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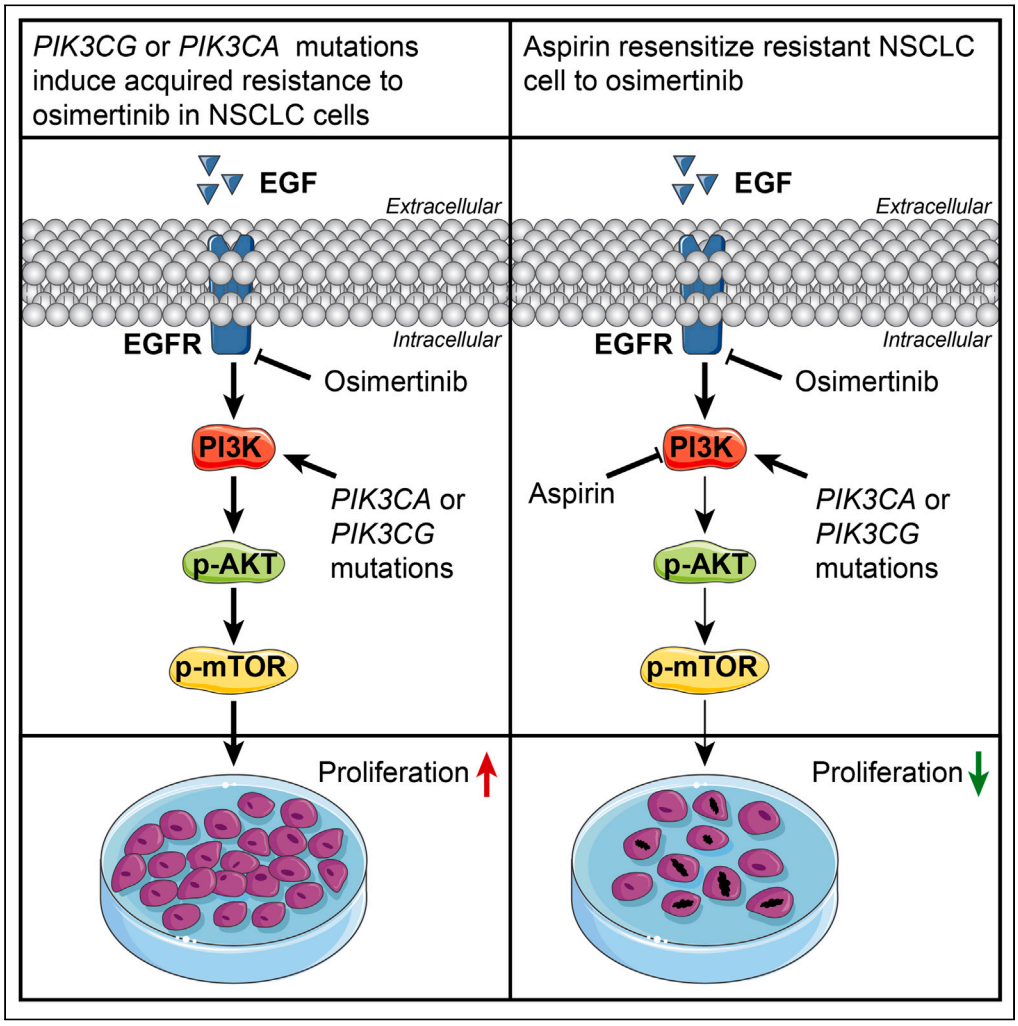

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Highlights

PIK3CG (L468M) mutation is discovered as a novel mechanism of resistance to osimertinib

Aspirin have a coordinating effect on the sensitization of osimertinib in NSCLC

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The potential therapeutic regimen for overcoming resistance to osimertinib due to rare mutations in NSCLC

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SUMMARY

The mechanisms of osimertinib resistance have not been well characterized. We conducted next-generation sequencing to recognize novel resistance mechanism and used cell line-derived xenograft (CDX) and patient-derived xenograft (PDX) models to evaluate the anti-proliferative effects of aspirin *in vivo* and *in vitro*. We observed that *PIK3CG* mutations led to acquired resistance to osimertinib in a patient and further confirmed that both *PIK3CG* and *PIK3CA* mutations caused osimertinib resistance. Mechanistically, the expression of PI3Kγ or PI3Kα was up-regulated after *PIK3CG* or *PIK3CA* lentivirus transfection, respectively, and which can be effectively suppressed by aspirin. Lastly, our results from *in vivo* studies indicate that aspirin can reverse osimertinib resistance caused by *PIK3CG* or *PIK3CA* mutations in both CDX and PDX models. Herein, we first confirmed that mutations in *PIK3CG* can lead to resistance to osimertinib, and the combined therapy may be a strategy to reverse *PIK3CG*/*PIK3CA* mutation-induced osimertinib resistance.

INTRODUCTION

Non-small-cell lung cancer (NSCLC) is the leading cause of cancer-related mortality worldwide, epidermal growth factor receptor (EGFR) mutations count for much in NSCLC.[1] Targeting mutant EGFR, tyrosine kinase inhibitors (TKIs) have been shown to provide strong survival benefits to patients.[2] The EGFR-TKI osimertinib, a third-generation drug, has a high remission rate for patients with NSCLC expressing EGFR-T790M mutations.[3] However, the efficacy of osimertinib is inevitably hindered by acquired resistance.[4] It is hypothesized that EGFR mutations C797S, G724S, L718Q, L792X, and M766Q are related to acquiring resistance on target mutations.[5] Due to the multifaceted resistance mechanisms of osimertinib, targeting EGFR mutations is simply insufficient, the latter pertains to MET and HER2 amplifications, MAPK signaling, SCLC, and epithelial-mesenchymal cell transformation (EMT). Nonetheless, known osimertinib resistance mechanisms can only explain the resistance of a small proportion of patients.[6]

Therefore, it is still necessary to further explore the resistance mechanism of osimertinib, even if this can only explain the cause of resistance in a few patients. An understanding of osimertinib-induced resistance is crucial in guiding subsequent treatments. The phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathway can be stimulated in lung cancer by PI3K catalytic subunit alpha (*PIK3CA*) mutations, such as *H1047R*, which was already proved as an important resistance mechanism of osimertinib.[7,8] Meanwhile, the activation of PI3K catalytic subunit gamma (*PIK3CG*) also proved to be closely related to cell proliferation in many tumors, such as AML cancers, hepatocellular carcinoma, and colorectal carcinoma.[9–11] However, it is still not clear whether *PIK3CG* mutation can induce osimertinib resistance, which needs further study. In this study, we observed that *PIK3CG L468M* mutations confer resistance to osimertinib in an NSCLC patient. Moreover, no effective approach to overcome or delay such two mutations induced resistance was ever found currently, thus clinically feasibility strategies are urgently needed.

