

Overexpression of Osimertinib-resistant ABCG2 in Non-small cell lung cancer cells

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Research Article

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Abstract

Objective: Osimertinib is one of the commonly used chemotherapeutic drugs for treatment of non-small cell lung cancer (NSCLC). Nonetheless, the eventual resistance developed by cancer cells to Osimertinib has led to limitations in its application. Accordingly, there is a significant need for identifying means of reversing Osimertinib resistance.

Method: The CCK-8 method was employed in detecting cell proliferation and toxicity. Western blot analysis detected protein expression, cell invasion analysis was performed using transwell assays, and the concentration of Osimertinib was determined using high performance liquid chromatography (HPLC).

Result: In this study, we constructed Osimertinib-resistant cells, thus indicating the vital role of ABCG2 expression in formation of drug-resistant cells. Down-regulating ABCG2 expression in drug-resistant cells can reduce the lung cancer cells' IC50, degree of proliferation and invasion. Combining use of ERK and PI3K inhibitors to inhibit ABCG2 expression is superior to employing a single inhibitor.

Conclusion: Inhibiting the expression of ABCG2 can reverse the resistance of NSCLC cells to Osimertinib. Overexpression of ABCG2 is caused by the coactivation of the MAPK and the PI3K/AKT pathways.

1. Introduction

Lung cancers are tumors that originate in the lungs. Despite great improvements in treatments, the lung cancer mortality rate continues to be significantly high ^[1]. Accordingly, lung cancer therapeutics continues to pose great challenges as lung cancer is still identified by a high morbidity rate and short periods of progression-free survival ^[2, 3]. America's latest "global lung cancer statistics for 2018", signify the highest levels of lung cancer morbidity and mortality on the global scale ^[4, 5]. Moreover, non-small cell lung cancer (NSCLC) constitutes the most common type of lung cancer by accounting for more than 85% of lung cancers based on the survey of Non-small-cell lung cancers: a heterogeneous set of diseases ^[4, 5].

Due to insufficiency of occult pathogenesis and early diagnostic methods, most patients are diagnosed at advanced stages. For instance, approximately 40–50% of patients, at the time of initial diagnosis, were diagnosed with distal metastasis, thereby forgoing the best chance of undergoing a radical operation ^[6–8]. Currently, the primary treatment for NSCLC is radio-chemotherapy. However, recent improvements in molecular detection technology has led to targeted therapy being widely used for the treatment of NSCLC owing to its numerous benefits. These include convenience of use, enhanced effects, and minimal side effects. Research on NSCLC targeted therapy has gradually become mainstream, especially concerning the use of tyrosine kinase inhibitors (TKI).

The epidermal growth factor receptor (EGFR) is a giant glycoprotein with tyrosine kinase activity ^[9]. Evidence has shown that the development of drug resistance in multiple tumors is related to the EGFR ^[10]. Following the EGF receptor's binding of a ligand, it undergoes a conformational change through

homodimerization or heterodimerization. This change induces the ubiquitination of intracellular tyrosine kinase residues, thereby activating downstream pathways and triggering signaling cascades [11]. More recently, EGFR inhibitors have become therapeutic targets for many tumors. Examples of these include the first-generation targeted therapy drugs gefitinib and erlotinib which see extensive application in the clinic [12]. A phase III clinical trial study showed that gefitinib can effectively improve the patient's objective remission rate, progression-free survival, and quality of life when compared to dual-drug platinum-containing regimens [13]. This is particularly true for patients carrying EGFR mutations; namely, the L858R point mutation or the exon 19 deletion [13]. However, most patients appear to develop resistance after one-year of therapy, and more than half of these patients were found to harbor the T790M mutation [14]. Consequently, the treatment of patients using Osimertinib is primarily used for patients diagnosed with gefitinib acquired resistance and who carry the T790M mutation [15].

