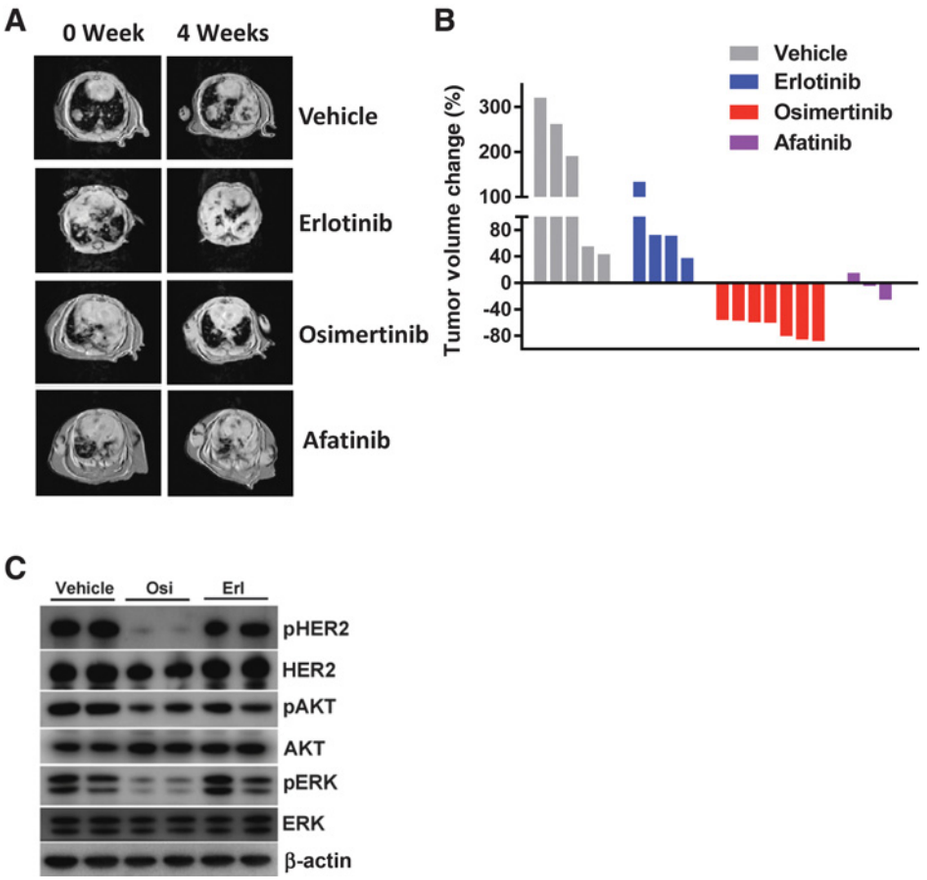


**Figure 2.**
Osimertinib induces tumor regression in wild-type HER2 GEMMs. **A,** Representative MRI image of HW mice before and after treatment with vehicle, erlotinib, osimertinib, or afatinib for 4 weeks. **B,** Waterfall plots show tumor volume change compared with before treatment, and each column represents one individual mouse. **C,** HW mice were treated with vehicle, osimertinib (Osi), or erlotinib (Erl) for 3 days, and tumor nodules were collected and lysates used in Western blot to detect pHER2 and downstream signaling. Two representative tumor samples from each group are shown.

survival (OS) compared with those treated with erlotinib (Fig. 3B and C). These 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 antitumor effect against HER2 in the HW tumor model.

### *In vivo* antitumor efficacy of osimertinib in EGFR$^{del19}$/HER2$^{wt}$ GEMMs

hHER2$^{wt}$ overexpression was proven to be an oncogenic driver in lung cancer based on the HW mouse 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 antitumor efficacy of osimertinib against hHER2 as a driver oncogene in the HW model, we further tested osimertinib against coexpression of h*HER2* in EGFR-mutant lung cancers.