Aspirin (acetylsalicylic acid), a classic anticoagulant, has become one of the most commonly used drugs. Several studies have provided considerable evidence demonstrating its potential for the anti-cancer effect, particularly in *PIK3CA*-mutated colorectal cancer.[12,13] Recent studies have shown that aspirin sensitizes osimertinib and EGFR-mutant patients with NSCLC who take aspirin and osimertinib are more likely to have

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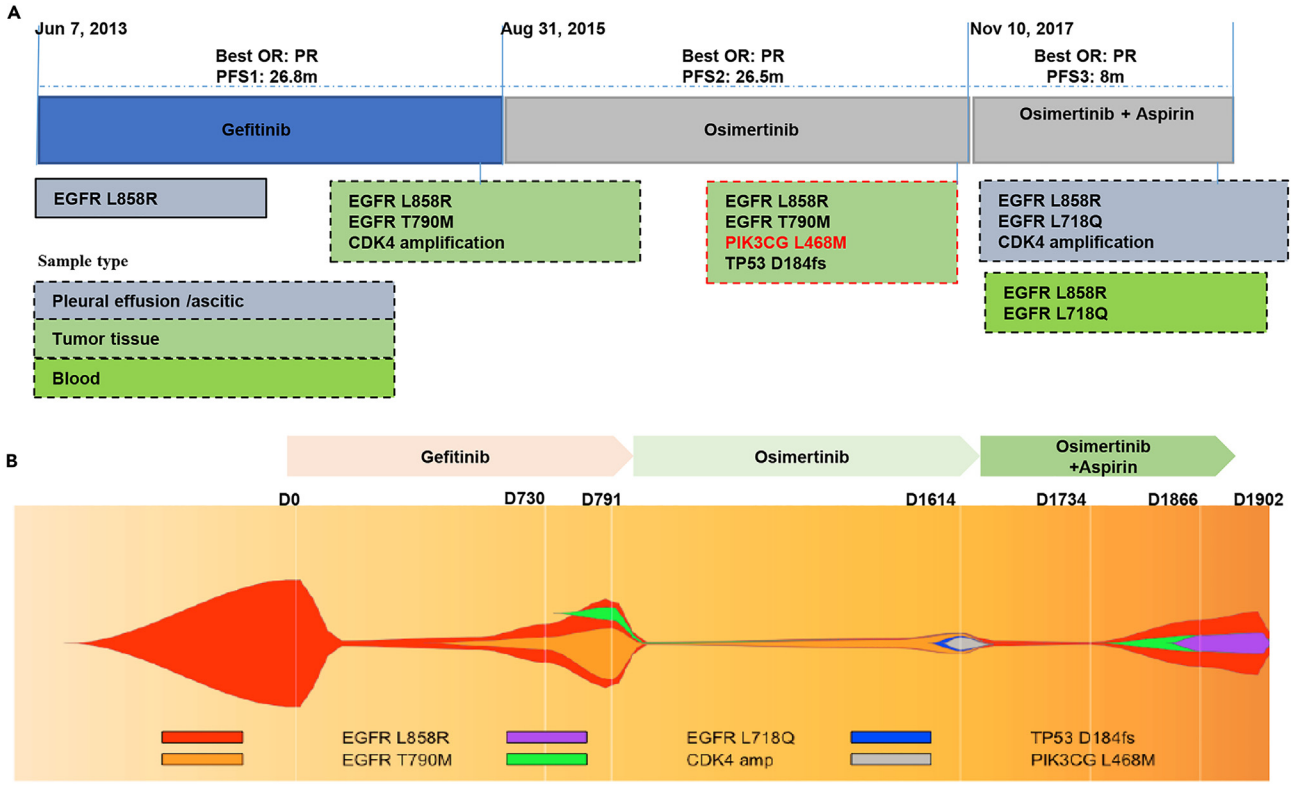

Figure 1. Acquired resistance to osimertinib due to *PIK3CG* mutation was identified by capture-based targeted ultra-deep sequencing (10000 ×)

(A) Schematic diagram of the treatments received by the patient and genetic alterations detected at each time point. Best overall objective response (OR) and progression-free survival (PFS) for each treatment (expressed in months) are also indicated. Genetic alterations aiding in identification of treatment options are highlighted. Abbreviations: Best OR, best overall objective response; PR, partial response; SD, stable disease; PFS, progression-free survival; and m, months.

(B) The clonal progression of major genetic alterations detected in formalin-fixed paraffin-embedded tissue biopsies or cell block and plasma samples. Time points are indicated in days. Different colors denote different genetic alterations.

better survival.[14,15] However, it is still not clear which patients would benefit better more from the concurrent aspirin use with osimertinib.

In this study, we reported that both *PIK3CG* and *PIK3CA* mutations could induce osimertinib resistance, and which could be effectively reversed by aspirin *in vitro* and *in vivo*. This study is not only identified an original resistance mechanisms of osimertinib, but also further proposed that aspirin is may be a clinically available treatment strategy to reverse resistance caused by *PIK3CA* and *PIK3CG* mutations.

RESULTS

PIK3CG and *PIK3CA* mutations cf. acquired resistance to osimertinib and advance the emergence of resistant cells

We reported one osimertinib-resistant patient in our previous study who experienced unstable angina pectoris, and was therefore prescribed 100 mg of aspirin daily co-administered with osimertinib, which effectively reversed osimertinib resistance (Figure 1A and Table S1).[14] Subsequently, capture-based targeted ultra-deep sequencing (10000 ×) performed on tumor biopsy samples at PD (according to Recist v1.1) revealed several mutations, including *EGFR* L858R, T790M, TP53, and *PIK3CG* L468M, with the overall clonal progression shown in Figure 1B and Data S1. Based on these results, we speculated that the acquired PIK3CG L468M mutation may lead to osimertinib resistance.

To validate whether the *PIK3CG* (which encodes for the catalytic subunit p110γ of class IA phosphatidylinositol 3-kinases [PI3K]) mutation could induce osimertinib resistance, *PIK3CG* (L468M) mutant viral vectors were transfected into osimertinib-sensitive Ba/F3-EGFR^{Del19}, Ba/F3-EGFR$^{Del19\text{-}T790M}$, HCC827, H3255,

