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*Original Article*

# NRP1 overexpression potentially enhances osimertinib resistance in NSCLC via activation of the PI3K/AKT signaling pathway

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**Abstract:** Resistance to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) is the main cause of mortality in lung cancer. This study aimed to investigate the roles of neuropilin 1 (NRP1) in non-small cell lung cancer (NSCLC). NRP1 expression was assessed in tumor tissues from patients with osimertinib-resistant (OR) NSCLC and osimertinib-responsive NSCLC as well as in patients with paracancerous NSCLC tissues who did not undergo radiotherapy or chemotherapy. *In vitro* experiments were conducted using five cell lines: BEAS-2B, HCC827, and PC9 cells, and the constructed OR cell lines, HCC827-OR and PC9-OR. HCC827-OR cells showing significant differences in osimertinib $IC_{50}$ were selected for further study. After investigating the effects of altering NRP1 expression on cell sensitivity to osimertinib, NRP1 expression was inhibited to further investigate changes in cell viability, proliferation, migration, invasion, and apoptosis in OR cells. Additionally, bioinformatics techniques were used to detect targets (Integrin β3) and signaling pathways (PI3K/AKT) downstream of NRP1; subsequent cell experiments verified their interactivity. Finally, an orthotopic mouse tumor model was constructed using HCC827-OR cells treated with a PI3K/AKT signaling pathway activator (740Y-P), allowing exploration of the role played by the PI3K/AKT signaling pathway via NRP1 regulation on NSCLC resistance both *in vivo* and *in vitro*. Results showed that NRP1 expression was significantly increased in the cells of patients with NSCLC-OR, and increased NRP1 expression reduced HCC827 cell sensitivity to osimertinib. Both *in vitro* and *in vivo* experiments showed that NRP1 deficiency mediated by NRP1 inhibitors inhibited HCC827-OR cell proliferation, migration, and invasion, promoted tumor cell apoptosis, and enhanced osimertinib efficacy. In contrast, 740Y-P partially inhibited the effects of NRP1 inhibitors combined with osimertinib on the PI3K/AKT signaling pathway and on tumor growth *in vivo* and *in vitro*. Cellular experimental results showed that NRP1 positively regulates the Integrin β3 expression and activation of the PI3K/AKT signaling pathway. Bioinformatics analysis showed that both NRP1 and Integrin β3 may jointly participate in regulating the PI3K/AKT signaling pathway. In conclusion, our findings suggest that elevated NRP1 expression in NSCLC tumor tissues may promote NSCLC resistance to osimertinib by activating the PI3K/AKT signaling pathway, and integrin β3 potentially being involved in this process. These insights may provide a novel strategy for combination therapy for OR NSCLC.

**Keywords:** Non-small cell lung cancer, resistant, neuropilin 1, EGFR tyrosine kinase inhibitors

## Introduction

Non-small-cell lung cancer (NSCLC) is the most common type of lung cancer [1]. Mutations in the epidermal growth factor receptor (EGFR), a transmembrane tyrosine kinase receptor [2], play a major role in the adenocarcinoma subtype of NSCLC [3]. Dysregulation of EGFR, which stimulates growth factor signaling, results in tumor cell proliferation, differentiation, migration, and invasion [4], and predicts advanced stages, metastasis, and poor overall survival

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rate in patients with NSCLC [5]. Studies have shown that anti-EGFR monoclonal antibodies targeting the extracellular domain of EGFR on the tumor cells' surface downregulate phosphorylated EGFR, promoting tumor cell death [6, 7], while epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) significantly improve survival and clinical outcomes for patients with EGFR-mutated NSCLC, and has become the recommended, standard first-line treatment option for these patients [8]. Currently, three generations of EGFR-TKIs have been approved for treating patients with different clinical settings of EGFR-mutated NSCLC. Among these, osimertinib, a third-generation EGFR-TKI, targets mutant receptors resistant to first- and second-generation EGFR-TKIs while minimizing toxicity [9], making it recommended as a first-line treatment for advanced NSCLC patients with active EGFR mutations [10]. However, similar to earlier-generation EGFR-TKIs, patients receiving osimertinib inevitably develop acquired resistance over time [11, 12]. The emergence of osimertinib-resistance (OR) poses a significant challenge to successful and long-lasting treatment for patients with EGFR-mutated NSCLC.

Neuropilins (NRPs) are transmembrane glycoprotein receptors [13]. NRP1 interacts with NRPs to induce angiogenesis. NRP1 is frequently overexpressed in lung cancer. For instance, Chen Z et al. discovered that the overexpression of NRP1 can enhance the proliferation and radiation resistance of NSCLC cells [14], while Juan Cong Dong et al. [15] and Lele Cong et al. [16] successively used shNRP1 and EG00229 as inhibitors of NRP1 to study their effects, which significantly enhanced the radiosensitivity of NSCLC cells both *in vivo* and *in vitro*. Furthermore, studies involving knockdown of NRP1 demonstrated significant inhibition of migration and invasion in NSCLC [17, 18]. Additionally, due to its role as a novel T-cell memory checkpoint, increased numbers of NRP1-CD8+ T cells in patients with NSCLC predict a better prognosis [19].

The phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway, which is one of the multiple subcellular signal cascades activated by mutated EGFR, remains active in various TKI-resistant cancers [20] and plays an important role in OR [21, 22]. 740Y-P, an agonist of PI3K, increases phosphorylation of PI3K and AKT, regulates the signaling of PI3K/AKT, and promotes glycolysis [23], proliferation [24], migration [25], invasion [26], oxidative stress, and ferroptosis [27] in NSCLC cells.

Integrin β3, also recognized as CD61 or platelet glycoprotein IIIa, plays pivotal roles in cell adhesion and signaling. It serves as a cell surface receptor protein [28] and is speculated to promote cell survival, invasion, and metastasis [29]. Additionally, it foster resistance to EGFR-TKI [30-32] in NSCLC. Integrin β3 is consistently highly expressed in lung cancer with acquired resistance to osimertinib [33], along with its mediated ternary compound comprising NRP1-integrin β3-KRASMUT, and its downstream signaling via PI3K-Akt acts as a primary resistance mechanism of KRASMUT NSCLC to cetuximab treatment [34].

In this study, the differential expression of NRP1 in patient with NSCLC-OR was investigated, cell and animal models were constructed, and bioinformatics analyses was conducted to explore the role of Integrin β3 and PI3K/AKT signaling pathway in the regulatory actions of NRP1 on NSCLC-OR.

## Materials and methods

### *Specimens*

Specimens of NSCLC and NSCLC-OR were harvested from 60 patients with NSCLC who received first-line osimertinib treatment while hospitalized in Huaian Hospital affiliated with Xuzhou Medical University. Among these patients, thirty (19 males, aged 60.68 ± 5.84 years; 11 females, aged 58.54 ± 5.60 years) showed osimertinib treatment resistance, whereas the other thirty (13 males, aged 62.85 ± 6.32 years; 17 females, aged 61.76 ± 6.51 years) responded well to the treatment. Paracancerous tissue specimens were obtained from an additional group of 30 (9 males, 49.78 ± 5.58 years old; 21 females, 53.24 ± 5.51 years old) patients with NSCLC, who had not received therapy at Huaian Hospital affiliated with Xuzhou Medical University. All patients ranged in age from 41 and 77 years of age. Samples were immediately stored in liquid nitrogen at -80°C. The number of human subjects totaled 90, and the date of sample collection ranged from October 2021 to October 2022. This study was approved by

the ethics committee of Huaian Hospital affiliated with Xuzhou Medical University (3208000052137-420). All patients provided informed consent.

*Immunohistochemistry (IHC) assay*

After fixing and deparaffinization, the slide-mounted tissues were immersed in EDTA buffer. Following blocking in 1% BSA, tissue sections were exposed to primary antibodies targeting NRP1 (ab81321, 1:400, Abcam, USA) and integrin β3 (ab179473, 1:100, Abcam), followed by washing and exposure to secondnary antibody (ab6721, 1:1000, Abcam). The sections were subsequently counterstained with DAPI before being visualization under a microscope at 400× (Nikon, Japan).