Osimertinib is an orally irreversible pyrimidinyl EGFR-TKI. It can selectively inhibit the effect of EGFR sensitive activating mutations such as those occurring in exons 19 and 21 [16, 17]. Moreover, studies have shown that Osimertinib is not only effective against these EGFR mutations, but also has a beneficial impact in the treatment of cancers with the EGFR T790M mutation that manifests as a result of treatment with first and second-generation EGFR-TKIs [18]. Unfortunately, following several months of treatment, the patients were observed to develop Osimertinib resistance [19]. The primary mechanisms were associated with Osimertinib acquired resistance: an *EGFR*-dependent mechanism and an *EGFR*-independent mechanism. The first and most important EGFR-dependent mechanism results from the manifestation of the *EGFR* C797S mutation [20]. The C797S and the T790M mutations occur in both forms; as cis and the trans mutations. Independent mechanisms include MET amplification, activation of HER-2, FGFR and MAPK, KRAS mutations, PI3KCA, BRAF, PTEN deletions, and SCLC transformation [19, 21]. After a period of treatment, drug resistance will eventually develop, and the specific drug mechanism that occurs is currently unclear [22].

The ATP binding cassette transporter G2 (ABCG2), is a semi-transporter protein encoded by the multidrug resistance protein gene *MDR1* [23]. ABCG2 utilizes the energy produced by ATP hydrolysis to transfer intracellular drugs and harmful substances out of cells to participate in the development of drug resistance to multiple tumors [24]. Studies have shown that ABCG2 activation is mainly through the activation of the E1b/cBcRP promoter which contains a phospho-cyclic-AMP (cAMP) and reaction elements (CER) [25]. The binding of the CER elements to the cAMP response element's binding proteins (P-CREB) leads to *ABCG2* transcription and protein expression. This, in turn, mediates the outflow of intracellular toxic substances and leads to acquired cell resistance [26].

Combined use of ERK and tyrosine kinase inhibitors in treating the progression of NSCLC cells has shown a synergistic treatment effect [27]. However, the unbearable drug toxicity greatly limits its clinical application [27]. Conversely, research into the use of efflux protein inhibitors with tyrosine kinase inhibitors as a combination therapy in the clinical setting has been poorly researched. Our previous research

suggests that NSCLC cells exhibit elevated ABCG2 expression which is stimulated by the third-generation tyrosine kinase inhibitor, Osimertinib. Therefore, we hypothesized that elevated ABCG2 expression contributed to acquired resistance to Osimertinib in NSCLC. In this study, we explore the therapeutic potential of using ABCG2 inhibitors together with Osimertinib through the regulation ABCG2 expression.

2. Materials And Methods

Cell culture

H1975, H1299, H1437 and A549 cells were purchased from the Chinese Academy of Sciences (China). Cells were cultured in RPMI-1640 (Gibco, USA) medium containing 10% FBS (Gibco, USA) at 37°C, 5% CO₂. Cells were maintained at an initial concentration of 0.5µmol/ML Osimertinib (MCE, USA) which was gradually increased to a concentration above 20µmol/ML. Cells were maintained at the highest concentration.

Cell proliferation test

Cells were collected when they reached the logarithmic growth phase and were then diluted to 2x10³ cells per ML. Next, 100µL of cells were placed in each well of a 96-well plate with each cell type designated three repeat wells. The cells were then placed in a 37°C, 5% CO₂ incubator for 24h. Medium was removed at 48h/72h/96h/120h and 180µL of a solution containing RPMI-1640 and 20µL CCK-8 (Biosharp, China) was added to each well before incubating the plates in a 37°C, 5% CO₂ incubator for 3h. The absorbance wavelength was detected at 450nm using a Multiskan GO (ThermoFisher) and a cell growth curve drawn.

Cytotoxicity test

500 cells per well were seeded onto a 96-well plate and cultured in RPMI-1640 medium supplemented with each concentration of Osimertinib. The plate was incubated in 37°C, 5% CO₂ and the medium changed after 72h to a mix of 180µL RPMI-1640 and 20µL CCK-8 solution which was added to each well. Plates were then incubated in a 37°C, 5% CO₂ incubator for 3h. Absorbance was then detected at 450nm using the Multiskan GO. This data was used to calculate the IC₅₀ value.

