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AXIN1/MYC Axis Mediated the Osimertinib Resistance in EGFR Mutant Non-Small Cell Lung Cancer Cells

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Osimertinib, a promising and approved third-generation epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI), is a standard strategy for EGFR-mutant non-small cell lung cancer (NSCLC) patients. However, developed resistance is unavoidable, which reduces its long-term effectiveness. In this study, RNA sequencing was performed to analyze differentially expressed genes (DEGs). The PrognoScan database and Gene Expression Profiling Interactive Analysis (GEPIA) were used to identify the key genes for clinical prognosis and gene correlation respectively. Protein expression was determined by western blot analysis. Cell viability assay and Ki67 staining were used to evaluate the effect of osimertinib on tumor cells. Finally, we screened out two hub genes, myelocytomatosis oncogene (*Myc*) and axis inhibition protein 1 (*Axin1*), upregulated in three osimertinib-resistant cell lines through RNA sequencing and bioinformatics analysis. Next, cell experiment confirmed that expression of C-MYC and AXIN1 were elevated in different EGFR mutant NSCLC cell lines with acquired resistance to osimertinib, compared with their corresponding parental cell lines. Furthermore, we demonstrated that AXIN1 upregulated the expression of C-MYC and mediated the acquired resistance of EGFR mutant NSCLC cells to osimertinib *in vitro*. In conclusion, AXIN1 affected the sensitivity of EGFR mutant NSCLC to osimertinib via regulating C-MYC expression *in vitro*. Targeting AXIN1/MYC signaling may be a potential new strategy for overcoming acquired resistance to osimertinib.

Keywords: AXIN1; EGFR; MYC; NSCLC; osimertinib
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Introduction

Lung cancer is the leading cause of cancer deaths worldwide, with approximately 350 deaths per day (Siegel et al. 2022). In recent years, the development of targeted therapy with small-molecule epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) has dramatically revolutionized the treatment landscape of epidermal growth factor receptor (EGFR)-mutant non-small cell lung cancer (NSCLC), but acquired drug resistance is an unavoidable problem. EGFR T790M was the most common resistance mechanism of 1st and 2nd generation TKIs (Schmid et al. 2020). Osimertinib, a 3rd generation EGFR-TKI, improved the outcomes of EGFR-mutant NSCLC patients and had been established as first-line therapy (Ramalingam et al. 2020). In addition, the ADAURA (ClinicalTrials.gov identifier NCT02511106) trial demonstrated the use of osimertinib in patients with resected NSCLC stage IB to IIIA harboring EGFR mutations, resulting in the approval of the drug as an adjuvant treatment (Wu

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et al. 2020). Unfortunately, effective therapeutic strategies are still lacking.

The acquired osimertinib resistance can be broadly divided into EGFR-dependent or EGFR-independent groups, such as mutations in C797S, the most common tertiary EGFR mutation (Mok et al. 2017), gene amplification of mesenchymal epithelial transition (*Met*) or Erb-b2 receptor tyrosine kinase 2 (*Erbb2*) (Takezawa et al. 2012; Ortiz-Cuaran et al. 2016), somatic mutations in the gene encoding the alpha catalytic subunit of phosphatidylinositol 3 kinase (*Pi3kca*), or the v-raf murine sarcoma viral oncogene homolog B (*Braf*) (Barnes et al. 2017). Some overlap when osimertinib is administered as front-line or second-line therapy. Simultaneously, the unknown mechanism is up to 50% (Leonetti et al. 2019), and the optimal management of patients constitutes a serious challenge after progression to osimertinib (Koulouris et al. 2022). Thus, mounting studies are conducted to figure out this problem. However, acquired resistance mechanisms to osimertinib are largely unknown and must be fully elucidated.

Over the past decade, RNA sequencing has significantly progressed and became new technologies and applications in cancer research, including biomarker discovery and drug resistance (Hong et al. 2020; Ergin et al. 2022). Furthermore, high-resolution mass spectrometry-based omics, such as proteomics, combined with computational sciences, provided an extremely powerful approach for studying biomedical questions (Tyanova and Cox 2018). In the present study, using these methods, we identified and verified genomic alterations associated with acquired resistance in three paired cell lines sensitive/resistant to osimertinib.