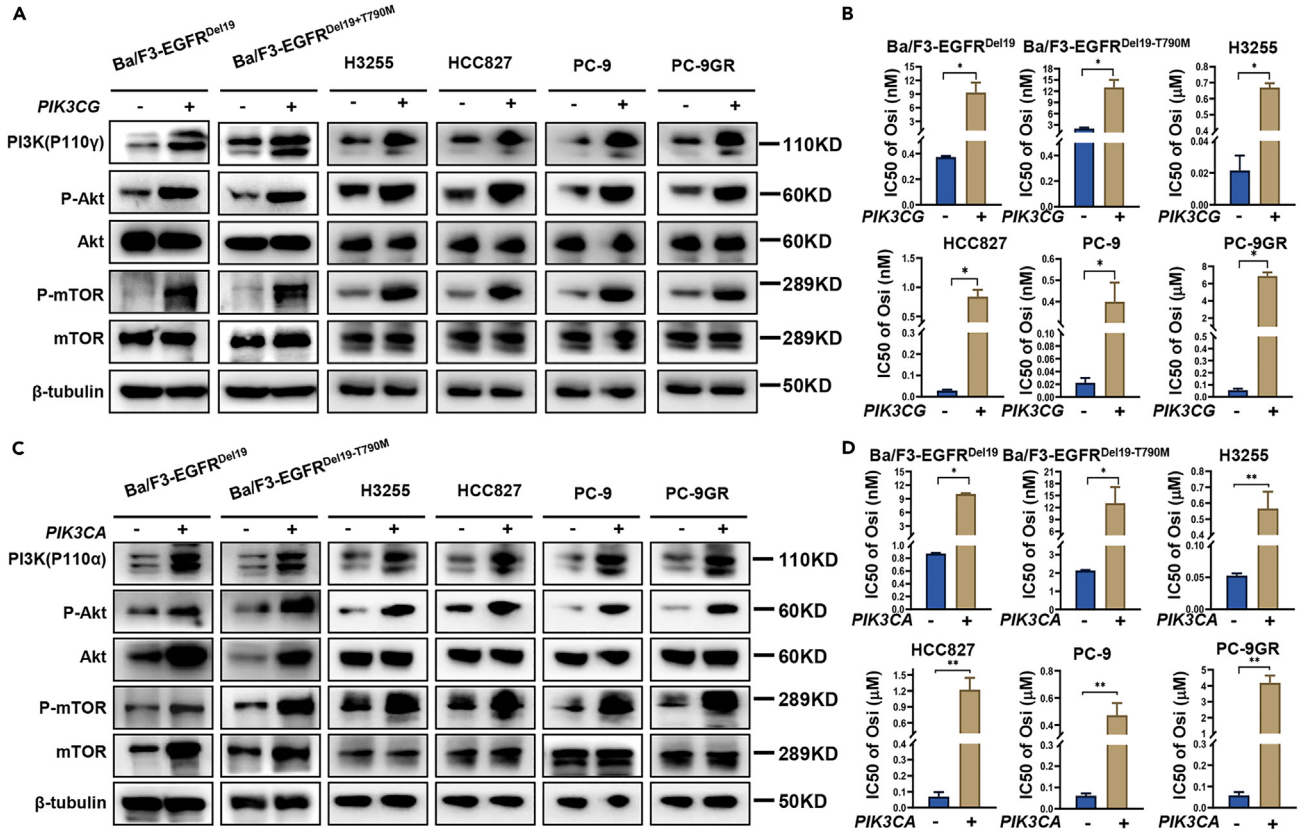

Figure 2. *PIK3CG* and *PIK3CA* mutations induced osimertinib resistance in EGFR-mutant NSCLC cell lines or Ba/F3 cells

(A) *PIK3CG* mutation increased PI3K (p110γ) and Akt/Bim signaling in Ba/F3-EGFR^{Del19}, Ba/F3-EGFR$^{Del19-T790M}$, HCC827, H3255, PC-9, and PC-9GR cells. Whole-cell protein lysates from mutant cells were subjected to immunoblotting to measure indicated proteins, with β-tubulin as a loading control. Similar results were obtained in three independent experiments.

(B) Cell viability was analyzed by CCK-8 assay in *PIK3CG* mutant (−/+) Ba/F3-EGFR^{Del19}, Ba/F3-EGFR$^{Del19-T790M}$, HCC827, H3255, PC-9, and PC-9GR cells treated with osimertinib alone. Histograms show the IC50 of osimertinib in the indicated treatment groups.

(C) *PIK3CA* mutation increased PI3K (p110α), and Akt/Bim signaling in Ba/F3-EGFR^{Del19}, Ba/F3-EGFR$^{Del19-T790M}$, HCC827, H3255, PC-9, and PC-9GR cells. Whole-cell protein lysates from mutant cells were subjected to immunoblotting to measure indicated proteins, with β-tubulin as a loading control. Similar results were obtained in three independent experiments.

(D) Cell viability was analyzed by CCK-8 assay in *PIK3CA* mutant (−/+) Ba/F3-EGFR^{Del19}, Ba/F3-EGFR$^{Del19-T790M}$, HCC827, H3255, PC-9, and PC-9GR cells treated with osimertinib alone. Histograms show the IC50 of osimertinib in the indicated treatment groups. Numbers in the figures are mean values with S.D.

PC-9, and PC-9GR cell lines. As shown in Figure 2A, induction of a *PIK3CG* mutation increased PI3K (p110γ) expression and activated the downstream protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway in *PIK3CG* mutant Ba/F3 and NSCLC cell lines. Furthermore, CCK-8 cell proliferation assay was performed in above-mentioned mutant cells. Here, we found that *PIK3CG* mutant cells all conferred osimertinib resistance effectively (Figure 2B). To the best of our knowledge, disturbances in the PI3K/Akt/mTOR pathway play a central role in lung cancer, with one of most common deregulations being *PIK3CA* (which encodes for the catalytic subunit p110α of PI3K) mutations,[16] which has already been confirmed to be an important resistance mechanism associated with osimertinib treatment, especially *PIK3CA* (H1047R) mutation.[17–19] We further assessed whether *PIK3CA* mutation also regulated acquired resistance to osimertinib in these cells. Subsequently, the *PIK3CA* (H1047R) mutant Ba/F3 and NSCLC cell lines were constructed, and we also observed increased PI3K (p110α) expression and Akt/mTOR signaling pathway activation in *PIK3CA* mutant cells (Figure 2C). As reported in previous clinical studies, we also found that *PIK3CA* mutations mediated significant acquired resistance to osimertinib in *PIK3CA* mutant Ba/F3 and NSCLC cell lines through CCK-8 cell proliferation assay (Figure 2D).

Together, these findings demonstrate that *PIK3CG* (L468M) and *PIK3CA* (H1047R) mutations all confer acquired resistance to osimertinib through PI3K/Akt/mTOR pathway-dependent mechanisms.