There were no available GEMMs that 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 antitumor 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$^{wt}$/CCSP-rtTA (Del19HW; DH) mice (Fig. 4A). In this model, lung tumors were co-driven by both EGFR$^{del19}$ and HER2$^{wt}$. Osimertinib efficiently inhibited DH tumors after 2 weeks (Fig. 4B). Erlotinib reduced tumors after 2 weeks as well, but tumors relapsed quickly after initial response (Fig. 4B). 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. 4C, top). After relapse, pEGFR was nearly undetectable while HER2 and pHER2 levels increased significantly (Fig. 4C, bottom). Furthermore, we monitored the long-term efficacy for osimertinib treatment in DH mice, and the PFS was significantly improved with osimertinib treatment (Fig. 4D). Taken together, these data indicated that osimertinib can also target HER2 together with an activating EGFR mutation.

### Antitumor 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$^{YVMA}$), the most frequent exon 20 insertion, were treated with osimertinib or erlotinib for 6 hours. Osimertinib

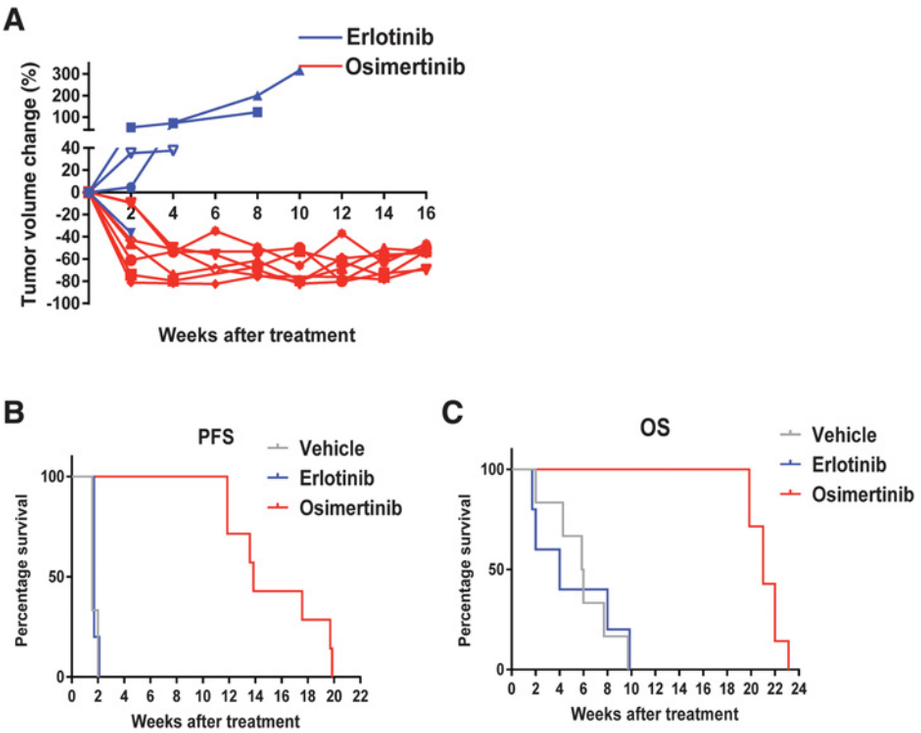


**Figure 3.**
Osimertinib demonstrates long-term survival benefit in wild-type HER2 lung cancer. **A,** Long-term monitoring of tumor volume change with erlotinib or osimertinib in HW mice. **B,** PFS of HW mice with each treatment. **C,** OS of HW mice with each treatment.