Materials and Methods

Cell culture and reagents

Human lung cancer cell lines H1975 and HCC827 were from the American Type Culture Collection (ATCC, Manassas, VA, USA). Osimertinib-resistant cell lines were established in our previous study (Li et al. 2019). Cells were cultured in RPMI-1640 (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Waltham, MA, USA) and 1% penicillin/streptomycin at 37°C in a humidified 5% CO_2 incubator. Osimertinib was purchased from MedChemExpress (Monmouth Junction, NJ, USA), and MG132 was from Selleck (Houston, TX, USA). Antibodies against C-MYC, AXIN1, and GAPDH were purchased from Abcam (Cambridge, UK).

Cell viability assay

Cell viability was determined by Cell Counting Kit-8 (CCK8; MedChemExpress) according to the manufacturer's instructions. Briefly, cells were seeded in a 96-well plate and treated as indicated for 48 h. Then, the medium was replenished and absorbances were measured on a Sunrise R microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm. The IC50 was calculated using Prism v. 8.00 (GraphPad Software, San Diego, CA, USA).

Ki67 staining

Cell proliferation was assessed by Ki67 staining assay. Briefly, cells were seeded in six-well plates and treated as indicated for 48 h. Then, cells were fixed and incubated overnight with Ki67. Cells were observed under a fluorescence microscope after incubation with a secondary antibody and 4,6-diamino-2-phenyl indole (DAPI).

siRNA transfection

Small interfering RNAs (siRNAs, RiboBio, Guangzhou, China) were used to silence endogenous AXIN1 expression (sequence: TTCTGAGGGAGT CTTCCGGG; GGATCCGTAAGCAGCACCGC) in PC-9GROR cells. For the evaluation of efficacy, cells were transfected with either 80 pmol siRNA or negative control siRNA (siNC) using Lipofectamine RNAiMAX (Thermo Fisher Scientific), following the manufacturer's instructions. At 72 h post-transfection, knockdown efficiency was determined by examining endogenous protein expression by western blot.

Lentivirus production and transduction

To generate cell lines overexpressing AXIN1, the human AXIN1 cDNA sequence (Genebank accession number: NM_003502) was searched for suitable target sequences. LentiCRISPRv2-AXIN-1 was designed and generated by Sino Biological (Suzhou, China). The transfection efficiency was determined by examining endogenous protein expression by western blot.

Western blot

Western blot was performed as previously described (Li et al. 2019). Briefly, cells were harvested from 6-well plates after washing with phosphate-buffered saline (PBS) and lysed for 30 min at 4°C in RIPA buffer (Sigma-Aldrich, Darmstadt, German). The BCA protein assay kit determined the quantitative protein analysis after centrifugation at 12,000 × *g* for 20 min at 4°C. Equal amounts of protein were submitted to gel electrophoresis for 2 h at 110 V, then transferred onto PVDF membranes (90 min, 200 mA) (Millipore, Darmstadt, German). Membranes were blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature and incubated overnight at 4°C with primary antibodies. Next, the membranes were washed and incubated with secondary antibodies for 1 h, then imaged with ChemiDoc Touch System (Bio-Rad, Hercules, CA, USA).

RNA sequencing

Transcriptome analysis was performed as previously reported (Li et al. 2022). The cell pellet was prepared and resuspended in TRIzol (Invitrogen, Waltham, MA, USA). Then, the total RNA was extracted using PureLink RNA Micro Kit (Thermo Fisher, 12183016) according to the

manufacturer's protocol. Differentially expressed genes (DEGs) were identified using R-package CORNAS41. A *P*-value < 0.05 and fold change > 1.5 or < 0.5 were set as the threshold for significant differential expression. Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis of the DEGs were performed using R package clusterProfiler42.