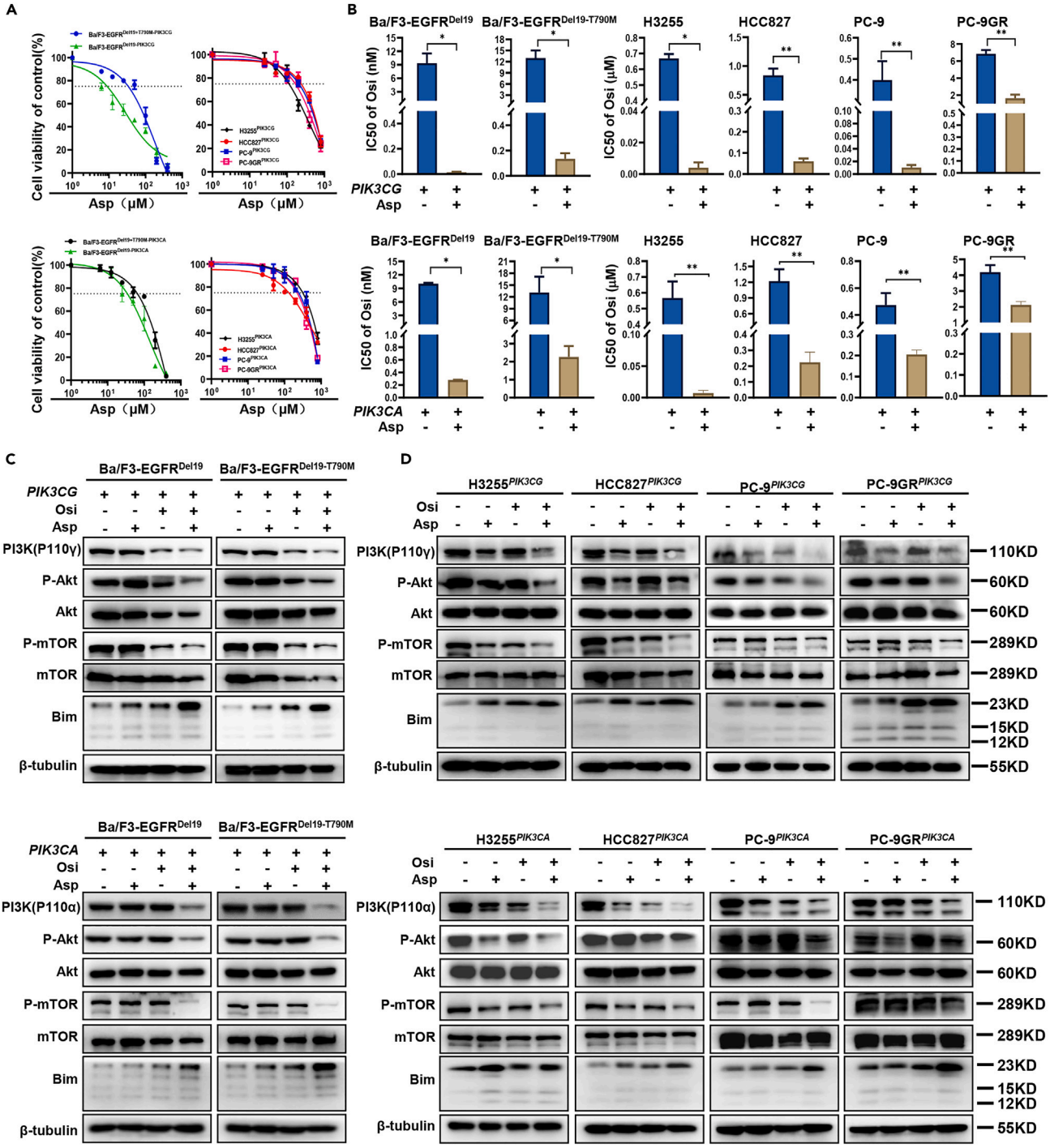

Figure 3. Aspirin re-sensitized *PIK3CG* and *PIK3CA* mutant Ba/F3 and NSCLC cells to osimertinib *in vitro*

(A) The effects of different doses of aspirin on various *PI3KCG* and *PI3KCA* mutant cell lines. Cell viabilities were examined 48 h after aspirin treatment. Assays were performed in triplicate.

(B) Cell viability was analyzed by CCK-8 assay in *PIK3CG* and *PIK3CA* mutant (−/+) Ba/F3-EGFR^{Del19}, Ba/F3-EGFR$^{Del19\text{-}T790M}$, HCC827, H3255, PC-9, and PC-9GR cells treated with osimertinib alone or in combination with aspirin treatment for 48 h. Histograms show the IC50 of osimertinib in the indicated treatment groups.

(C) Aspirin in combination with osimertinib increased apoptosis in *PIK3CG* and *PIK3CA* mutant Ba/F3-EGFR^{Del19}, Ba/F3-EGFR$^{Del19\text{-}T790M}$ cells. Images are representative of three independent experiments. *$p < 0.05$; **$p < 0.01$. Asp: aspirin; Osi: osimertinib. Ctrl: control.

Figure 3. *Continued*

(D) Aspirin decreased PI3K (p110γ), PI3K (p110α) and Akt/mTOR signaling, and activated Bim-related apoptosis signaling in *PIK3CG* and *PIK3CA* mutant HCC827, H3255, PC-9, and PC-9GR cells. Whole-cell protein lysates from *PIK3CG* and *PIK3CA* mutant cells with different treatments were subjected to immunoblotting to measure indicated proteins, with β-tubulin used as a loading control. Similar results were obtained in three independent experiments. Numbers in the figures are mean values with S.D.

Aspirin re-sensitizes *PIK3CG* and *PIK3CA* mutant Ba/F3 and NSCLC cell lines to osimertinib

We further investigated whether aspirin re-sensitizes resistant cells to osimertinib. We confirmed that 25 and 200 μmol/L aspirin slightly decreased the viability of *PIK3CG* or *PIK3CA* mutant Ba/F3 and NSCLC cell lines, respectively (Figure 3A). Therefore, the *PIK3CG* and *PIK3CA* mutant Ba/F3-EGFR^{Del19} and Ba/F3-EGFR$^{Del19\text{-}T790M}$ cells, which are osimertinib-resistant cells, were treated with osimertinib and aspirin (25 μmol/L) simultaneously. Strikingly, these osimertinib-resistant cells could be re-sensitized to osimertinib by aspirin (Figure 3B). Similar results were observed in *PIK3CG* and *PIK3CA* mutant NSCLC cell lines, which could be effectively reversed by treatment with 200 μmol/L aspirin (Figure 3B). To further evaluate the molecular mechanisms regulated by aspirin in re-sensitizing osimertinib resistance, we explored the expression of the PI3K/Akt/mTOR pathway. Here, we revealed that aspirin significantly decreased PI3K (p110γ) and PI3K (p110α) expression, inhibited the Akt/mTOR pathway, and activated the Akt/Bcl-2-like protein 11 (Bim) signaling pathway in *PIK3CG* and *PIK3CA* mutant Ba/F3 (Figure 3C). Meanwhile, similar findings have also been observed in NSCLC cells (Figure 3D). Overall, these results indicate that aspirin can reverse *PIK3CG* and *PIK3CA* mutation-induced osimertinib resistance through a PI3K inhibition-dependent mechanism.