*Cell culture*

Human NSCLC cell lines HCC827 (RRID:CVCL_2063, CRL-2868), PC9 cells (RRID:CVCL_B260, ZY-H210) and human bronchial epithelial cell line BEAS-2B (RRID:CVCL_0168, CRL-3588) were obtained from ATCC (USA). Cells were incubated in the RPMI-1640 medium (SH30027.01, Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) in an atmosphere of 5% $CO_2$ at 37°C. The osimertinib-resistant (OR) HCC827 cell line was established by treating cells with increasing doses of osimertinib (2.5, 5, 10, 20, 30, 40, 60, 80, and 120 μM) for a duration of 14 d. HCC827- and PC9-OR cells were maintained in culture with the presence of 2.5 μM osimertinib. In the control group experiment, the cells were cultured in a normal medium. The effects of treatment were evaluated after 48 h (sufficient time for cell counts to double, reaching confluence).

Based on pre-experimental results (**Figure 2**), we selected a concentration of 2.5 μM Osimertinib, between the $IC_{50}$ values of HCC827 cells (0.01 μM) and HCC827-OR cells (29.39 μM), which was also suitable for PC9 cells (1.18 μM; 15.45 μM), and we chose to evaluate subsequent experiments at 48 hours post-treatment for subsequent experiments.

*Cell transfection*

Cells seeded at 1 × $10^6$/well in six-well plates reached a confluency of 60-80% upon transfection.

For siRNA, 3 μL siRNA (10 μM) was diluted in 150 μL Opti-MEM® Medium and 9 μL Lipofectamine® RNAiMAX Reagent (13778150, Invitrogen™) was diluted in 150 μL Opti-MEM® Medium. The diluted siRNA and diluted Lipofectamine® RNAiMAX Reagent solutions were then mixed together (1:1 ratio). After incubating for 5 min at room temperature, the siRNA-lipid complexes were added to cells (250 μL/well), and the cells were then incubated for 3 d at 37°C.

For pcDNA, The diluted Lipofectamine™ 3000 Reagent (L3000075, Invitrogen™) and the diluted DNA (250 μL OptiMEM™ medium and 10 μl LP3000™ Reagent-mix for every 5 μg DNA) were prepared according to manufacturer's instructions. After incubating for 15 min at room temperature, DNA-lipid complexes composed of diluted DNA mixed in a ratio of 1:1 with Diluted Lipofectamine™ 3000 Reagent were added to cells (250 μL/well). Then, the cells were incubated for 3 d at 37°C.

*RT-qPCR*

The total cellular RNA samples were obtained using a MolPure® Cell/Tissue Total RNA Kit (19221ES60, Yeasen, China). The total RNA (2 μg) was reverse transcribed into cDNA using a cDNA synthesis kit (cat. log. K1622, Thermo Fisher Scientific, USA). PCR was conducted using Hieff ®qPCR SYBR Green Master Mix (cat. log. 04913914001; Roche, Basel, Switzerland) on an ABI 7900 system (Applied Biosystems). mRNA expression was normalized to GAPDH and calculated using the $2^{-\Delta\Delta CT}$ method. The PCR primers used were as follows: NRP1 forward 5'-GAAGTTCCTCTCCAACTTCCC-3' and reverse 5'-AGGAAACAGCACAACGCAAA-3'; integrin β3 forward 5'-ACCAGTAACCTGCGGATTGG-3' and reverse 5'-TCCGTGACACACTCTGCTTC-3'; and GAPDH forward 5'-GGTCACCAGGGCTGCTTTTA-3' and reverse 5'-CCCGTTCTCAGCCATGTAGT-3'.

*Western blot*

After removing the culture medium and washing once with phosphate-buffered saline (PBS), M-PER™ (1 mL/$10^7$ cells) mammalian protein extraction reagent (78501, Thermo Fisher Scientific™) was added to the cell dish which was gently shaken for 5 min. Lysates were collected, transferred to microcentrifuge tubes, and clarified by centrifugation at

12000×g for 5 min to obtain total protein solutions. Protein concentrations were determined using a BCA assay kit (Abcam) and adjusted to 500 μg/mL. Forty micrograms of protein were separated by running a 12% SDS-PAGE at 120 V. Proteins were then transferred onto PVDF membranes, and subsequently blocked with 5% skimmed milk. The membranes were then incubated with 6 mL of diluted primary antibodies at 4°C overnight, and then washed and incubated with secondary antibodies at room temperature for 1 h. Finally, proteins were imaged using an ECL kit (cat. log. 32209, Pierce, USA).

*Co-IP assays*

Untreated HCC827-OR cells were homogenized in an IP lysis buffer (Pierce™ IP Cracking buffer, 87788: Halt™ Protease Inhibitor Cocktail, 78430: Halt™ Phosphatase Inhibitor Cocktail, 78428 = 100:1:1, Thermo Fisher Scientific). After being collected and centrifuged, the cell lysates were then incubated with primary antibodies. After adding Protein A+G agarose beads, the samples were pelleted, washed, and underwent western blotting.

*Immunofluorescence*

Cells were fixed with 4% paraformaldehyde, permeabilized, and blocked with 5% FBS. Subsequently, cells were then incubated with primary antibodies against NRP1 or integrin β3, followed by incubation with the secondnary antibody. Finally, the cells were counterstained with DAPI and images were captured using an immunofluorescence microscope (Zeiss, Germany).

*Cell counting kit-8 (CCK-8) assay*

After transfection, the cell suspension was plated into a 96-well plate (100 μL/well, 3 × $10^3$ cells/well) and incubated at 37°C overnight. For cells incubated for 24, 48, 72, and 96 h, 10 μL of CCK-8 reagent was added. The cells were then incubated for another hour and optical density values were detected using a microplate reader (Bio-rad, USA) at a wavelength of 450 nm. $IC_{50}$ values were determined using Graphpad Prism v.9.5.1.

*Colony formation assay*

The cells were plated into a six-well plate at a density of 1 × $10^6$ cells/well in 5% $CO_2$ at 37°C. After 3 weeks, the cells were fixed in 4% paraformaldehyde and stained using a Giemsa staining kit (ab150670, Abcam). Finally, colony images were captured using a microscope (Olympus).

*Wound healing*

Once reaching confluence, the cells were washed in PBS and centrifuged at 2000 rpm for 5 min. Supernatants were discarded and cells were re-suspended in serum-free medium with 0.2% BSA. Cell density was adjusted to reach a concentration of approximately 1 × $10^6$ cells/mL. Cells were plated into a 24-well plate at 200 μL/well. When the cell monolayers reached over 80% confluency, scratches on them would be created by gently dragging across their surfaces with the tip end of pipette tips having volumes equal to two hundred microliters (200 μL). Subsequently, the scratched monolayers would be supplemented with serum-free medium and cultured for twenty-four hours. Finally, the scratches on these monolayers could be visualized under an Olympus microscope.

*Transwell assay*

At the end of the incubation period, the cells were washed in PBS and centrifuged at 2000 rpm for 5 min. The supernatant was discarded, and cells were re-suspended in serum-free medium with 0.2% BSA. Cell density was adjusted to 1 × $10^6$ cells/mL. Cells were seeded into a 24-well plate at 200 μL/well, the upper chambers precoated with 50 μL/$cm^2$ Matrigel (354262, Corning, USA) when detecting invasion. The lower chambers were added medium containing 10% FBS (800 μL/well). Cells in the upper chambers were removed and cultured for 48 h, then the invading cells were stained using Giemsa reagent (Beyotime, Shangha, China) under low light conditions. Finally, five regions of interest were visualized under a microscope.