High performance liquid chromatography(HPLC):

Cells were placed in Osimertinib for 24h prior to collection. Collected cells were rinsed three times and use PBS supplement to 1ml. The total number of cells was quantified and the cells collected in a cryotube prior to undergoing five freeze/ thaw cycles in liquid nitrogen for 6 minutes per cycle. Next, the liquid chromatography mobile phase (ammonium acetate: ACN, 55:45) was added, the mixture shaken and mixed for 2 minutes. The mixture was then centrifuged and 500µl of supernatant removed from each tube. With a detection wavelength of 251nm for Osimertinib, the wavelength of each mixture was detected and the concentration of Osimertinib calculated.

Western-blot assay:

Cells were stimulated using Osimertinib prior to collection of the total protein from each cell type. The BCA method was used to detect the protein concentration. Next, 20µg of protein was added to 8% SDS-PAGE to separate the proteins and to analyze them using antibodies (Cell Signaling Technology, USA). The final detection was performed using ECL reagents (YaMei, China).

T790M mutation model construction

1×10⁵ cells/well were seeded onto a 24-well plate. After 24h, the cells had proliferated to a concentration of 2×10⁵ cells/well. The medium was replaced with 2ml fresh RPMI-1640 medium containing 6µg/ml polybrene and supplemented with an appropriate amount of virus suspension. The mixture was incubated at 37°C for 4 hours, then 2ml of fresh medium was added to dilute the polybrene. Cultivation continued for 24 hours, while replacing the virus-containing medium with fresh medium 72–96 hours post-transfection. 5µg/ml puromycin was added for stable selection for one week, and a stable transfected cell line was obtained.

shRNA transfection:

The interfering RNA siABCG2 and the sh-control were chemically synthesized by Invitrogen. Cells were then collected and a cell suspension made and counted. Each well of a 6-well plate was subsequently inoculated with 2ml of 2×10⁵ cells and cultivated to 50%-60% confluency. Next, the cell suspension was washed with serum-free medium. 4.0µg of shABCG2 and sh-control was then diluted in 250µl of RPMI-1640 serum-free medium and 10µl of Lipofectamine 2000 was diluted in 250µl medium, then left to stand for 5 minutes. The diluted Lipofectamine 2000 was mixed with DNA for 25 minutes, then the medium was replaced in the 6-well plate with 2ml serum-free medium. 500 µl of the above mixture was added to the 6-well plate and the cells were cultivated in a 37°C, 5% CO₂ incubator for 6 hours. Next, the medium was changed to a 10% serum-containing medium and culturing continued for 48 hours.

Transwell assay:

The Matrigel (BD Biosciences USA) was added to the transwell chamber (Corning, USA) and incubated at 37°C, 5% CO₂ overnight. 1×10⁴ cells were added to the upper chamber of the transwell chamber, and 500µL RPMI-1640 medium with 10% FBS were added to the lower chamber. These were then incubated at 37°C, 5% CO₂ for 24 hours. The cells in the upper chamber of the transwell were then wiped with a cotton swab and the chamber was immersed in methanol for 30 minutes followed by 1% crystal violet for 20 minutes. The chamber was then washed with PBS and the cells counted under the microscope.

Apoptosis detection:

6µM of the ABCG2 inhibitor Fumitremorgin C was added to cultured cells for 24h. Subsequently, 10µM of Osimertinib was added for 2h. The cells were collected, washed three times with PBS, then fixed with fixative for 10min. The fixative was removed and the cells washed three times with PBS for 3 minutes

each time. The cells were immersed in Hoechst 33258 staining solution for 5 minutes, removed from the staining solution, before adding a single drop of anti-fluorescence quencher and observing them under a fluorescence microscope. Five fields of cells in each group were counted under the microscope. Counts were made of the number of apoptotic cells and the total cells, respectively. The average value was taken and the apoptosis rate calculated.

Statistical analysis:

The aforementioned experiments were repeated three times. The results of the experiments were presented as the mean \pm SEM (standard error of the mean). Student's paired t-test and one-way ANOVA were used to compare the experimental findings. Statistical significance was considered at p-values of < 0.05 , < 0.01 , and < 0.001 which were indicated in the figures using a single asterisk, two asterisks, and three asterisks, respectively. All data were analyzed using SPSS 21.0.