Aspirin plus osimertinib potentiates osimertinib-induced anti-tumor activity in *PIK3CG* mutant CDX tumor

Based on the previous findings, we evaluated whether aspirin plus osimertinib was effectively in cell line-derived xenograft (CDX) models established from PC-9^{PIK3CG} cells and patient-derived xenograft (PDX) models with PIK3CA mutations. A synergistic effect was observed upon co-administration of osimertinib (10 mg/kg) and aspirin (20 mg/kg), which significantly reduced tumor size in CDX mouse models (Figure 4A). After 4 weeks of drug administration, combination therapy induced a significant decrease in tumor volume compared to the other three treatment groups (control, osimertinib alone, and aspirin alone groups) (Figures 4B and 4C). During the study, no obvious weight loss was observed in all four groups (Figure 4D). IHC staining revealed significantly reduced expression of PI3K (p110γ) and Ki67 in the combination therapy group, indicating that co-administration of aspirin with osimertinib significantly inhibited cell proliferation (Figure 4E). Besides, we further observed combination of aspirin and osimertinib inhibited PI3K (p110γ) expression and Akt/mTOR signaling pathway in *PIK3CG* mutant CDX tumors (Figure 4F). Taken together, these results suggested that the therapeutic benefits associated with the combinatorial use of aspirin and osimertinib are applicable to *PIK3CG* (L468M) mutations *in vivo*.

Co-treatment of aspirin potentiates the anti-proliferative effects of osimertinib in *PIK3CA* mutant NSCLC PDX tumor

Finally, using aspirin combined with osimertinib treatment schedules we evaluated the effect of this combinational therapy against PDX tumors with *PIK3CA* (H1047R) mutation. In treatment during the initial phase, osimertinib (10 mg/kg) and aspirin (20 mg/kg) alone slightly delayed tumor growth compared to tumors in control group, indicating that the PDX tumors were intrinsically resistant to osimertinib. Importantly, mice treated with osimertinib and aspirin had a significantly reduced rate of xenograft tumor growth and weight (Figures 5A, 5B, and 5C). Similarly, we also observed no obvious weight loss in all four treatment groups, no apparent adverse events were observed during these treatments (Figure 5D). Furthermore, IHC staining revealed reduced expression of PI3K(p110α) and Ki67 in the combination group and western blot also showed combination of aspirin and osimertinib inhibited PI3K (p110α) expression and Akt/mTOR signaling pathway in *PIK3CA* mutant PDX tumors (Figures 5E and 5F). Taken together, these results also suggested that the therapeutic benefits of inhibiting *PIK3CA* (H1047R) mutant PDX tumors associated with the combinatorial use of aspirin and osimertinib.

DISCUSSION

Despite the remarkable and durable responses achieved with osimertinib treatment, a majority of treated NSCLC patients experience an inevitable disease progression.[4,20] EGFR-dependent and EGFR-independent mechanisms contribute to acquired resistance to osimertinib, and each known osimertinib resistance

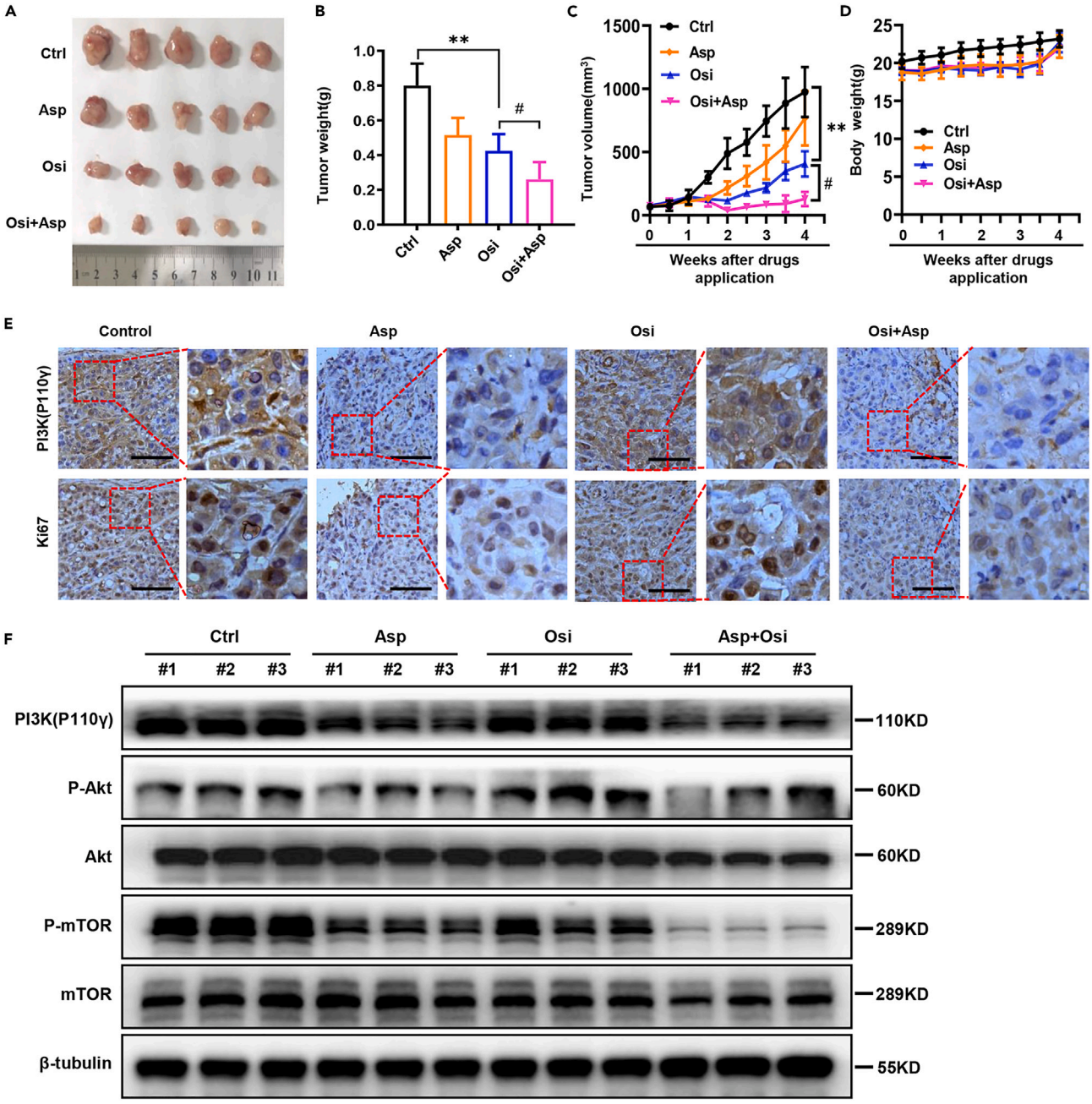

Figure 4. Aspirin sensitized osimertinib anti-tumor ability in *PIK3CG* mutant cell-derived xenograft mouse models

(A) Macroscopic appearance of PC-9^{PIK3CG}-derived tumors at 4 weeks after drug administration.