*Flow cytometry*

Apoptosis was visualized using an AV-FITC kit (V13242; Thermo Fisher Scientific). At the end of the incubation period, cells were washed in cold PBS and centrifuged at 2000 rpm for 5 min. Supernatants were discarded and cells were re-suspended in 1X annexin-binding buffer. After measuring cell density, cells were

diluted to approximately 1 × $10^6$ cells/mL in annexin-binding buffer. Fifteen microliter of Annexin V conjugate and 1.5 μL of 100 μg/mL PI working solution were added to each sample containing 100 μL cell suspension. Cells were then incubated for 15 min at room temperature, washed with 1X annexin-binding buffer, and mounted on slides. Apoptosis rates were subsequently determined using flow cytometry (Biosciences, USA).

*Xenograft assay*

Twenty-four BALB/c nude mice (6-8 weeks, 18-22 g) were obtained from the Shanghai Laboratory Animal Company (Shanghai, China). The mice were randomly divided into four groups: control, osimertinib, osimertinib+NRP1 inhibitor, and osimertinib+NRP1 inhibitor+740Y-P group. Each mouse was subcutaneously injected with 3 × $10^4$ HCC827-OR cells. Tumor size was measured every 3 d using the formula $V = lw^2/2$. On day 21 of modeling, when the long diameter of the tumor exceeded 10 mm, osimertinib was administered orally at a dose of 5 mg/kg once a day in the osimertinib group. In the NRP1 inhibitor group, si-NRP1 at a concentration of 24 μg/mL was injected via tail vein. In group 740Y-P, an injection of 2 μg/μL of compound 740Y-P was given via tail vein. The control group received normal saline injections (100 μL per mouse) every three days. At day 42, all mice were sacrificed and tumors were collected for analysis. This study was approved by the Experimental Animal Board of Huaian Hospital affiliated with Xuzhou Medical University (3208000052137-421).

*Bioinformatics analyses*

NRP1- and integrin β3 (ITGB3)-related signaling pathways were analyzed using the KEGG database (https://www.genome.jp/kegg/), while genes interacting with NRP1 were analyzed using the STRING database (https://cn.string-db.org/).

*Statistical analysis*

All data obtained from all triplicate experiments were analyzed using GraphPad v.9.5.1 software and presented as means ± standard deviation values. Differences among multiple groups were analyzed using analysis of variance, the least significant difference test was used for post hoc analysis, and unpaired t-test was used for two group comparisons. Statistical significance was set at $P<0.05$.

## Results

*NRP1 is overexpressed in patients with OR-resistant NSCLC*

As a nerve fiber protein, NRP1 plays an important role in nerve and vascular development as well as immune regulation. To verify the oncogenic function of NRP1 in lung cancer, we assessed its expression in patients with NSCLC, and found that NRP1 mRNA expression was markedly increased in patients with NSCLC, and subtype analysis showed that NRP1 expression was elevated in EGFR-TKI-resistant NSCLC (**Figure 1A**). Similarly, there was a marked elevation of NRP1 protein expression in patients with OR-resistant NSCLC (**Figure 1B**). Moreover, IHC scores were higher in patients with OR-resistant NSCLC (**Figure 1C**).

*NRP1 is overexpressed in OR-resistant NSCLC cells*

Compared to parental HCC827 and PC9 cells, HCC827-OR and PC9-OR cells exhibited significantly enhanced dose- and time-dependent resistance to osimertinib (**Figure 2A-D**). We detected NRP1 expression in NSCLC cells. In addition, NRP1 mRNA expression was markedly increased in OR NSCLC cells (**Figure 2E**), which is consistent with the western blot results (**Figure 2F**). Notably, NRP1 expression was significantly elevated in NSCLC cells (**Figure 2G**), indicating successful transfection of these cells. Overexpression of NRP1 promoted the viability of simertinib-treated NSCLC cells (**Figure 2H**). NRP1 expression markedly decreased in HCC827-OR cells treated with siRNA (**Figure 2I**), suggesting that the cells were successfully transfected. NRP1-knockdown inhibited HCC827-OR cell viability (**Figure 2J**).

*NRP1 deficiency alleviates chemoresistance of HCC827 cells*

The knockdown of NRP1 markedly decreased the cell viability of HCC827-OR cells (**Figure 3A**). Moreover, there was a marked decrease in

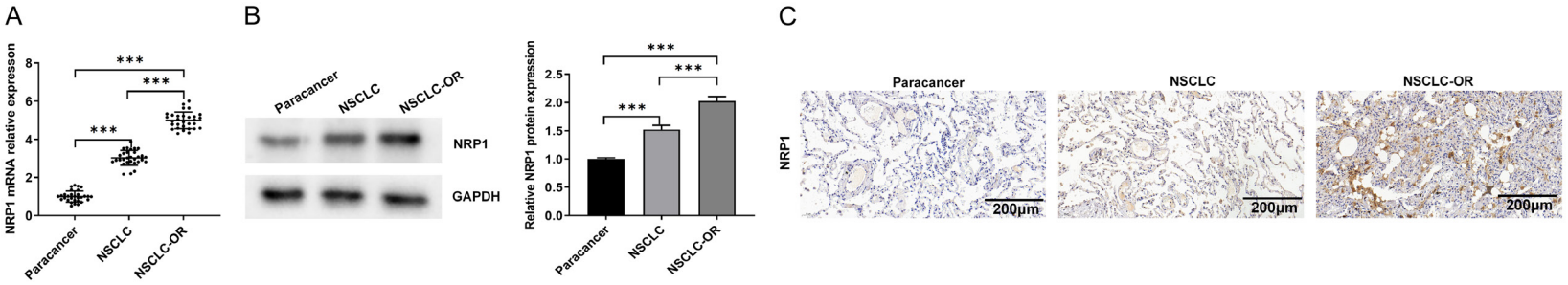


**Figure 1.** NRP1 is overexpressed in OR-resistant NSCLC patients. A. NRP1 mRNA expression in NSCLC patients determined using RT-qPCR (30 participants in each group). B. NRP1 protein expression in NSCLC patients was determined using a western blot. C. NRP1 expression in NSCLC patients was determined using IHC (200X). ***$P$<0.001.