3. Results

3.1 The increased expression of ABCG2 in Osimertinib-resistant cells

We tested the toxic effect of Osimertinib on NSCLC cells, and the results indicated that in each wildtype group the half-inhibitory concentration (IC₅₀) of Osimertinib was approximately 1–3 μ mol. Moreover, in the T790M mutant H1975 cells, the IC₅₀ was approximately 1 nmol, and the cells were stimulated by increasing the concentration of Osimertinib (0.5 μ mol – 20 μ mol) to make the cells resistant. This led to a significant increase in the IC₅₀ value of Osimertinib and of the cells becoming resistant (Fig. 1). When compared with the parental cells, the proliferation rate of the drug-resistant cells had accelerated (Fig. 2A). Cell invasion is significantly increased in (Figure 2B). Subsequently, the protein expression levels in the cells were detected using Western-blot analysis. It is worth noting that the expression of ABCG2 in the drug-resistant cells was significantly increased (Fig. 1C). Therefore, we have reason to believe that the expression of ABCG2 is related to acquired Osimertinib-resistance in NSCLC cells.

3.2 The increase in ABCG2 expression in Osimertinib-resistant cells led to a decrease in the intracellular Osimertinib concentration

The intracellular Osimertinib concentration was detected using HPLC to verify the increase in the ABCG2 protein pumping Osimertinib out of the cell, thereby leading to drug resistance. First, Osimertinib was configured to a concentration of 0.05 μ M, 0.1 μ M, and 0.2 μ M (Fig. 3). Next, the peak area was detected by HPLC and the concentration was taken as the abscissa and the absorption peak area was used as the ordinate to plot a standard curve. The curve of $y = 21458266.86x + 69104.4$ was obtained. After stimulation with Osimertinib, the cells were collected and split before using HPLC to detect the

concentration of Osimertinib. The area calculated was entered into the equation to calculate the concentration of Osimertinib. This concentration was then used to calculate the concentration of Osimertinib in a single cell. The results showed that the concentration of Osimertinib in drug-resistant cells was lower than that in the parental cells (Fig. 4). Therefore, we believe that the increase in ABCG2 expression led to the increased excretion of Osimertinib out of the cell and that this contributed to the development of drug resistance.

3.3 Establish a wild-type cell T790M mutation model

To explore the fail mechanism of Osimertinib treated first-generation tyrosine kinase-resistant cells, we transfected cells with a lentiviral to transform them into T790M mutant cells. Thus, these cells mimicked the first-generation tyrosine kinase resistant cells. Stable expression strains were selected using puromycin. Following RNA extraction from the cells, gene sequencing was performed to confirm that the cells contained the mutation site (Fig. 5A). The drug toxicity test results showed that the half-inhibitory concentration of cells was approximately 40-110nmol (Fig. 5B). As previously mentioned, by increasing the dose the cells were induced to develop resistance to Osimertinib. The IC₅₀ of the drug-resistant cells was significantly increased (Fig. 5B), the cell proliferation speed was accelerated (Fig. 5C), the degree of invasion was enhanced (Fig. 6B), and the expression of ABCG2 in drug-resistant cells was significantly increased in keeping with the findings on the above drug-resistant cells (Fig. 6C). Therefore, we suspect that the overexpression of ABCG2 is also involved in the acquired resistance to Osimertinib in the first generation of tyrosine kinase-resistant NSCLC cells.

3.4 Inhibition of ABCG2 expression can partially reverse resistance to Osimertinib

To confirm that the cancer cells' resistance to Osimertinib was the result of increased expression of ABCG2, we used ABCG2 shRNA to down-regulate the expression of ABCG2 in the drug-resistant cells (Fig. 7). The IC₅₀ of the cells was significantly reduced and the sensitivity nearly recovered to equal that of the parental cells (Fig. 6). Fumitremorgin C is a specific ABCG2 inhibitor which was set up at multiple concentration gradients to help establish the optimum inhibitor concentration. Cell viability was then detected and our results showed that at Fumitremorgin C concentrations that are lower than 10 μ M, almost no toxicity to cells. Furthermore, following 2h incubation with 6 μ M Fumitremorgin C, the IC₅₀ of resistant cells to Osimertinib was significantly reduced. Accordingly, these findings demonstrated that inhibiting ABCG2 expression can reverse the acquired resistance to Osimertinib in NSCLC cells. In addition, up-regulating the expression of ABCG2 in non-drug-resistant cells will cause the cells to develop resistance to Osimertinib. The Hoechst 33258 stain enabled the detection of NSCLC cells undergoing apoptosis following incubation with 6 μ M Fumitremorgin C for 24h. When compared to control cells, the apoptosis levels of Osimertinib-resistant cells was deemed significant (Fig. 6). Thus, these data demonstrate that ABCG2 is involved in Osimertinib resistance and that inhibiting the expression of ABCG2 may be used as a means of overcoming Osimertinib resistance in NSCLC.