(B and C) Weight (g) and volume (mm^3) of PC-9^{PIK3CG} tumors treated with osimertinib, aspirin, and combined therapy. **, $p < 0.01$ when compared with the control; #, $p < 0.05$ as compared with osimertinib alone.

(D) PC-9^{PIK3CG} nude mouse body weight was measured after indicated treatment.

(E) The representative intensity images for each IHC score of PI3K (p110γ) and Ki67 staining in PC-9^{PIK3CG} tumor tissues are shown. Scale bars, 100 μm. Asp: aspirin; Osi: osimertinib; Ctrl: control.

(F) Western blot showed aspirin decreased PI3K (p110γ) and Akt/mTOR signaling in *PIK3CG* and *PIK3CA* mutant Ba/F3-EGFR^{Del19}, Ba/F3-EGFR$^{Del19\text{-}T790M}$, HCC827, H3255, PC-9, and PC-9GR cells. Whole-cell protein lysates from *PIK3CG* mutant CDX tumors with different treatments were subjected to immunoblotting to measure indicated proteins, with β-tubulin used as a loading control. Numbers in the figures are mean values with SEM.

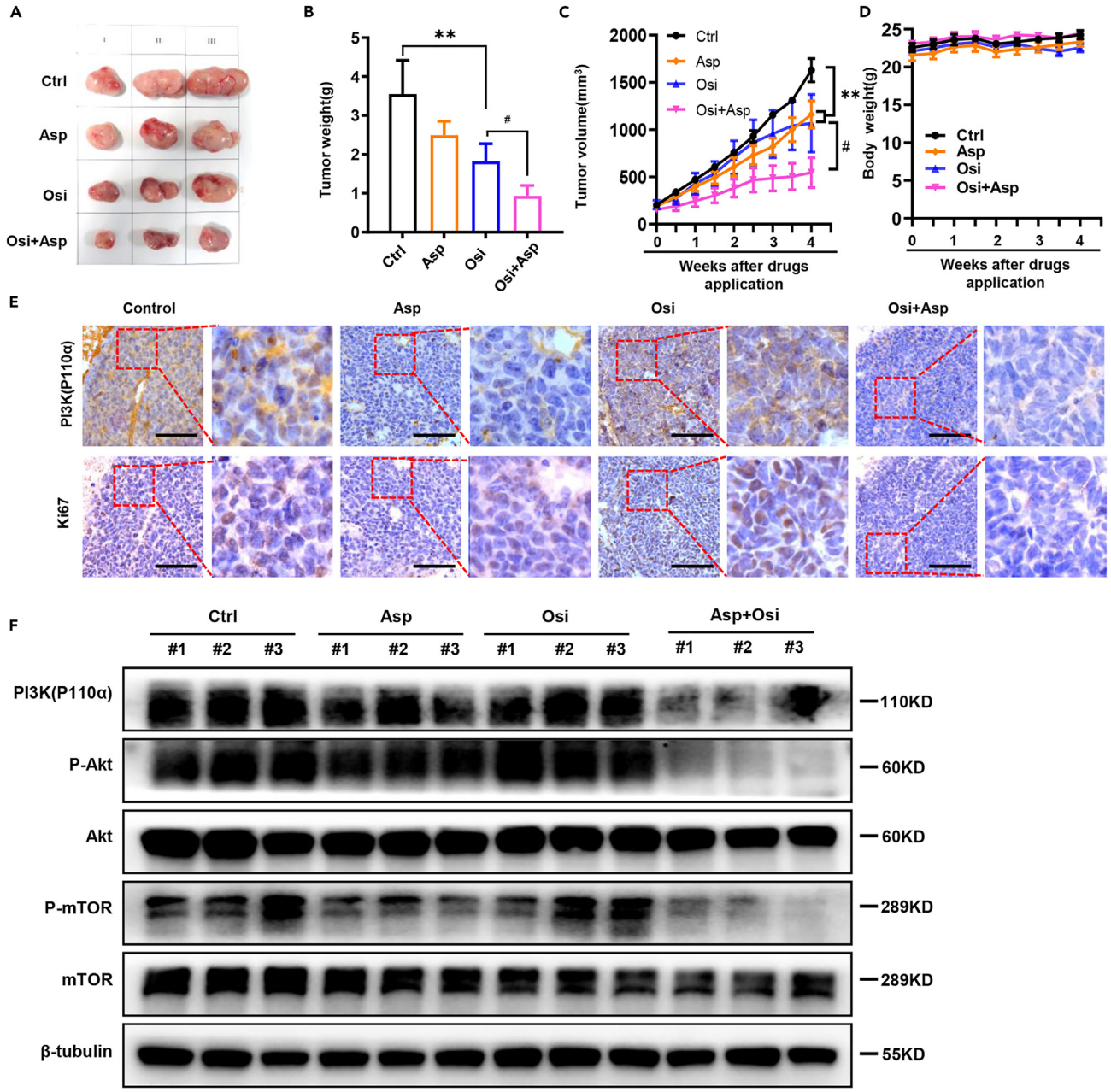

Figure 5. Aspirin enhanced osimertinib anti-tumor ability in *PIK3CA* mutant patient-derived xenograft mouse models

(A) Macroscopic appearance of *PIK3CA* mutant patient-derived tumors at 4 weeks after drug administration.

(B and C) Weight (g) and volume (mm^3) of patient-derived tumors treated with osimertinib, aspirin, and combined therapy. **, $p < 0.01$ when compared with the control; #, $p < 0.05$ as compared with osimertinib alone.

(D) The nude mouse body weight was measured after indicated treatment, and the data were shown as mean ± SEM (n = 3).

(E) The representative intensity images for each IHC score of PI3K (p110α) and Ki67 staining in *PIK3CA* mutant patient-derived tumor tissues are shown. Scale bars, 100 μm. Asp: aspirin; Osi: osimertinib; Ctrl: control. Western blot showed aspirin decreased PI3K (p110α) and Akt/mTOR signaling in *PIK3CG* and *PIK3CA* mutant Ba/F3-$EGFR^{Del19}$, Ba/F3-$EGFR^{Del19\text{-}T790M}$, HCC827, H3255, PC-9, and PC-9GR cells. (F) Whole-cell protein lysates from *PIK3CA* mutant PDX tumors with different treatments were subjected to immunoblotting to measure indicated proteins, with β-tubulin used as a loading control. Numbers in the figures are mean values with SEM.

mechanism accounts for a small proportion.[21,22] Thus, osimertinib's resistance mechanism must be further investigated, even if the mechanism can only explain the cause of resistance in a few patients. In this study, we report that the *PIK3CG* mutation is a rare mutation conferring acquired resistance to osimertinib *in vitro*

and *in vivo*. In addition, since no standard treatment regimen is currently available for osimertinib resistance, most clinicians prescribe cytotoxic chemotherapy as the third- and/or subsequent-line of therapy.[23] Hence, further efforts are needed to explore innovative treatment strategies to combat osimertinib resistance. Notably, there have been reported some strategies to overcome the resistance of osimertinib, but relevant results are still difficult to enter clinical application in a short period of time.[24–28] Interestingly, we found that *PIK3CA* and *PIK3CG* mutation-induced osimertinib resistance could be reversed with aspirin treatment, which had anti-proliferative and pro-apoptotic effects. Meanwhile, aspirin has been reported to be effective in delaying acquired resistance to osimertinib in pre-clinical models and is associated with improved survival in patients with NSCLC.[14,15] However, we have discovered that not every patient will benefit from this combinatorial therapeutic regimen. Further identification of the patients with *PIK3CA* or *PIK3CG* mutations, who may benefit from aspirin and osimertinib therapies, is an important scientific question that requires investigation.