Protein–protein interaction (PPI) analysis

The interaction network among these genes was analyzed by the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (https://string-db.org/).

PrognoScan database analysis

The correlation between the core genes and overall survival (OS) in lung cancer was analyzed by Kaplan-Meier plotter (http://www.kmplot.com) (Shi et al. 2022). PrognoScan searches for relationships between gene expression and patient prognosis across a large collection of publicly available cancer microarray datasets. The hazard ratio (HR) and Cox *P*-value were calculated and displayed on the plot.

Gene correlation analysis in GEPIA (Gene Expression Profiling Interactive Analysis)

The online database GEPIA (http://gepia.cancer-pku.cn/index.html) was used to confirm the significantly correlated genes (Shi et al. 2022). Gene expression correlation analysis was performed for given sets of TCGA expression data. AXIN1 was used for the x-axis, and MYC was represented on the y-axis.

Data analysis

Statistical analysis was performed using GraphPad Prism 8.0. Data are expressed as mean ± standard deviation (SD). Student t-test was generally used to analyze the differences between the two groups. A *P*-value < 0.05 was considered statistically significant.

Results

Identification of key genes in EGFR mutant lung cancer cells with osimertinib resistance

To figure out the potential resistance mechanism to osimertinib, a high-throughput transcriptome sequencing (RNA-Seq) was performed in HCC827 and HCC827OR cells, and analysis was conducted with our previous data (PC-9GR vs. PC-9GROR, and H1975 vs. H1975OR) (Li et al. 2022). We obtained the RNA-seq datasets and screened out 886 DEGs from all three datasets. The coverage and overlap of genes were illustrated in the Venn diagram (Fig. 1A). Based on these genes, we performed KEGG pathway enrichment analysis and found that metabolic pathways, pathways in cancer and human T-cell leukemia virus 1 infection are the top three pathways (Fig. 1B). Considering the correlation with tumor drug resistance, we focused on pathways in cancer and found 31 genes enriched in this pathway.

Next, we constructed a PPI network to explore how these DEGs interact with each other, and it turned out that seven genes (*Nfkbia*, *Keap1*, *Rela*, *Hras*, *Jun*, *Myc*, and *Axin1*) which are located at the center of the network diagram have higher connectivity and may be the hub genes (Fig. 1C). The heatmap showed that 31 DEGs among the three paired cell lines are upregulated in PC-9GROR,