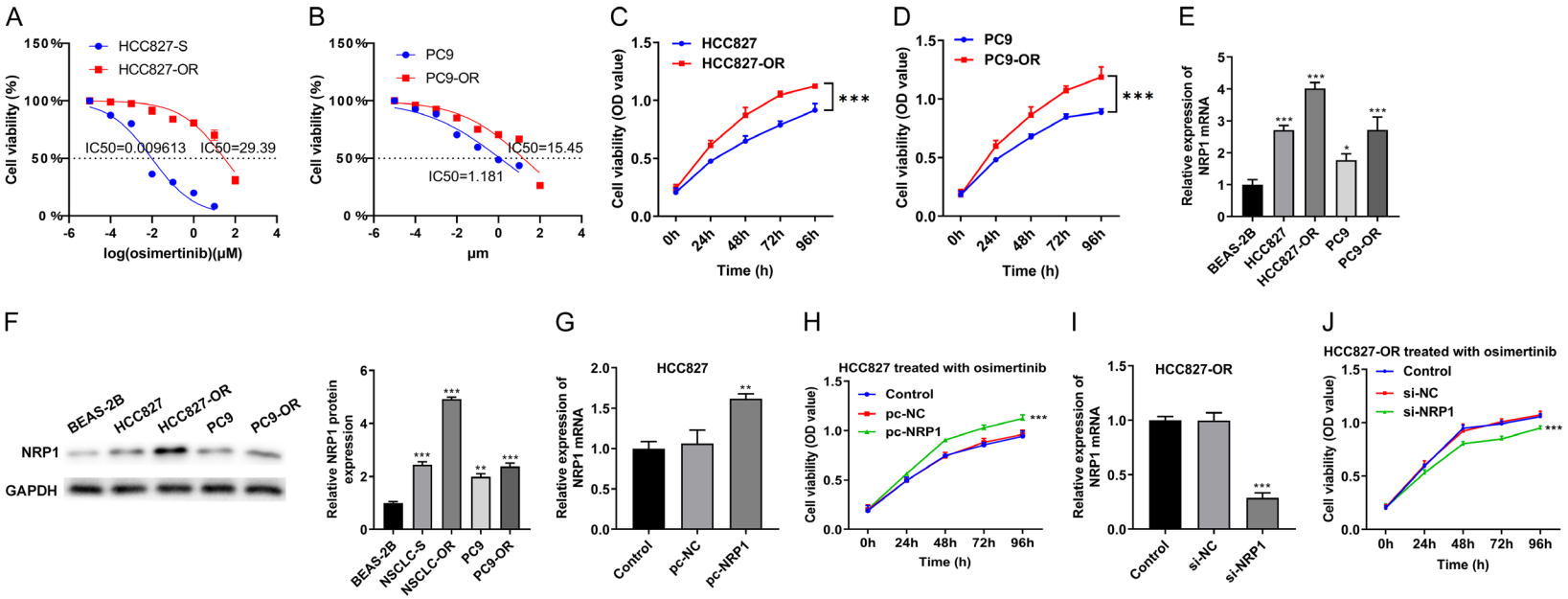


**Figure 2.** NRP1 is overexpressed in OR-resistant NSCLC cells. A-D. $IC_{50}$ values determined using CCK-8 assay. E. NRP1 mRNA expression in NSCLC cells was determined using RT-qPCR. F. NRP1 protein expression in NSCLC cells was determined using a western blot. G. NRP1 mRNA expression in NSCLC cells was determined using RT-qPCR. H. Cell viability was determined using CCK-8 assay. I. NRP1 mRNA expression in NSCLC cells was determined using RT-qPCR. J. Cell viability was determined using CCK-8 assay. *$P$<0.05, **$P$<0.01, ***$P$<0.001.

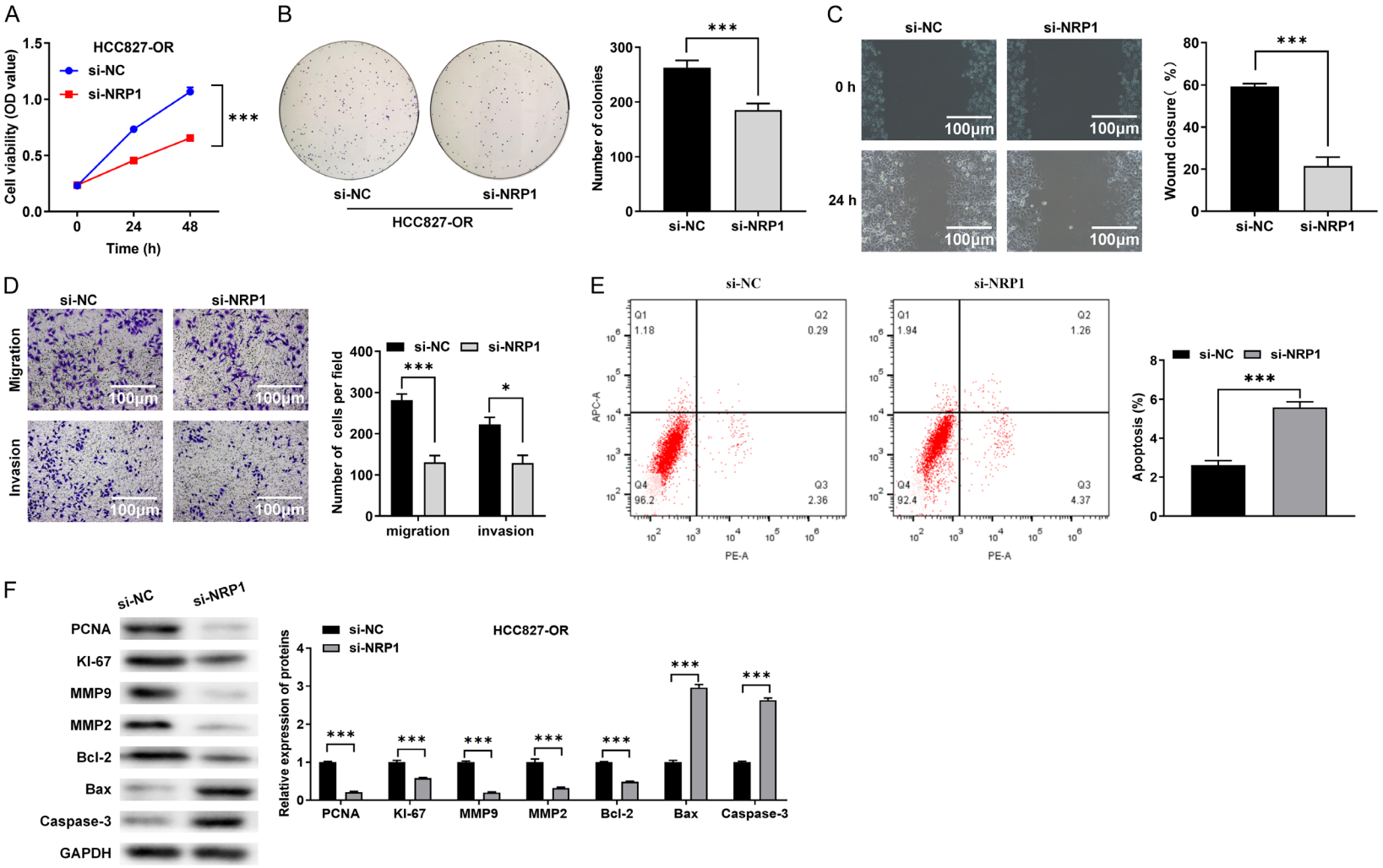


**Figure 3.** NRP1 inhibitor-mediated NRP1 deficiency alleviated chemoresistance of HCC827 cells. A. Cell viability was determined using CCK-8 assay. B. Cell proliferation was determined using a colony formation assay. C. Cell migrative ability determined using wound healing assay (200X). D. Cell migrative and invasive ability determined using transwell assay (200X). E. Cell apoptosis was determined using flow cytometry. F. Protein expression in NSCLC cells was determined using a western blot. *$P$<0.05, ***$P$<0.001.

the number of HCC827-OR cell colonies following NRP1 knockdown (**Figure 3B**). The migratory and invasive abilities of HCC827-OR cells were significantly suppressed by NRP1 knockdown (**Figure 3C**, **3D**). Additionally, NRP1 knockdown led to a significant increase in the apoptosis rate of HCC827-OR cells (**Figure 3E**). To validate this observation, the protein expression of markers of proliferation (PCNA and Ki-67), migration (MMP2 and MMP9), and apoptosis (Bax, Bcl-2, Caspase-3) was determined. NRP1 knockdown resulted in decreased protein expression of PCNA, Ki-67, MMP2, MMP9, and Bcl-2 (**Figure 3F**) while it increased the expression levels of Bax and Caspase-3.

*NRP1 knockdown enhances HCC827-OR cell chemosensitivity to osimertinib*

Combined administration of osimertinib and NRP1 knockdown resulted in a more significant decrease in integrin β3 expression compared to osimertinib alone (**Figure 4A**, **4B**). Osimertinib significantly reduced the viability of HCC827-OR cells, and this effect was even more potent in the NRP1 inhibitor+osimertinib group (**Figure 4C**). The combined treatment of NRP1 inhibitor and osimertinib markedly decreased the number of observed colonies formed by HCC827-OR cells compared to treatment with only osimertinib (**Figure 4D**). Furthermore, the combination of NRP1 inhibitor and osimertinib more efficiently inhibited migration and invasion of HCC827-OR cells than osimertinib alone (**Figure 4E**, **4F**). Additionally, the combined administration of NRP1 inhibitor and osimertinib remarkably enhanced apoptosis in HCC827-OR cells compared to treatment with only osimertinib (**Figure 4G**). Compared to treatment with only osimertinib, NRP1 inhibitor+osimertinib significantly decreased protein expression levels of PCNA, Ki-67, MMP2, MMP9, and Bcl-2 while upregulating Bax and Caspase-3 (**Figure 4H**).