Numerous studies have recently been published on the molecular mechanisms associated with resistance acquisition and potential treatment strategies capable of reversing or delaying osimertinib resistance.[21,29] Several *in vitro* studies have implicated the promotion of proliferation and escape from apoptosis as two important mechanisms that lead to EGFR-TKI resistance in NSCLC cell lines.[14,30] *PIK3CA* and *PIK3CG* both encode catalytic subunits of PI3K, which generate phospholipid second messengers (p110α or p110γ) and activate Akt/PKB and PDK1 signaling pathways by recruiting them to cell membranes.[31] Consequently, *PIK3CG* and *PIK3CA* mutations could induce osimertinib resistance due to uncontrolled proliferation and apoptotic escape via abnormal activation of the PI3K/Akt pathway.[14,30] Furthermore, we have demonstrated that aspirin can effectively inhibit both catalytic subunits of PI3K (p110α or p110γ) expression in this study. This brave perspective is different from that reported in previous research, which is that aspirin mainly depends on COX-related signaling pathways to induce anti-proliferative and pro-apoptotic effects.[13,32,33] *PIK3CG* L468M mutations are associated with disease progression, and the efficacy of aspirin in these patients may be attributed to the detection of *PIK3CG* L468M mutations prior to aspirin and osimertinib treatment. Moreover, *PIK3CA* H1047R mutations can also affect the status of PI3K. In our current study, combinatorial use of aspirin and osimertinib was capable of re-sensitizing both *PIK3CA* and *PIK3CG* mutant cell lines and xenograft models to osimertinib, thus reversing osimertinib resistance. Based on these data, we hypothesize that *PIK3CG* and *PIK3CA* mutation-mediated osimertinib resistance could be inhibited through aspirin treatment.

Besides, aspirin combination therapy has several characteristics such as having few side effects and being cost-effective. Furthermore, the dose used in this study (20 mg/kg/day) can be translated to a clinical dose of 100 mg/day which is taken for decreasing risk factor of cardiovascular disease purposes in 50 kg humans,[34] and the results in our study showed that aspirin is effective at low concentrations and is worthy of being widely used in clinical practice. We have developed two prospective clinical trials (ClinicalTrials.gov, NST: 03543683 and NST: 03532698), to observe the clinical effects of aspirin combined with osimertinib in treating untreated EGFR mutation NSCLC patients. These trials would help us further understand whether *PIK3CA* and *PIK3CG* mutant patients would benefit more from these combination therapies and develop a combination therapeutic strategy involving aspirin and osimertinib for prolonging survival in patients with NSCLC based on solid clinical evidence.

Overall, we report for the first time that osimertinib resistance induced by *PIK3CG* and *PIK3CA* mutation is reversed by aspirin, which may serve as a treatment strategy for NSCLC patients who develop osimertinib resistance caused by *PIK3CG* and *PIK3CA* mutations.

Limitations of the study

Our study results are limited by some limitations. Although we have demonstrated that aspirin can reverse acquired resistance to osimertinib caused by *PIK3CG* and *PIK3CA* mutations *in vivo* and *in vitro*, these findings are based on pre-clinical studies and thus require more rigorous clinical testing. Meanwhile, these osimertinib-resistant patients had neither *PIK3CA* nor *PIK3CG* mutations, whether aspirin can also play the same role in re-sensitizing osimertinib and its mechanism deserve further discussion.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107105.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.H.; methodology, Y.H.; validation, R.H.; data curation, R.H., C.L., Y.W., C.H., Y.Z., and C.L.; writing—original draft preparation, R.H. and C.Z.; writing—review and editing, R.H. and C.Z.; visualization, R.H. and C.L.; supervision, Y.H.; project administration, Y.H.

DECLARATION OF INTERESTS

The authors have no conflicts of interest to declare.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-PI3K (P110α)	Cell Signaling Technology	Cat# 4255S; RRID:AB_659888
Rabbit anti-PI3K (P110γ)	Cell Signaling Technology	Cat# 5405S; RRID:AB_1904087
Rabbit anti-Akt	Cell Signaling Technology	Cat# 9272S; RRID:AB_329827
Rabbit anti-phospho (Ser473)-Akt	Cell Signaling Technology	Cat# 4060S; RRID:AB_2315049
Rabbit anti-mTOR	Cell Signaling Technology	Cat# 2983; RRID:AB_2105622
Rabbit anti-phospho(Ser2448)-mTOR	Cell Signaling Technology	Cat# 5536; RRID:AB_10691552
Rabbit anti-β-Tubulin	Cell Signaling Technology	Cat# 2128
Rabbit anti-Bim	Cell Signaling Technology	Cat# 2933; RRID:AB_1030947
Rabbit anti-Ki67	Cell Signaling Technology	Cat# 34330
anti-rabbit goat IgG(HRP)	Sino Biological	Cat# SSA004
Bacterial and virus strains		
Lenti-NC-GFP viruses	Gene-Chem	N/A
Lenti-PIK3CG (L468M)-GFP viruses	Gene-Chem	N/A
Lenti-PIK3CA (H1047R)-GFP viruses	Gene-Chem	N/A
Biological samples		
Patient lung cancer tissue	Daping hospital	N/A
Patient-derived xenografts (PDX)	Shanghai LIDE Biotechnology	https://www.lidebiotech.com/
Chemicals, peptides, and recombinant proteins		
osimertinib	Selleck Chemicals	Cat#S7297
aspirin	Selleck Chemicals	Cat#S3017
DMSO	Sigma-Aldrich	Cat#276855
Lipofectamine 3000 reagent	Thermo Fisher Scientific	Cat#L30000-015
Critical commercial assays		
QIAamp DNA FFPE Tissue Kit	Qiagen	Cat#56404
QIAamp Circulating Nucleic Acid Kit	Qiagen	Cat#55114
Cell Counting Kit-8	MedChemExpress	Cat#96992
BCA Protein Assay Kit	EMD Millipore	Cat#71285-3
DAB display reagent Kit	ZSGB-Bio	Cat#ZLI-9018
Experimental models: Cell lines		
H3255	FineTest	C473
HCC827	Guangzhou Cellcook Biotech	CC0215
PC-9	Guangzhou Cellcook Biotech	CC0204
PC-9GR	This paper	N/A
Ba/F3-EGFR^{Del19}	Cobioer Biosciences	CBP73045
Ba/F3-EGFR$^{Del19\text{-}T790M}$	Cobioer Biosciences	CBP73046
Experimental models: Organisms/strains		
BALB/C nu/nu mice	Beijing Huafukang Biotechnology	http://hfkbio.com/

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
GraphPad Prism version 9	GraphPad Software	https://www.graphpad-prism.cn/
ImageJ	Schneider et al.[7]	https://imagej.nih.gov/ij/
Deposited data		
Patient 1	This paper, deposited in National Genomics Data Center (NGDC) with accession number PRJCA017500	HRA004777

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yong He(heyong@tmmu.edu.cn).