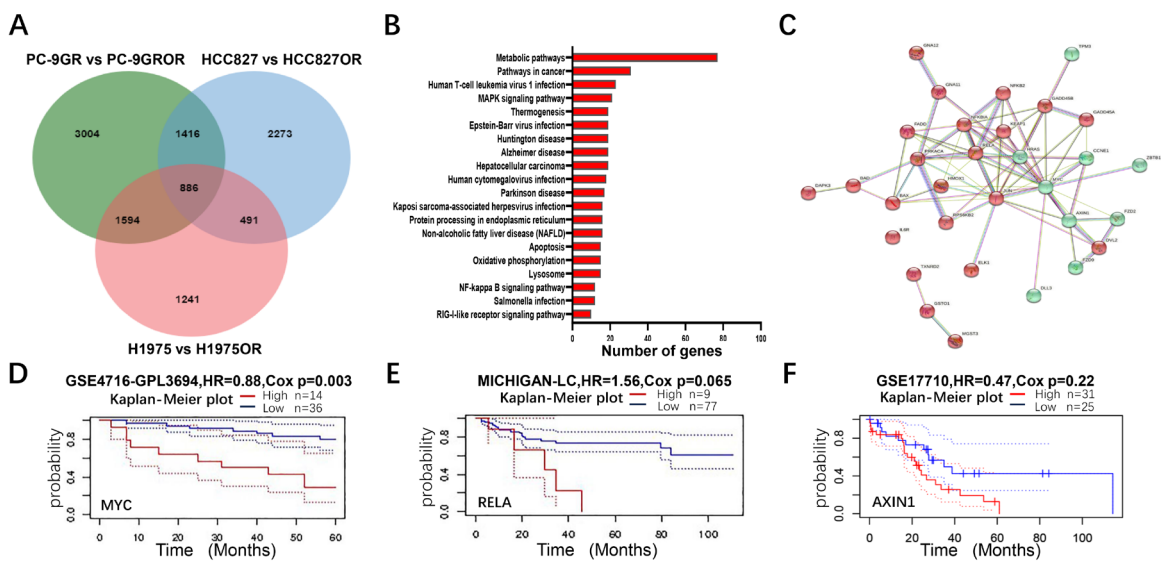

Fig. 1. The screening of hub genes among three paired osimertinib-sensitive/resistant cancer cells.
(A) Venn diagram demonstrates overlapped differentially expressed genes (DEGs) among three paired cells. (B) Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis of the common DEGs. (C) Protein–protein interaction network of the genes enriched in pathways in cancer. Kaplan-Meier curves for overall survival probability of MYC (D), RELA (E), and AXIN1 (F) in lung cancer, analyzed by the PrognoScan database.

HCC827OR, and H1975OR cells, respectively (Supplementary Fig. S1). Furthermore, we did overall survival (OS) analyses of all these candidate genes in lung cancer using PrognoScan database, which indicated that four genes (*Nfkbia*, *Keap1*, *Hras*, and *Jun*) are positively correlated with the prognosis of NSCLC patients (Supplementary Fig. S2), while *Myc*, *Rela*, and *Axin1* negatively affect the OS, and *Myc* has the most significant *P*-value (Fig. 1D-F). Coincidentally, a previous study demonstrated that targeting *c-Myc* could overcome the acquired resistance of EGFR mutant NSCLC cells to osimertinib (Zhu et al. 2021). These results showed that *Myc* is a crucial upregulated gene in osimertinib-resistant EGFR mutant lung cancer cells.

Cell experiments verified the upregulation of protein C-MYC in osimertinib-resistant lung cancer cells

We next performed cell experiments to confirm whether protein C-MYC was upregulated in osimertinib-resistant lung cancer cells. As shown in Fig. 2A, western blot analysis showed that the level of protein C-MYC in PC-9GROR, HCC827OR, and H1975OR cells was elevated compared with their corresponding parental cell lines. Likewise, the immunofluorescence staining showed increased expression of C-MYC in PC-9GROR cells compared to PC-9GR cells (Fig. 2B). To clarify the mechanism of why osimertinib-resistant cancer cells have a high level of C-MYC expression, we determined whether the degradation of C-MYC was different. As expected, compared with PC-9GR cells, protein C-MYC degraded slowly in PC-9GROR cells in the presence of the protein synthesis inhibitor cycloheximide (CHX) (Fig. 2C). Furthermore, the addition of MG132, a proteasome inhibitor that prevents protein degradation, increased the expression of C-MYC (Fig. 2D). Taken together, we verified that the level of protein C-MYC was elevated in osimertinib-resistant cancer cells due to slower degradation.

AXIN1 positively affected the expression of C-MYC

The PPI network diagram shows that AXIN1, NFKBIA, and JUN interact with MYC to a higher degree (Fig. 3A), but only AXIN1 had a positive correlation with the OS. Also, it was reported that the Wnt target genes *Axin2* and *c-Myc* showed a positive trend (Ren et al. 2018). Thus, we used the online database GEPIA, which was based on the TCGA database, to study the gene correlation, and it turned out that the expression of MYC was positively correlated with AXIN1 (Fig. 3B). To confirm that AXIN1 was upregulated in osimertinib-resistant cancer cells, we performed a western blot analysis of the three paired cell strains, and it turned out that the expression of AXIN1 in PC-9GROR, HCC827OR, and H1975OR cells was elevated (Fig. 3C). Next, to explore whether AXIN1 regulates MYC, we constructed AXIN1 overexpression cell lines using PC-9GR and found that the expression of AXIN1 and C-MYC were elevated (Fig. 3D). Likewise, knockdown of AXIN1 in PC-9GROR cells resulted in reduced levels of AXIN1 and C-MYC simultaneously (Fig. 3E). Then, we performed a CHX experiment in these cell lines. Western blot analysis showed that overexpression of AXIN1 resulted in a slowed degradation of C-MYC in PC-9GR cells (Fig. 3F), while knockdown of AXIN1 in PC-9GROR cells accelerated the degradation of C-MYC (Fig. 3G). These results demonstrated that AXIN1 affected C-MYC expression positively by regulating its degradation.