*NRP1 interacts with integrin β3 in HCC827-OR cells*

The NRP1 inhibitor significantly suppressed integrin β3 mRNA expression in HCC827-OR cells treated with osimertinib (**Figure 5A**). Furthermore, NRP1 inhibition markedly decreased integrin β3 protein expression (**Figure 5B**). A co-IP assay using untreated HCC827-OR cells further confirmed the interaction between NRP1 and integrin β3 (**Figure 5C**). The NRP1 inhibitor significantly decreased NRP1 and integrin β3 positive fluorescent signal (**Figure 5D**).

*ITGB3 regulates PI3K/AKT signaling in an NRP1-dependent manner*

KEGG analysis revealed that NRP1 activates the PI3K/AKT signaling pathway (**Figure 6A**). STRING database analysis revealed that both NRP1 and ITGB3 interact with proteins involved in PI3K/AKT signaling (**Figure 6B**). Osimertinib slightly downregulated p-PI3K/AKT (**Figure 6C**), but it was markedly downregulated by the combination of the NRP1 inhibitor and osimertinib treatment (**Figure 6C**). Moreover, treatment with 740Y-P attenuated the effects of the NRP1 inhibitor and osimertinib.

*740Y-P treatment promotes NSCLC cell aggressiveness*

To confirm the role of NRP1/PI3K/AKT signaling in NSCLC, we investigated the cellular functions of osimertinib+NRP1 inhibitor and osimertinib+NRP1 inhibitor+740Y-P. We observed that 740Y-P treatment significantly increased the proliferative, migratory, and invasive abilities of lung cancer cells (**Figure 7A-D**) while inhibiting tumor cell apoptosis (**Figure 7E**). Furthermore, 740Y-P treatment upregulated the protein level expression of PCNA, Ki-67, MMP2, MMP9, Bcl-2, Bax, and Caspase-3 (**Figure 7F**).

*NRP1 knockdown enhances lung cancer cell osimertinib sensitivity by modulating PI3K/AKT signaling*

As shown in **Figure 8A-C**, administration of osimeretinib had no significant effect on tumor size, volume, and weight, indicating OR. However, si-NRP1+osimertinib reduced tumor size, volume, and weight (**Figure 8A-C**). Downregulation of p-PI3K/AKT, PCNA, Ki-67, MMP2, MMP9, and Bcl-2 along with the upregulation of Bax and Caspase-3 (**Figure 8E**, **8F**) were significantly alleviated by 740Y-P. These results indicate that NRP1 inhibition increases tumor cell sensitivity to osimertinib, promoting tumor cell apoptosis and inhibiting tumor progression, and that these effects are mediated by silencing PI3K/AKT signaling.

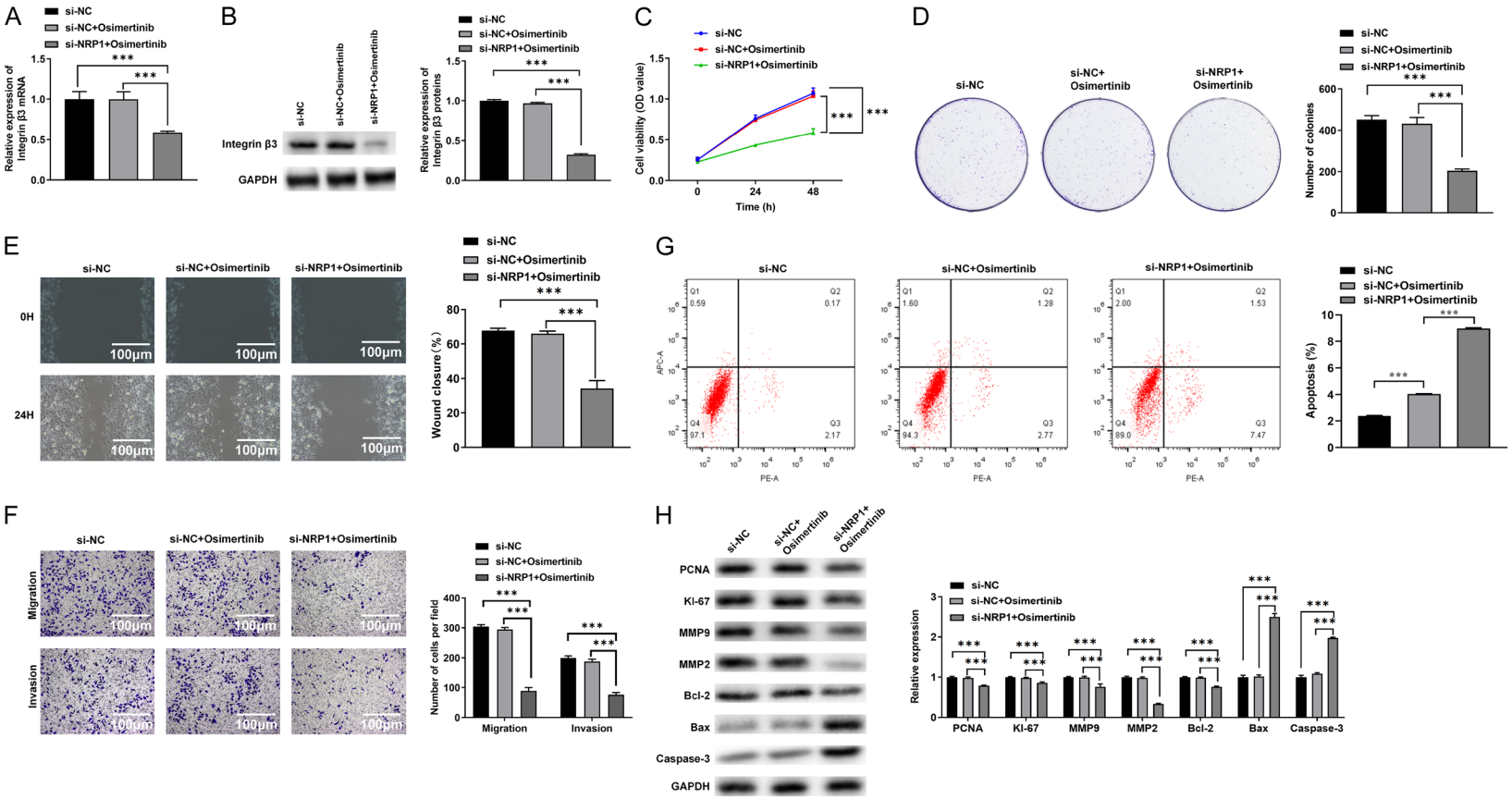


**Figure 4.** NRP1 inhibitor enhances the chemosensitivity of HCC827-OR cells to Osimertinib. A. Integrin β3 mRNA expression in NSCLC cells determined using RT-qPCR. B. Integrin β3 protein expression in NSCLC cells was determined using western blot. C. Cell viability was determined using CCK-8 assay. D. Cell proliferation was determined using a colony formation assay. E. Cell migrative ability determined using wound healing assay (200X). F. Cell invasive ability determined using transwell assay (200X). G. Cell apoptosis was determined using flow cytometry. H. Protein expression in NSCLC cells was determined using a western blot. **P*<0.05, ****P*<0.001.