inhibited pHER2 at 500 nmol/L, although erlotinib did not (Fig. 5A). We also performed a 3-day proliferation assay and found osimertinib suppressed Ba/F3-HER2$^{YVMA}$ at a much lower concentration ($GI_{50}$ = 44 nmol/L) compared with erlotinib (Fig. 5B). However, HER2$^{YVMA}$ ($GI_{50}$ = 44 nmol/L) was less sensitive than HER2$^{wt}$ ($GI_{50}$ = 10.4 nmol/L). 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$^{G776insV_G/C}$, were treated with osimertinib for various times, from 6 hours to 5 days. Osimertinib significantly inhibited pHER2 at 500 nmol/L at all time points. Of note, total HER2 levels significantly increased with osimertinib treatment in a dose-dependent manner after 1 day (Fig. 5C, lanes 1–3). Downstream phosphorylation of AKT and ERK was also suppressed by osimertinib treatment (Fig. 5C, lanes 1–3). Interestingly, MYC was also inhibited by osimertinib at all time points, and p21 was reduced by osimertinib after 5 days (Fig. 5C, lanes 1–3). Considering both MYC and p21 are downstream targets of the BETi 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. 5C, lanes 4–6). Moreover, combination treatment downregulated total HER2 and MYC levels while upregulating p21 (Fig. 5C, lanes 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. 5D).

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

Tet-op-hHER2$^{YVMA}$/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 2 weeks, neither osimertinib nor JQ1 alone showed efficacy, but combination treatment led to significant tumor regression (Fig. 6A). After 4 weeks, only 3 out of 8 mice showed tumor regression with osimertinib treatment (Fig. 6B). In contrast, JQ1 and osimertinib combination showed a better antitumor benefit than single treatments (Fig. 6B). Moreover, the long-term treatment study indicated that PFS was greatly improved with JQ1 and osimertinib combination compared with single-agent treatments in SH26 mice (Fig. 6C). 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, whereas *HER2* exon 20 insertions were resistant to osimertinib single-treatment *in vivo*. Our findings demonstrate for the first time that the BETi JQ1 could