3.5 The increase in ABCG2 expression in Osimertinib resistant cells is associated with the activation of the MAPK and the PI3K/AKT pathways

The association of ABCG2 activation to signaling pathways was investigated because of the association of ABCG2 activation with many factors. Specifically, the most reported of these factors are the activation of the MAPK and the PI3K/AKT pathways. The results of the Western blot analysis performed in this study demonstrated that the MAPK and the PI3K/AKT pathways were associated with increased protein levels in drug-resistant cells (Fig. 7). Subsequently, these pathways were examined with or without the ERK inhibitor PD98059 and the PI3K inhibitor Buparlisib using the A549 and H1975 cells. The findings indicated that both PI3K and ERK inhibition will result in a reduction of ABCG2 expression. Thus, it can be concluded that ABCG2 expression is caused by MAPK PI3K/AKT co-activation.

4. Discussion

EGFR is an important regulator of cell growth which is widely expressed in tumors when unregulated or mutated [28, 29]. Increased expression of EGFR leads to the activation of downstream pathways to induce cell proliferation, invasion and drug resistance [28, 29]. With the continued developments in molecular technology and the improvements seen in targeted therapy, EGFR has evolved into an important target [30]. Tyrosine kinase inhibitors are one of the main treatment regimens for patients with EGFR mutations [31], and their resistance mechanisms have become the focus of current research.

In our study, we demonstrated how NSCLC developed resistance to Osimertinib following the continuous increasing of the Osimertinib concentration. The analyzed data indicated the significant increase in the IC50 value of the drug-resistant cells. Moreover, cell proliferation was accelerated and the invasion ability of the cancer cells was enhanced. It is also worth noting that the ABCG2 expression in the drug-resistant cells was increased. ABCG2 is considered to be related to drug resistance in a variety of tumors [32], and its overexpression can result in the excretion of intracellular toxic substances; including a variety of chemotherapeutic drugs [33]. Thus, by reducing the intracellular concentration of toxic drugs, ABCG2 overexpression can cause the development of drug resistance [34]. Studies have identified an array of ABCG2 substrates, including mitoxantrone, camptothecin derivatives, methotrexate, and more [35]. In addition, many TKIs, including imatinib and gefitinib, are also the substrates of ABCG2 [36].

Research findings have also reported that tyrosine kinase inhibitors can act as both substrates or inhibitors of the transporter ABCG2 and that this distinction is dependent on their concentration [37]. At high concentrations, TKIs prevent the phosphorylation of downstream pathways through the inhibition of the ATP binding site of the receptor tyrosine kinases. Moreover, this results in the inhibition of cell proliferation and survival. Conversely, at low concentrations, Osimertinib is transported out of the cell as a substrate of the ABCG2 transporter, thereby leading to development of Osimertinib resistance [38]. A study

of the factors affecting the accumulation of Osimertinib in the brain showed that ABCG2 is related to the accumulation of Osimertinib, thus indicating that Osimertinib is the substrate of ABCG2 [39]. To prove that ABCG2 excretes Osimertinib from drug-resistant cells, we detected the concentration of Osimertinib in the cells using HPLC. The HPLC findings showed that the concentration of Osimertinib in drug-resistant cells decreased compared with parental cells, thus indicating that Osimertinib excretion was increased in drug-resistant cells. Therefore, we have reason to believe that Osimertinib resistance in NSCLC is related to drug efflux.