AXIN1 mediated the resistance of PC-9GR cancer cells to osimertinib

To answer whether AXIN1 affected the sensitivity of EGFR-mutant lung cancer cells to osimertinib, we first conducted cell viability CCK-8 assay. As shown in Fig. 4A, overexpression of AXIN1 reduced the sensitivity of PC-9GR cells to osimertinib. Meanwhile, in PC-9GROR cells, the knockdown of AXIN1 increased the sensitivity (Fig. 4B). Then, we performed Ki67 staining to determine further the role of AXIN1 in the resistance of PC-9GR cancer cells to osimertinib. As shown in Fig. 4C, osimertinib treatment significantly decreased the percentage of Ki67-positive cells in PC-9GR cancer cells, but it showed little effect when AXIN1 was overexpressed. In contrast, the administration of osimertinib showed significant inhibition of cell proliferation when AXIN1 was knocked down in PC-9GROR cells, although it could not inhibit the proliferation of PC-9GROR cells in the control group (Fig. 4D). Thus, these results implied that AXIN1 could mediate the resistance of EGFR mutant NSCLC cells to osimertinib.

Discussion

The current study identified key gene alterations in three paired cell lines sensitive/resistant to osimertinib, including first- and second-line treatment. The common feature is that *Axin1* and *Myc* increased in osimertinib-resistant cell lines in comparison with their corresponding parental cell lines according to the RNA sequencing. We confirmed this phenomenon by examining protein expression and sought the relationship between gene alterations and drug resistance. Thus, our study provides a novel finding for overcoming acquired resistance to osimertinib.

The resistance mechanisms of 1st and 2nd generation TKIs have been fully studied, and EGFR T790M is the most common mechanism (Wu and Shih 2018). In recent years, osimertinib has been widely used in EGFR mutant NSCLC patients in first- or second-line settings. Mounting studies have revealed some potential resistance mechanisms to osimertinib, such as resistance mutations in *Egfr*, activation of alternate *Rtks*, *Ras*/*Raf* mutations, *Pi3kca* mutations, and Exon 16-Skipping *Her2* (Le et al. 2018; Oxnard et al. 2018; Yang et al. 2018; Hsu et al. 2020). To figure out other unknown mechanisms, the present study provides new insight into gene alterations between osimertinib-sensitive and resistant cells via RNA sequencing (Fig. 1A), a vital tool utilized in many cancer research aspects (Hong et al. 2020). Bioinformatics has a promising potential to drive