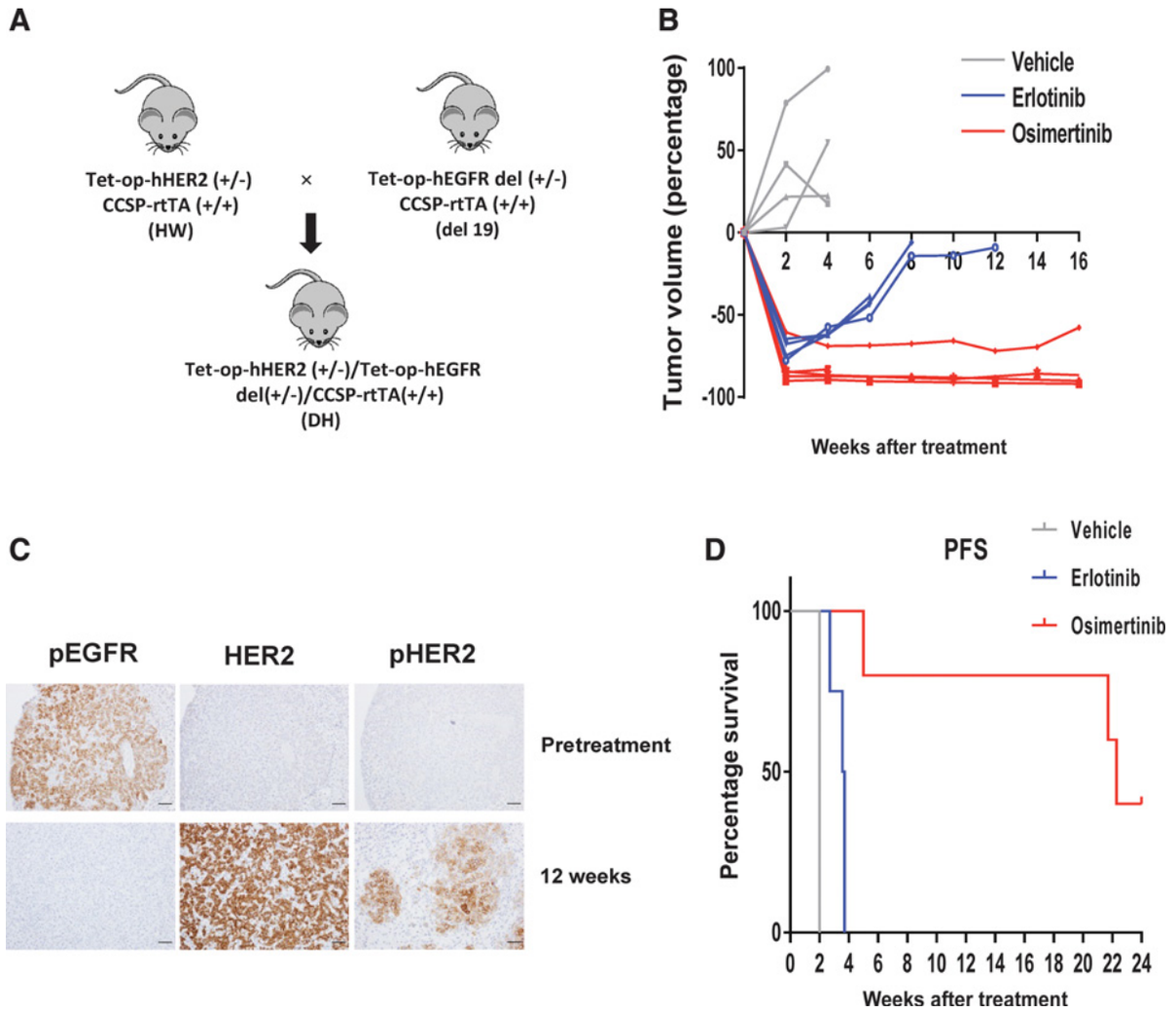


**Figure 4.**

Osimertinib induces regression in lung tumors codriven by both $EGFR^{del\ 19}$ and $HER2^{wt}$. **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 IHC. Scale bar, 100 μm. **D,** PFS of DH mice treated with vehicle, erlotinib, or osimertinib.

synergize with osimertinib to inhibit tumor growth of *HER2*-mutated lung cancers. Our study implicates the need to subdivide the patients with lung cancer carrying aberrant HER2 for osimertinib.

Irreversible dual EGFR/HER2 inhibitors (HKI) 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 preclinical 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. 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