The activation of ABCG2 is primarily through the MAPK and PI3K/AKT pathways. A study has shown that the phosphatidylinositol 3-kinase/serine-threonine kinase (PI3K/AKT) pathway activator IGF-1R can activate the ABCG2 signaling pathway [40]. Moreover, the findings also indicated that the MAPK signaling pathway, another downstream pathway of EGFR, can also lead to the expression of ABCG2 [41]. In this experiment, we found that the expression of ABCG2 was reduced after down-regulating the expression of ERK and PI3K. Furthermore, we also established that the expression of ABCG2 was the lowest following the co-inhibition of ERK and PI3K. Accordingly, we propose that the increase in ABCG2 expression is related to both the activation of the MAPK pathway and of the PI3K/AKT pathway.

Osimertinib is effective in the treatment of NSCLC, but it is limited by multidrug resistance in the later stages of treatment [19]. The mechanism of drug resistance is highly complex. This complexity is mainly born of the amplification of MET/HER2 or activation of the Bypass pathway and novel fusion events and histological/phenotypic transformations that are observed [19, 42], and more. Our experimental results show that the overexpression of multidrug resistance protein also plays an vital role in Osimertinib resistance.

In this study, we selected H1975 cells which harbor Osimertinib-sensitive T790M mutations. We also tested the A549 and H1299 cells that do not contain this mutation site. Rather, the latter two cells were transfected with a lentivirus which resulted in their transfection into T790M mutant cells (the secondary mutation of EGFR caused by gefitinib resistance). The above cells all showed increased expression of ABCG2 during their resistance to Osimertinib. HPLC analysis proved the decrease in the concentration of Osimertinib in the resistant cells, together with the elevation in the rate of proliferation and the cell's invasion ability. This resistance was subsequently reversed by down-regulating ABCG2 in the drug-resistant cells while also leading to the cells losing their sensitivity after overexpression of ABCG2 in non-resistant cells. Therefore, ABCG2 is one of the factors contributing to the development of Osimertinib resistance in NSCLC cells.

In general, the expression of ABCG2 induced the development of resistance of NSCLC cells to a variety of chemotherapy drugs. David Westover et al. showed that ABCG2 mediates the resistance of NSCLC to the second-line drug irinotecan [43]. Other studies reported that overexpression of ABCG2 causes NSCLC to develop resistance to the commonly used chemotherapy drug topotecan [44]. Bin Ke et al. showed that inhibiting the expression of ABCG2 can reverse the resistance of NSCLC to cisplatin [45]. In addition, a variety of EGFR TKIs are considered to be substrates of ABCG2, and our research shows that ABCG2 is

related to the resistance of Osimertinib to NSCLC. Therefore, regulation of ABCG2 is crucial to enhancing treatment and reducing the drug resistance of NSCLC cells.

5. Conclusions

In this study, we summarized the mechanism of Osimertinib resistance found in NSCLC. Our results show that inhibition of ABCG2 is expected to be a means of reversing the resistance to Osimertinib that can manifest in NSCLC patients. Based on this study, a combination of ABCG2 inhibitors and Osimertinib may overcome resistance to Osimertinib in NSCLC cells. These findings provide a theoretical basis for clinical treatment.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for Publication

This paper has not been previously published and is also not under consideration for publication elsewhere. Each worker participating in this study was asked to sign an informed consent after information was given about the study goals and program. After this manuscript was done, all authors had read it and agreed that this work be published; they also accepted responsibility for the manuscript's contents.

Availability of data and materials

The data used and analyzed during the current study are available from the corresponding author on reasonable request.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Authors' Contributions

Xiaojun Ge : Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Mei Wang and Junyao Jiang: contributed to Cell culture, Induced resistance, the data analysis and data interpretation.

Yuxuan Du and Shuaimei Liu: contributed to detection of the protein expression intracellular, cells apoptosis and proliferation, and more.