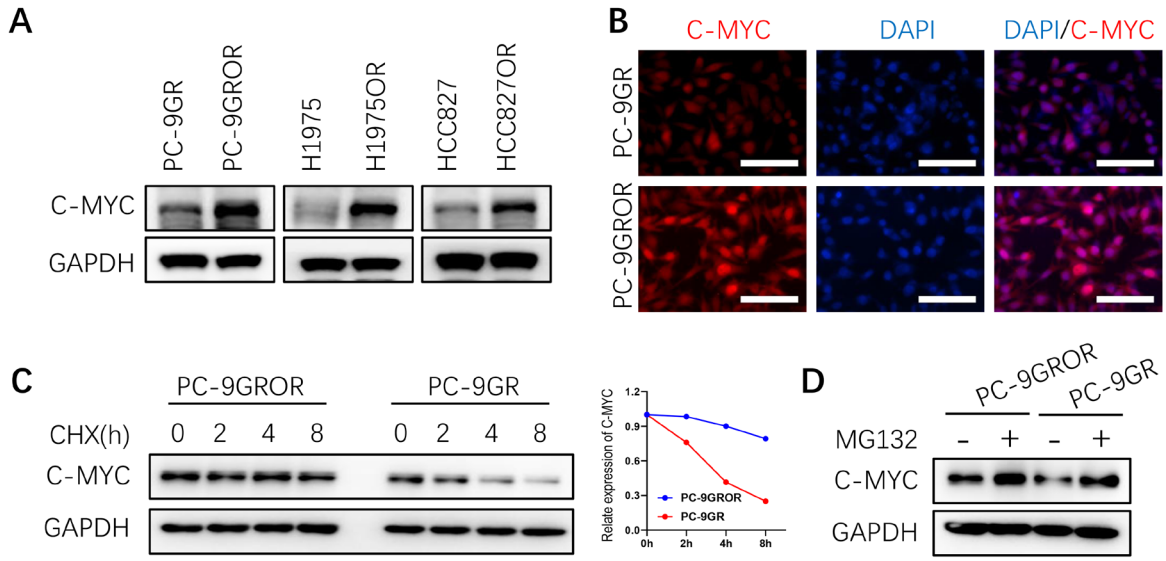

Fig. 2. The expression of C-MYC was upregulated in osimertinib-resistant cancer cells.
(A) Cell lysates from PC-9GR, PC-9GROR, HCC827, HCC827OR, H1975, and H1975OR were immunoblotted with antibodies against C-MYC. (B) Immunofluorescence staining of C-MYC from PC-9GR and PC-9GROR cells. Scale bars: 50 μm. (C) Western blot showing the expression of C-MYC in PC-9GROR and PC-9GR cells treated with 10 μM cycloheximide (CHX) at indicated intervals, and the level of C-MYC was quantified using ImageJ software. (D) Western blot analysis of C-MYC in PC-9GROR and PC-9GR cells in the presence of MG132.