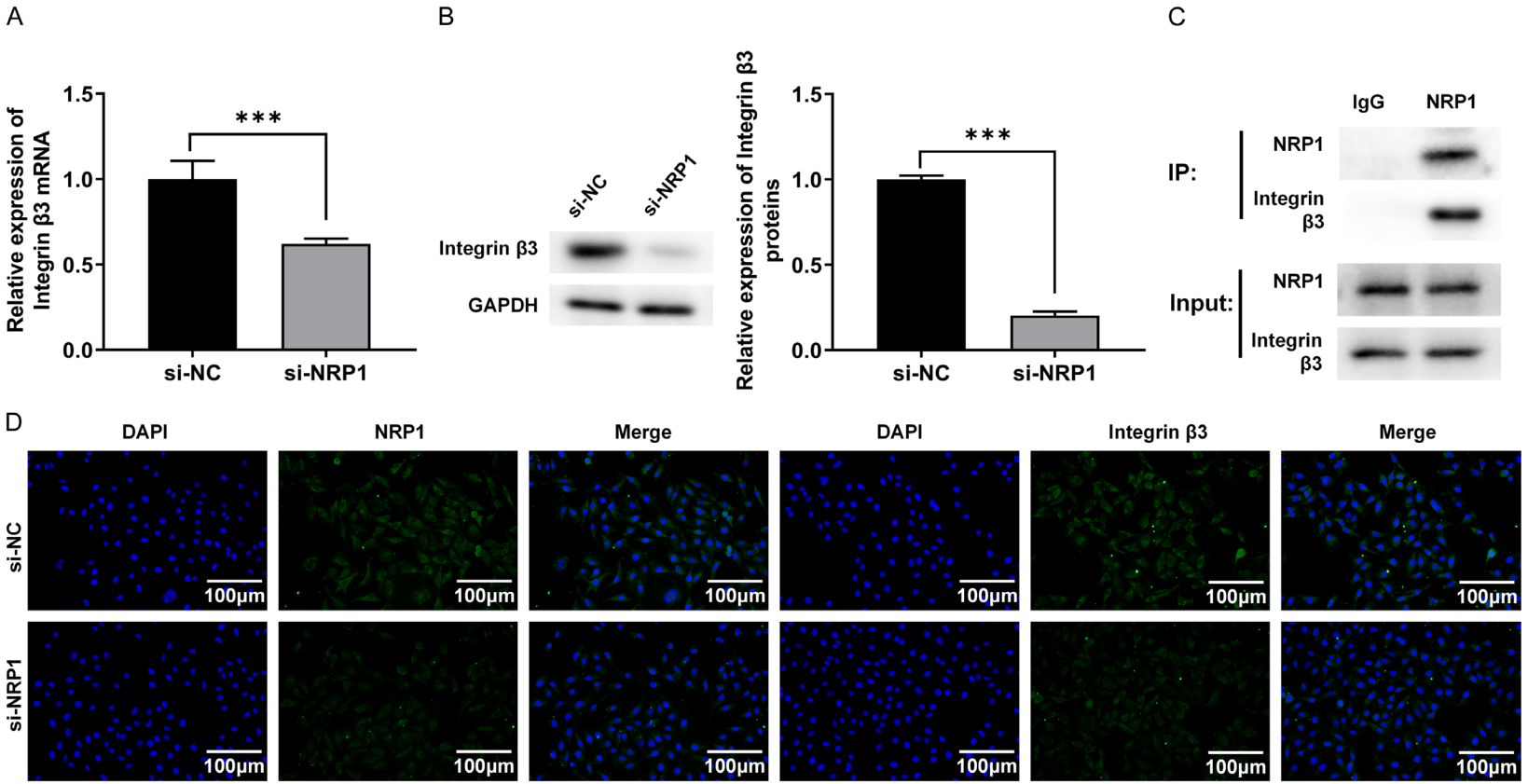


**Figure 5.** NRP1 interacts with Integrin β3 in HCC827-OR cells. A. Integrin β3 mRNA expression in NSCLC cells determined using RT-qPCR. B. Integrin β3 protein expression in NSCLC cells was determined using western blot. C. The interaction between NRP1 and Integrin β3 was detected using Co-IP assay. D. NRP1 and Integrin β3 expression were determined using an immunofluorescence assay (200X). ***$P$<0.001.

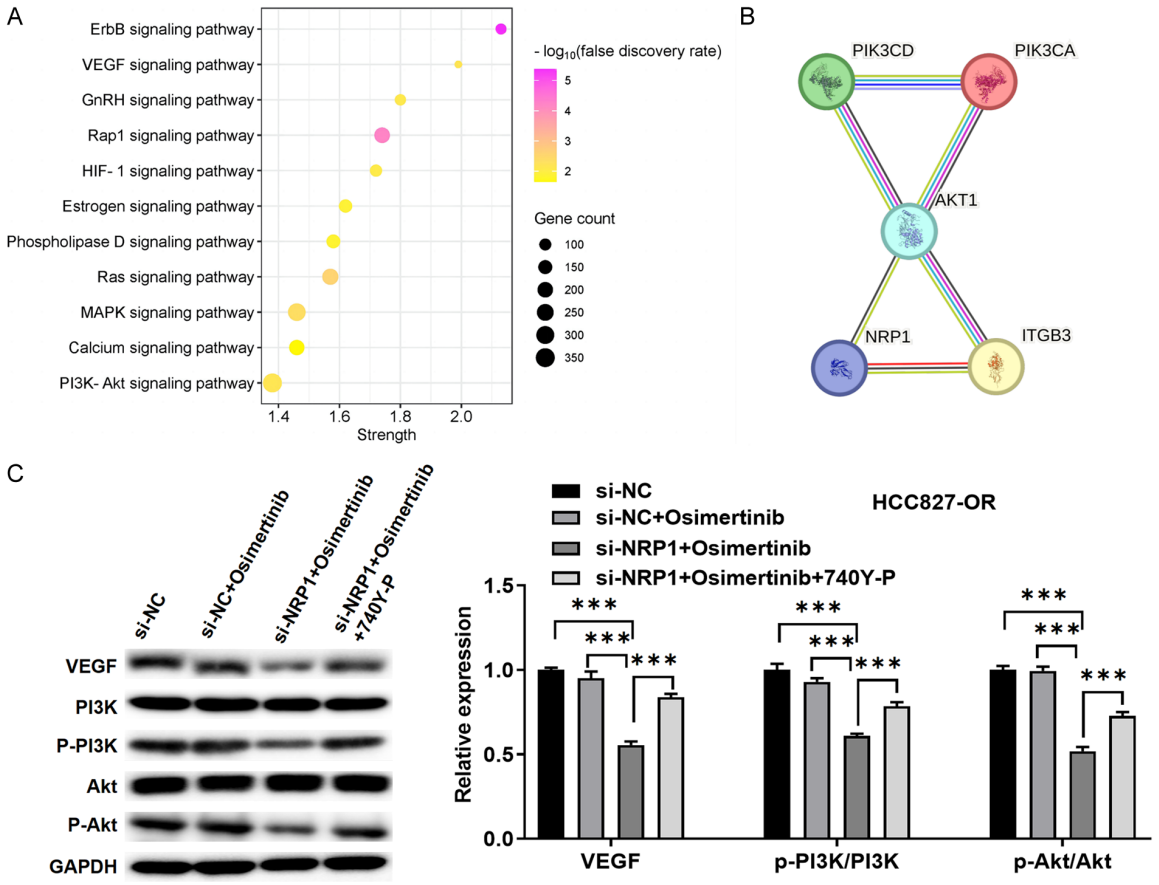


**Figure 6.** Integrin β3 regulates PI3K/AKT signaling in an NRP1-dependent manner. A. The interaction between NRP1 and PI3K/AKT signaling was analyzed using KEGG. B. The interaction between NRP1 and PI3K/AKT signaling was analyzed using STRING. C. The protein expression of p-PI3K/AKT was determined using a western blot. $^{***}P<0.001$, $^{\#\#\#}P<0.001$, $^{\$\$\$}P<0.001$.

Additionally, si-NRP1+osimertinib suppressed both NRP1 and ITGB3 expression (**Figure 8D**). This suppression was abrogated by 740Y-P, indicating that downregulating NRP1 expression may downregulate ITGB3 expression by silencing PI3K/AKT signaling.