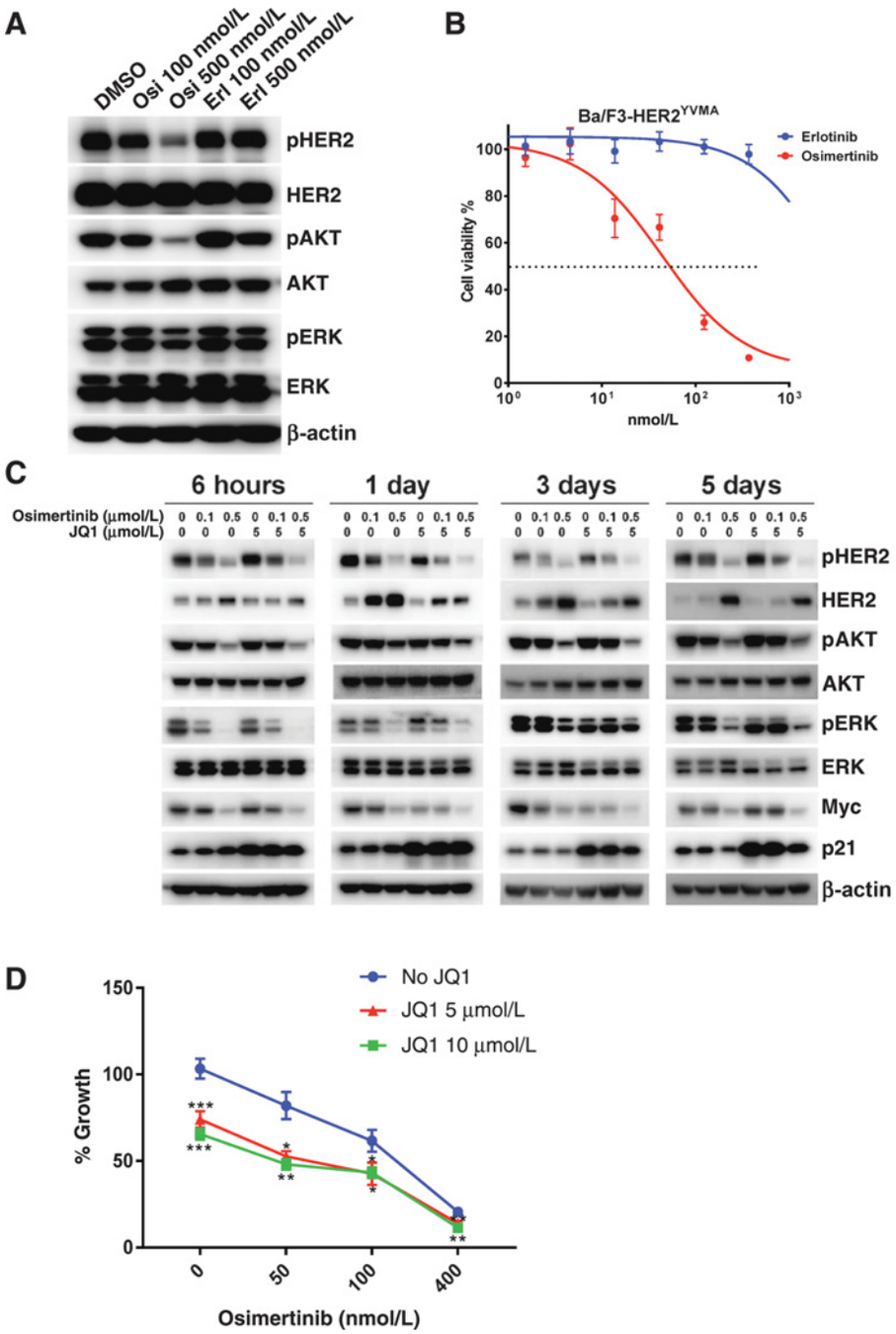


**Figure 5.**
Combined osimertinib and JQ1 therapy suppresses NSCLC with *HER2* exon 20 insertions *in vitro*. **A,** Ba/F3-HER2$^{YVMA}$ cells stably expressing human *HER2* exon 20 insertion (YVMA) were treated with either osimertinib (Osi) or erlotinib (Erl) for 6 hours at indicated concentrations, and then pHER2 was detected. **B,** Ba/F3-HER2$^{YVMA}$ cells were plated into 96-well plates and treated with osimertinib and erlotinib for 3 days. Growth inhibition rate ($GI_{50}$) was calculated based on the CCK-8 assay. **C,** H1781 cells were treated with osimertinib and/or JQ1 for indicated time, and then HER2 signaling was evaluated. **D,** H1781 cells were treated with osimertinib and/or JQ1 for 5 days, and growth rate was measured with CCK-8.

two novel HER2 strains provided the first available GEMM tools to test therapeutics against *HER2*$^{wt}$ 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 antitumor response in the HW mouse 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 the clinic for patients with *HER2* amplification. It would be interesting to address whether BRD4 inhibitors can overcome the resistance to osimertinib in the HW mouse model.

The potent efficacy of osimertinib against *HER2*$^{wt}$ 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