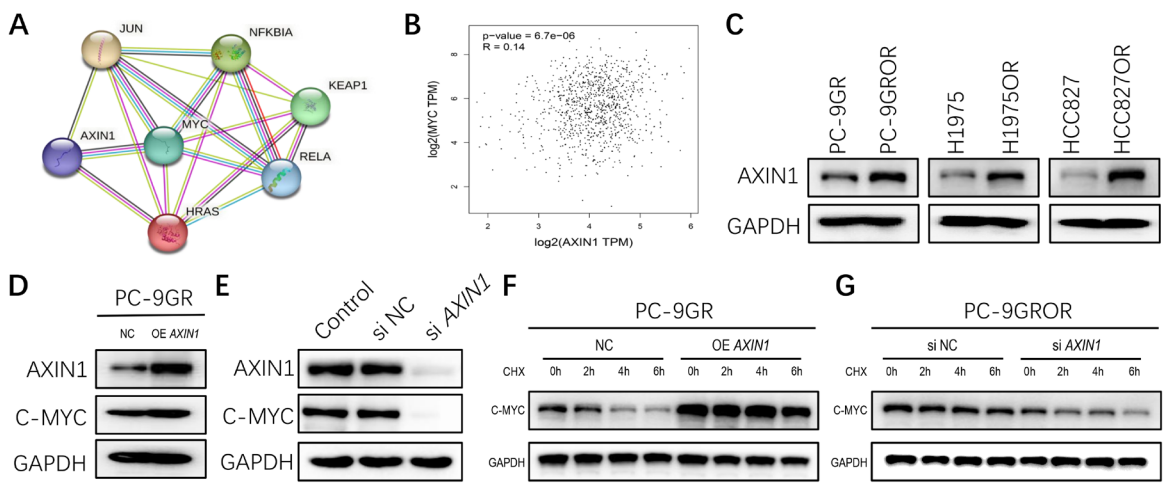

Fig. 3. AXIN1 was upregulated in osimertinib-resistant cancer cells and affected the expression of C-MYC.
(A) Protein-protein interaction network of the hub genes (*NFKBIA, KEAP1, RELA, HRAS, JUN, MYC, AXIN1*). (B) Scatter plot of MYC and AXIN1 based on the online database GEPIA, R = 0.14, $P < 0.01$. (C) Cell lysates from PC-9GR, PC-9GROR, H1975, H1975OR, HCC827, and HCC827OR were immunoblotted with antibodies against AXIN1. (D) Western blot analysis of AXIN1 and C-MYC expression after virus-mediated overexpression of AXIN1 in PC-9GR cells. (E) Western blot showing the expression of AXIN1 and C-MYC after siRNA-mediated knockdown of AXIN1. (F) Western blot analysis of C-MYC in PC-9GR and PC-9GR (OE AXIN1) cells treated with 10 μM cycloheximide (CHX) at indicated intervals. (G) Western blot showing C-MYC expression in PC-9GROR and PC-9GROR (si AXIN1) cells treated with 10 μM CHX at indicated intervals.

cancer research and the development of new therapies (Jiang et al. 2022). Thus, combining bioinformatic analysis, we further identified the hub genes and found that *Myc* may be a pivotal gene associated with osimertinib resistance (Fig. 1).

The *Myc* gene, consisting of 3 paralogs, *C-myc*, *N-myc* and *L-myc*, was considered a human cancer driver gene and a target for treating cancer (Duffy et al. 2021). Coincidentally, Zhu et al. (2021) demonstrated that osimertinib decreased C-MYC levels via enhancing protein degradation in EGFR-mutant NSCLC cell lines. Still, the levels of C-MYC were substantially elevated in osimertinib-resis-