## Discussion

In this study, NRP1 was overexpressed in patients with OR NSCLC and cell lines, and downregulation of NRP1 silenced the PI3K/AKT signaling pathway and enhanced sensitivity to osimertinib *in vivo* and *in vitro*. Furthermore, activation of the PI3K/AKT signaling pathway by 740Y-P counteracted the effects of NRP1 downregulation on NSCLC and ITGB3. These results suggest that abnormal expression of NRP1 in NSCLC-OR cells may reduce their sensitivity to osimertinib by activating the PI3K/AKT signaling pathway, upregulating ITGB3, and promoting progression of NSCLC.

Acquired EGFR-TKI resistance can be induced by the T790M mutation, tumor cell polarization, or proliferation [35]. A recent study has shown that fourth-generation EGFR-TKIs have been developed to target the EGFR T790M mutation induced by first- and second-generation inhibitors, as well as the C797S mutation induced by third-generation inhibitors (e.g., osimertinib) [36]. However, secondary drug resistance in tumors is induced by multifactorial networks [37], suggesting that we need to further refine the patient information when obtaining clinical samples for detection in order to distinguish between different resistance factors and facilitate the subsequent use of appropriate drugs. Notably, correlation analysis showed that female patients receiving first-line

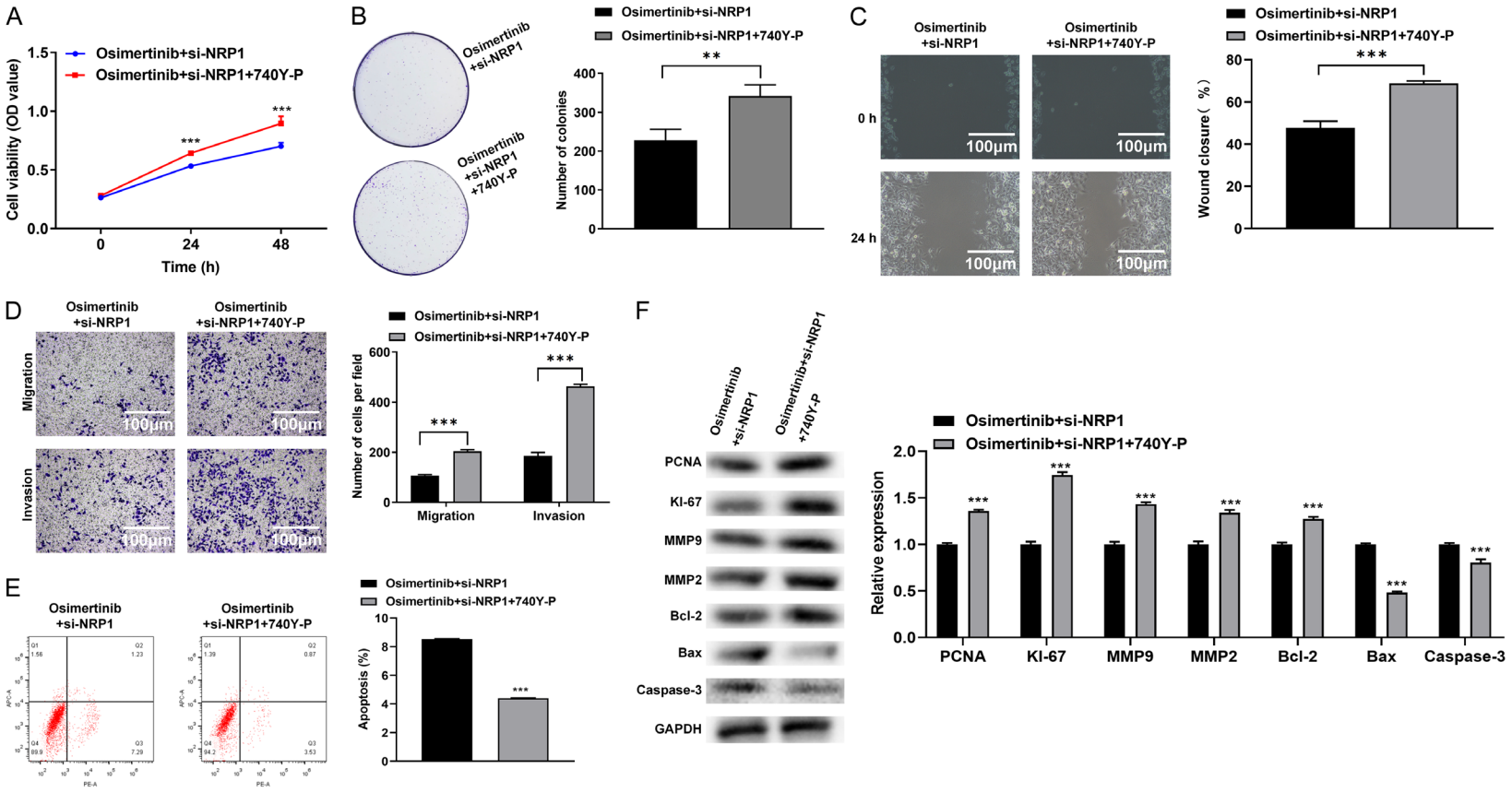


**Figure 7.** The 740Y-P treatment promotes the aggressiveness of NSCLC cells. A. Cell viability determined using CCK-8 assay. B. Cell proliferation was determined using a colony formation assay. C. Cell migrative ability determined using wound healing assay (200X). D. Cell invasive ability determined using transwell assay (200X). E. Cell apoptosis was determined using flow cytometry. F. Protein expression in NSCLC cells was determined using a western blot. $^{**}P<0.01$, $^{***}P<0.001$.