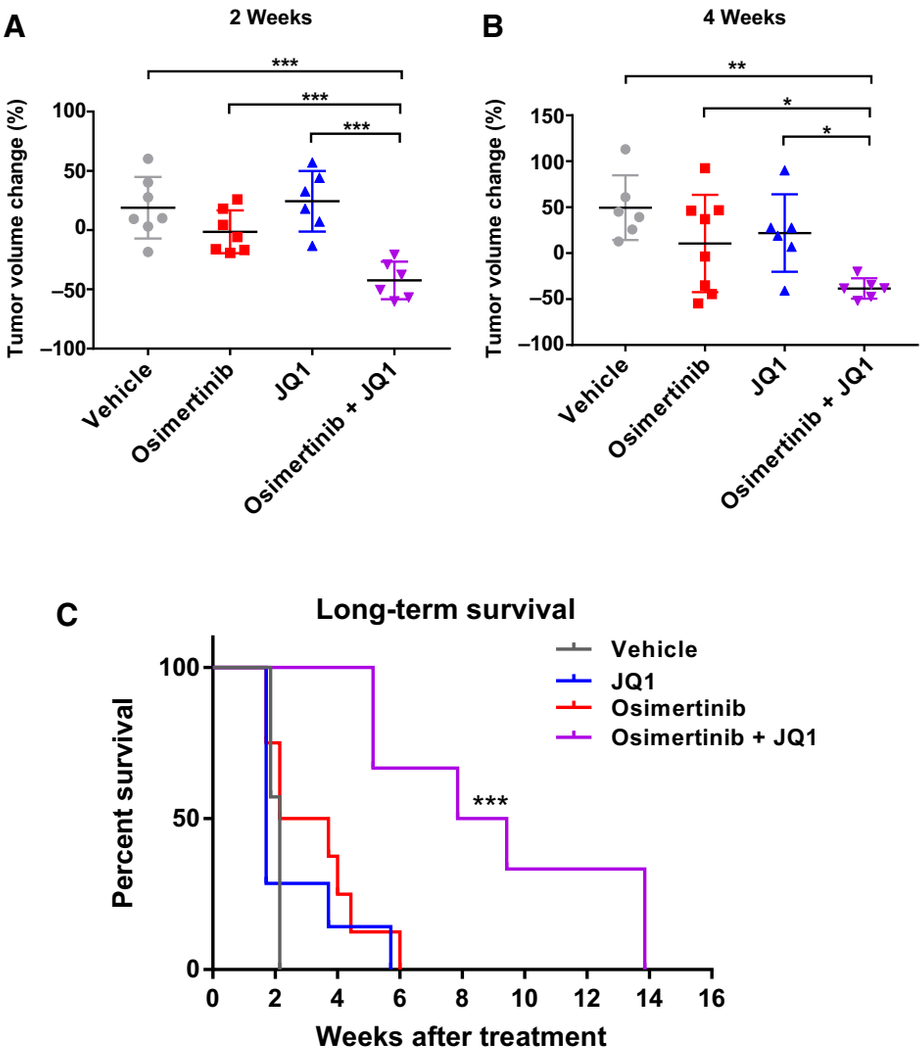


**Figure 6.**
Osimertinib and JQ1 combination induces tumor regression and long-term survival benefit in HER2$^{YVMA}$ GEMMs. **A** and **B,** SH26 mice were treated with osimertinib, JQ1, or their combination for 2 weeks (**A**) or for 4 weeks (**B**), and tumor volume change was calculated compared with before treatment based on MRI quantification. **C,** PFS of SH26 mice treated with vehicle, osimertinib, JQ1, or combination. *P* values reported were considered statistically significant: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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*$^{wt}$, 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 the BETi JQ1 could synergize with multiple TKIs in different cancer types (29–31). In a subset of acute myelogenous leukemia (AML), JQ1 synergized with the FLT3 TKI ponatinib to attenuate c-Myc, Bcl-2, and CDK4/6 and increase p21, BIM, and cPARP, thus inducing significant AML apoptosis (29). In HER2-driven breast cancer, the 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. BETi are an important group of epigenetic readers, and currently, multiple BETi are under investigation in clinical trials for different cancer types, including lung cancer. It will be interesting to understand the mechanism of the BETi–TKI synergy at 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% to 10% of all EGFR mutations in lung cancer (37, 48). Most EGFR mutations, including exon 19 deletion and L858R mutation, are sensitizing mutations that 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 the 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 combination 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.

## Disclosure of Potential Conflicts of Interest

K. Wong reports receiving commercial research grants from AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** S. Liu, S. Li, X. Wang, T. Chen, M.M. Quinn, P. Gao, H. Ji, K-K. Wong
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** S. Liu, S. Li, J. Hai, T. Chen, D.A.E. Cross, K-K. Wong
**Writing, review, and/or revision of the manuscript:** S. Liu, S. Li, J. Hai, X. Wang, D.A.E. Cross, K-K. Wong
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** Y. Zhang

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