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Figures

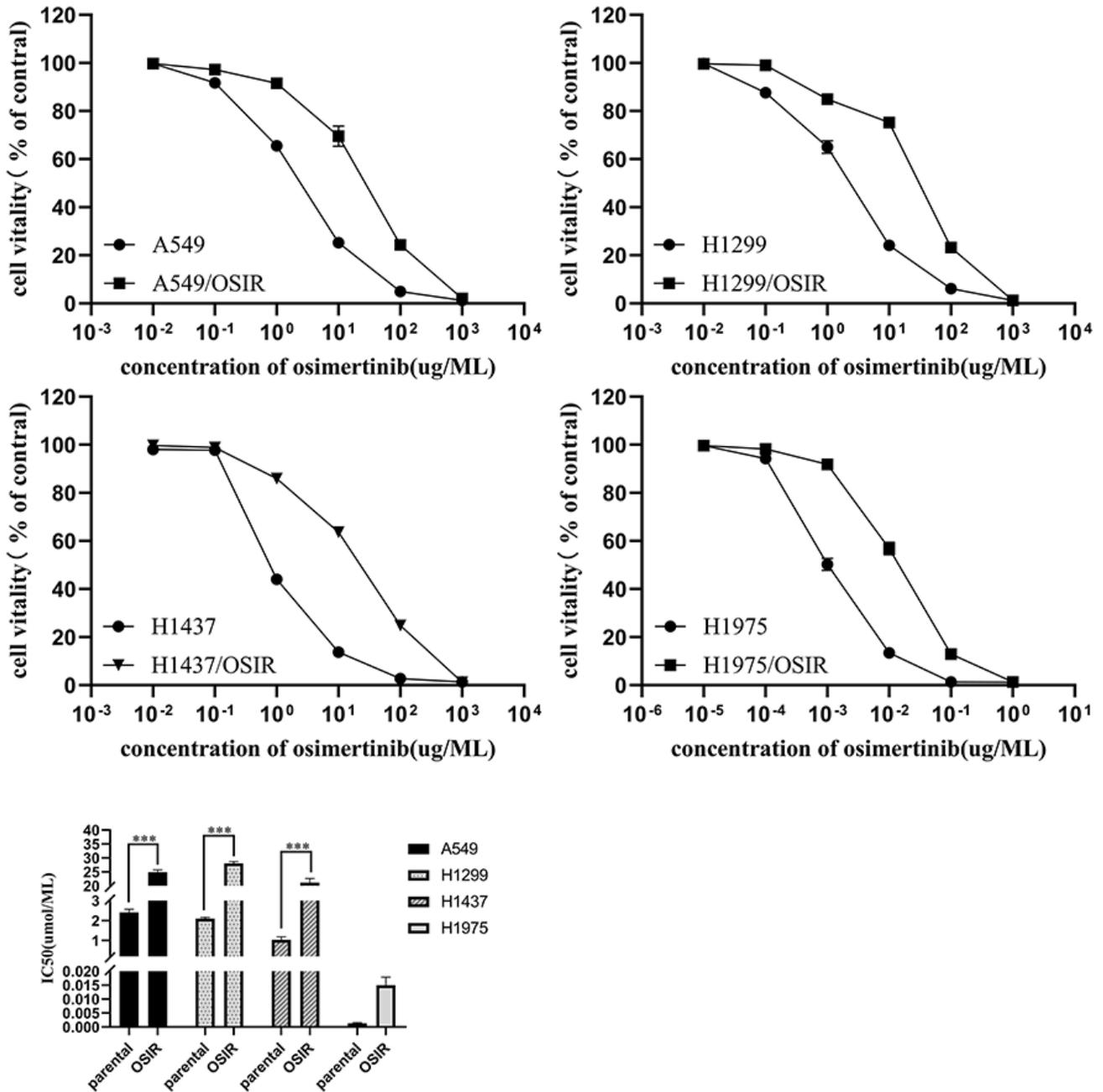


Figure 1

The IC₅₀ of the parental and the Osimertinib resistant cells. The IC₅₀ of resistant cells is significantly increased and is more than 10 times that of the parental cells. The error bars equate to the mean ± S.E.M. (***) signifies an IC₅₀ value for the drug resistant group that is significantly higher than the parental group at p<0.005.