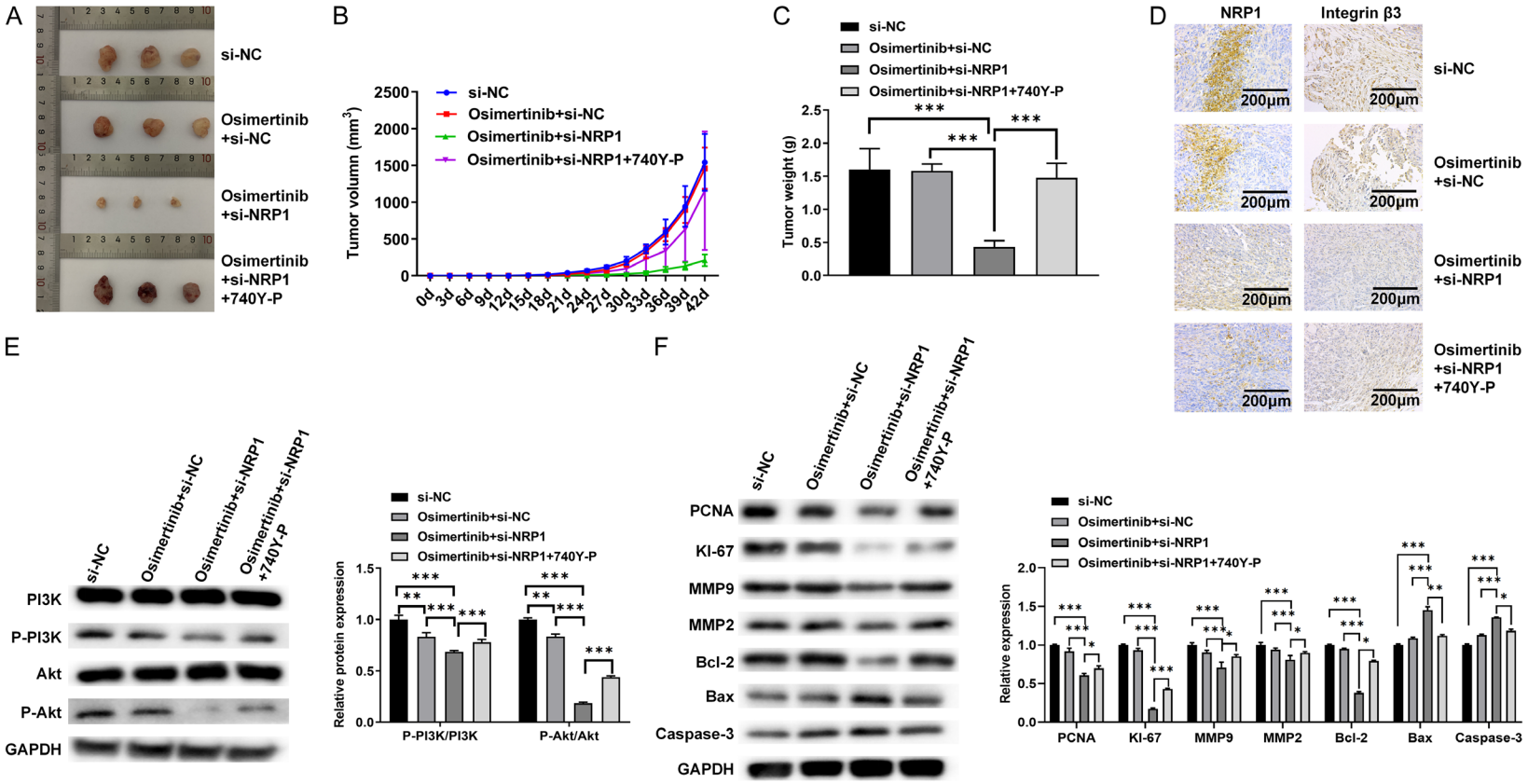


**Figure 8.** NRP1 inhibitor enhances the Osimertinib sensitivity of lung cancer cells via regulating PI3K/AKT signaling. The tumor size (A), volume (B), and weight (C) were determined using Xenograft assay. (D) NRP1 and Integrin β3 expression were determined using IHC (200X). (E) The protein expression of p-PI3K/AKT was determined using a western blot. (F) The protein expression was determined using a western blot. $^{**}P<0.01$, $^{***}P<0.001$, $^{\#}P<0.05$, $^{\#\#\#}P<0.001$, $^{\$}P<0.05$, $^{\$\$\$}P<0.001$.

EGFR-TKIs correlates with a superior response to osimertinib [38-40], which is consistent with our sample statistics. Additionally, a retrospective analysis by Kato et al. reported that lower activity of osimertinib in younger patients compared to older patients [41]; Herein, most participants were over 65 years old (23/31), and the conclusion was controversial [42], suggesting the need to investigate the effect of sex and age on OR.

Chenxi et al. [43] reported that in Calu-1 cells, the apoptosis rate of the control group was 1%, while the si-NRP1 group only showed a slight increase to 2.2%, which was 120% higher than that of the control group (1%), but proliferation was significantly inhibited. Similar to Chenxi et al.'s findings, this study found that si-NRP1 (5.63%) increased the apoptosis rate of HCC827-OR cells by 112% compared with the control group (2.65%), and proliferation was also significantly inhibited. Furthermore, Chenxi et al.'s results demonstrated that apatinib+ NRP1 siRNA treatment led to a 71% increase in apoptosis rate compared with apatinib alone (9.6% vs 5.6%) in Calu-1 cells, while in this study, si-NRP1+osimertinib treatment resulted in a higher increase of 122% in apoptosis rate compared with si-NC+osimertinib treatment (9% vs 4.05%) in HCC827 cells. Combining the results of Chenxi et al. and this study, although the change range of apoptosis was similar to that of cell viability and proliferation, the numerical presentation still showed the characteristics of low apoptosis, suggesting that osimertinib, as an inhibitor, may exhibit greater efficacy in inhibiting tumor development rather than exerting a pronounced cytotoxic effect on cancer cells.

As NRPs are multifunctional, knockdown of NRP1 affects many biological effects, impacting cell cycle, cell proliferation, and migration [13]. The present study demonstrated that NRP1 knockdown disrupted NSCLC cell proliferation, migration, and apoptosis, as well as increased the the sensitivity of drug-resistant cells to osimertinib. Additionally, it was found that approximately 10-12% of patients who progressed to EGFR-TKIs had mutations in cell-cycle regulators [44], which has been confirmed to correlate with poor outcomes in patients with NSCLC treated with osimertinib [45]. Francesco et al. discovered that overexpression of cyclin D1 induces leads to high resistance against acute osimertinib treatment [46], suggesting the crucial role of cyclins in NRP1 OR-regulation and warranting further investigation.

Studies have shown that NRP1 interacts with oncogenic signaling pathways, including TGF-β/Smad, SEMA3A, and TNPO1/FUBP1 signaling, resulting in tumor cell proliferation, polarization, and immune evasion [47, 48]. This study demonstrated that NRP1 functions as an oncogene in NSCLC and may act through the PI3K/AKT signaling pathway related to EGFR [49]. Active PI3K/AKT signaling enhances tumor cell proliferation, immunosuppression, and chemoresistance in lung cancer [50-52]. PI3K/AKT is a marker of tumor cell proliferation, and its activation enhances the morbidity of NSCLC cells [53]. In this study, we interfered with the effect of downregulating NRP1 on NSCLC using 740Y-P. These studies showed that 740Y-P, as a PI3K agonist by increasing the phosphorylation of PI3K and AKT. It regulates PI3K/AKT signaling and promotes glycolysis [23], proliferation [24], migration [25], invasion [26], oxidative stress, and ferroptosis [27] in NSCLC cells, suggesting that there are other aspects of the regulatory effects of NRP1 on NSCLC via the PI3K/AKT signaling pathway that remain to be explored. We also found an interaction between NRP1 and integrin β3. The activation of PI3K/AKT signaling interferes with the expression of both NRP1 and integrin β3, while Kim et al.'s research indicated that the ternary compound NRP1-integrin β3-KRASMUT along with its downstream signal PI3K-Akt may be the main resistance mechanism for KRASMUT NSCLC against cetuximab treatment [34], indicating the necessity of further exploration of the integrin β3 mechanism in NSCLC-OR. In addition, this study only used HCC827-OR cells to verify the interaction between NRP1 and Integrin β3 without controlling for normal HCC827 cells, thus it can only demonstrate an interaction between NRPI and Integrin β2 specifically within HCC827-OR cells. The specificity of the interaction between NRP1 and Integrin β3 in OR cells still needs to be further verified.

Dong et al. [15] conducted in vitro studies through the A549 cell line and in vivo studies on the subcutaneous (s.c) tumor model of nude

mice, and found the enhancing effect of NRP1 inhibitors on the radiosensitivity of NSCLC. The results of this study showed that NRP1 inhibitors increased the sensitivity of NSCLC to Osimertinib *in vitro* and *in vivo*. In addition, it is interesting to note that both NSCLC cell lines in this study, HCC827 and PC9, showed the same upregulation of NRP1 after Osimertinib treatment. However, in the study by Dong et al., A549 and H460, which are also NSCLC cell lines, exhibited opposite changes in NRP1 expression after 10 Gy X-ray irradiation. Specifically, A549 cells with initially high expression of NRP1 demonstrated upregulated NRP1 expression, while H460 cells with initially low expression of NRP1 displayed decreased NRP1 expression. Furthermore, A549 cells with upregulated NRP1 expression exhibited a higher clone formation rate and a lower apoptosis rate, indicating radioresistance. The differences observed among these cell lines suggest that although NRP1 plays a similar role in different NSCLCs, there may be specific regulatory mechanisms involved.

In conclusion, NRP1 is found to be upregulated in NSCLC, and targeting NRP1 in NSCLC-OR cells can modulate their sensitivity to osimertinib through the PI3K/AKT signaling pathway. Additionally, ITGB3 may play a role in this regulatory process. Further investigation into the NRP1-ITGB3-PI3K/AKT mechanism of osimertinib resistance is warranted for identifying key determinants of EGFR TKI resistance.

## Disclosure of conflict of interest

None.

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