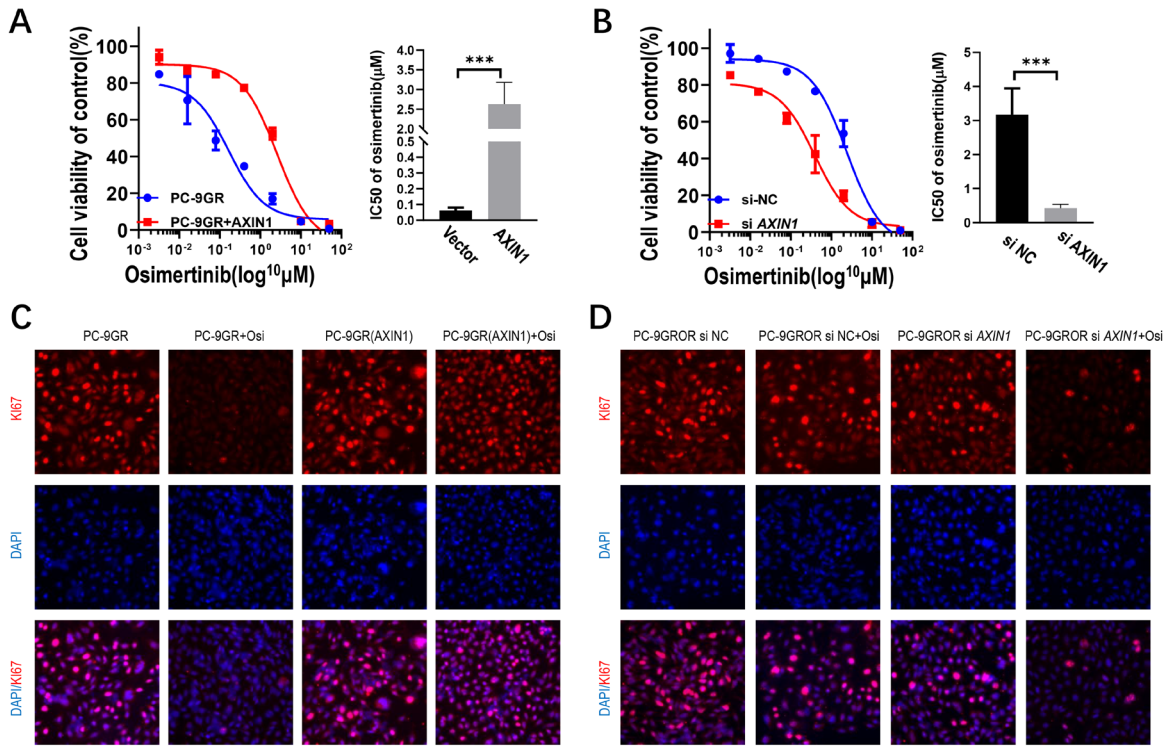

Fig. 4. AXIN1 affected the anti-tumor efficacy of osimertinib.
(A) Cell viability CCK-8 assay for PC-9GR cells transfected with control or AXIN1 virus plasmid and treated with osimertinib for 48 h. Histogram shows IC50 values in the indicated groups, $^{***}P < 0.01$. (B) Cell viability CCK-8 assay for PC-9GROR cells transfected with control or AXIN1 siRNAs and treated with osimertinib for 48 h. Histogram shows IC50 values in the indicated groups, $^{***}P < 0.01$. (C) Ki67 incorporation on PC-9GR and PC-9GR (OE AXIN1) cells treated with osimertinib. Scale bars: 100 μm. (D) Ki67 incorporation on PC-9GROR cells transfected with control or AXIN1 siRNAs and treated with osimertinib or not. Scale bars: 100 μm.

tant cell lines. They could not be reduced any further by osimertinib, indicating that targeting *c-Myc* could overcome the acquired resistance of EGFR mutant NSCLC cells to the third-generation EGFR tyrosine kinase inhibitor (Zhu et al. 2021). In line with this, we found that the level of protein C-MYC is higher in three different EGFR mutant NSCLC cell lines with acquired resistance to osimertinib compared with their corresponding parental cell lines (Fig. 2A). Furthermore, C-MYC degradation also became slow in PC-9GROR cells (Fig. 2C). Although these results showed that C-MYC inhibition would be a powerful approach to overcoming acquired resistance to osimertinib, direct targeting of C-MYC has been challenging for decades. Alternatively, indirect targeting of MYC has been widely explored to achieve desirable anti-tumor effects (Chen et al. 2018). Thus, we expect to find a potential strategy to target MYC.

We know that the Wnt/β-catenin signaling pathway plays a crucial role in cancer progression, and inhibition of β-catenin target genes, such as *Myc* and *Axin2*, may be a promising strategy for cancer treatment (Zhang et al. 2020). Of note, AXIN1 and AXIN2 are thought to be equivalent suppressors of canonical Wnt signaling (Figeac and Zammit 2015). The scaffold protein AXIN1 is a platform for properly functioning multiple proteins (Mariotti et al. 2017). The present study shows that AXIN1 was highly associated with MYC from the PPI diagram (Fig. 3A). More importantly, the upregulation of AXIN1 was also associated with a worse OS (Fig. 1F). In consensus with this, a previous study demonstrated that preserved AXIN expression is associated with an aggressive phenotype in invasive breast carcinomas (Irini et al. 2013). Therefore, we conducted gene correlation analysis based on the GEPIA database and found that MYC was positively correlated with AXIN1 (Fig. 3B). Then, we showed that AXIN1 affected the expression of C-MYC by regulating its degradation (Fig. 3F, G). Furthermore, CCK-8 assay and Ki67 staining showed that AXIN1 affected the sensitivity of EGFR-mutant lung cancer cells to osimertinib (Fig. 4). These results indicate that targeting AXIN1/MYC may be a promising strategy to overcome osimertinib resistance.

In conclusion, this study demonstrated that MYC and AXIN1 are upregulated in three osimertinib-resistant lung cancer cell lines. Specifically, AXIN1 mediated the resistance of EGFR-mutant cancer cells to osimertinib by regulating the degradation of MYC. Future studies must investigate the underlying mechanism and confirm this effect in other EGFR-mutant cancer cell lines.

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Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Files

Please find supplementary file(s);
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