Materials availability

All unique reagents generated in this study are available upon reasonable request from the lead contact.

Data and code availability

The original sequencing data related to Figure 1, Table S1 and Data S1 has been deposited in the Genome Sequence Archive for Human (GSA-Human: HRA004777) database. The project has been registered in BioProject (https://bigd.big.ac.cn/bioproject/) in National Genomics Data Center (NGDC: PRJCA017500). Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Ethics statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Daping Hospital for studies involving humans (Ethical review of medical research of Daping Hospital 2019-168). All subjects enrolled in the study gave written informed consent. Written informed consent was obtained from patients prior to publication of this article. Animal studies were conducted under protocols approved by the Ethics Committee of Daping Hospital on the Use and Care of Animals (AMUWEC20211187).

Mice

Briefly, a total of 1×10^6 PC-9^{PIK3CG} cells were injected subcutaneously into the left forelimb of 6-week-old female BALB/c nu/nu mice (Laboratory Animal Center of Army Medical University). Each group of mice with transplanted tumors was then randomly assigned to four groups. Drug treatment was initiated on day 5 and continued until the average tumor volume reached 50 mm^3 on day 7. Osimertinib (10 mg/kg) or aspirin (20 mg/kg) was intragastrically administrated every day. In accordance with institutional guidelines, these mice were housed in individual ventilated cages. In a treatment program, tumor sizes and body weights are measured every two days, and the tumor volume is calculated as follows: (length × $width^2$)/2. They were monitored for 28 days until euthanasia. Animal experiments were performed with the approval of the Committee on Animal Experimentation of the Army Medical University (Chongqing, China).

NSCLC PDX models were generated by Shanghai LIDE Biotechnology Co., Ltd. (Shanghai, China) with informed consent obtained from the patient (LD1-0025-200739). Samples were obtained from a 56-year-old female patient who had acquired resistance to osimertinib. The presence of EGFR-19 deletion and *PIK3CA* (H1047R) mutations were confirmed by next-generation sequencing (NGS) detection. Collected tumor tissue was cut into 5 mm sections and implanted subcutaneously into BALB/c nu/nu mice (n = 3). Once tumor volumes reached 200 mm^3, mouse were randomly assigned to four groups. Phosphate-buffered saline (PBS) (200μl), osimertinib (10mg/kg), aspirin (20mg/kg), or osimertinib and aspirin combinatorial treatments were intragastrically administered. In accordance with institutional guidelines, all mice were housed

in individual ventilated cages and monitored for four weeks before sacrifice. The dosage of aspirin and osimertinib administered was based on previous studies.[14]

METHOD DETAILS

Cell lines and reagents

Human lung cancer cell lines HCC827, H3255, and PC-9 were purchased from the American Type Culture Collection (ATCC). PC-9GR (gefitinib resistance due to T790M mutation) was created through chronic exposure of the drug at gradual dose increments. Ba/F3-EGFR^{Del19} and Ba/F3-EGFR$^{Del19\text{-}T790M}$ cell lines were generated by and obtained from Cobioer Biosciences Co., Ltd. (Nanjing, China). Osimertinib (S7297) and aspirin (S3017) were obtained from Selleck Chemicals.

DNA preparation, targeted sequencing and analysis

DNA from formalin-fixed paraffin-embedded (FFPE) tumor biopsy and ascitic cell pellet was extracted using QIAamp DNA FFPE tissue kit (Qiagen). Circulating cell-free DNA (cfDNA) was recovered from 4-5ml of plasma using QIAamp Circulating Nucleic Acid kit (Qiagen). Capture-based targeted sequencing using a panel consisting of 168 cancer-related genes and subsequent sequencing analysis were performed as previously described.[35] All detection service was performed by Burning Rock Biotech(Guangzhou, China).

Lentivirus production and transduction

Lenti-*PIK3CA* (H1047R)-GFP, Lenti-*PIK3CG* (L468M)-GFP, and Lenti-NC viruses were purchased from GeneChem Co., Ltd. (Shanghai, China). HCC827, H3255, PC-9, PC-9GR, Ba/F3-EGFR^{Del19}, and Ba/F3-EGFR$^{Del19\text{-}T790M}$ cells were transfected with ViraPower packaging mix using Lipofectamine 3000 reagent (Invitrogen, USA) based on the manufacturer's instructions.

Cell viability assay

Tumor cells were cultured in 96 well plates at a density of 3000 cells/well. To test the efficacy of drugs and their combinations, aspirin concentrations were varied in the media. Following 48 hours (h) of culture, The Cell Counting Kit-8 was used to assess cell viability (CCK-8; MedChemExpress, Monmouth Junction, NJ, USA) assay. The assay was performed as described in the product description. Three independent experiments were carried out.

Western blot analysis

Tumor cells grown and were treated as indicated, then total protein of cells were harvested by scraping and were quantitated by BCA Protein Assay Kit (EMD Millipore, MA, USA). The following primary antibodies were used: Antibodies against PI3K (P110α) (#4255S), PI3K (P110γ) (#5405S), Bim (#2933S), Akt (#9272S), phospho (Ser473)-Akt (#4060S), mTOR (#2983), phospho(Ser2448)-mTOR (#5536), and β-tubulin (#2128) were obtained from Cell Signaling Technology. The secondary antibody was a goat-anti-rabbit antibody conjugated with horseradish peroxidase. An antibody against Tubulin was used to determine equal protein loading. Three independent experiments were carried out to obtain the results.

Hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining

Tumor tissues were fixed and embedded with paraffin. Then, 3-mm formation-fixed paraffin-embedded slides were prepared for H&E and IHC staining. Briefly, tumor sections were deparaffinized, tissue incubated with specific antibody, anti-rabbit goat IgG labeled with HRP (ZSGB-Bio, China) was used as the secondary antibody. Staining was performed using the protocol from the DAB display reagent kit and the primary antibody Ki67 as a marker for cell proliferation.

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism version 9.0 software packages were used for data analyses. Data are presented as mean ± SEM or mean ± SD. Statistical significance was determined by t tests (two-tailed) for two groups.