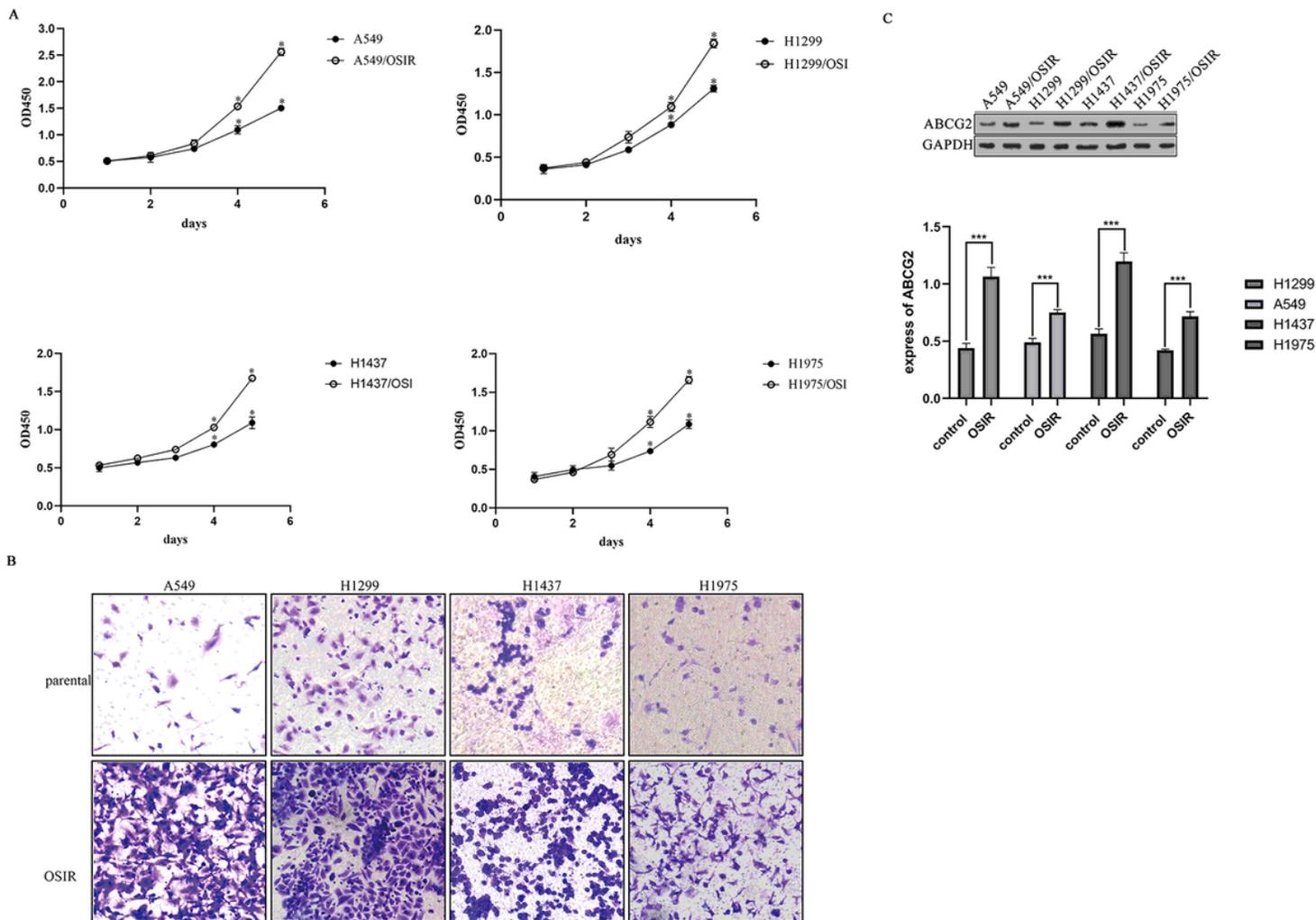


Figure 2

The proliferation, invasion and protein expression of each cell. A. CCK-8 method for detection of cell proliferation. B. The transwell assay for identifying the invasion of parental cells and drug-resistant cells. C. Western-blot analysis used to detect the expression of ABCG2. The error bars equate to the mean \pm S.E.M. (***) signifies an IC50 value for the drug resistant group that is significantly higher than the parental group at $p < 0.005$.

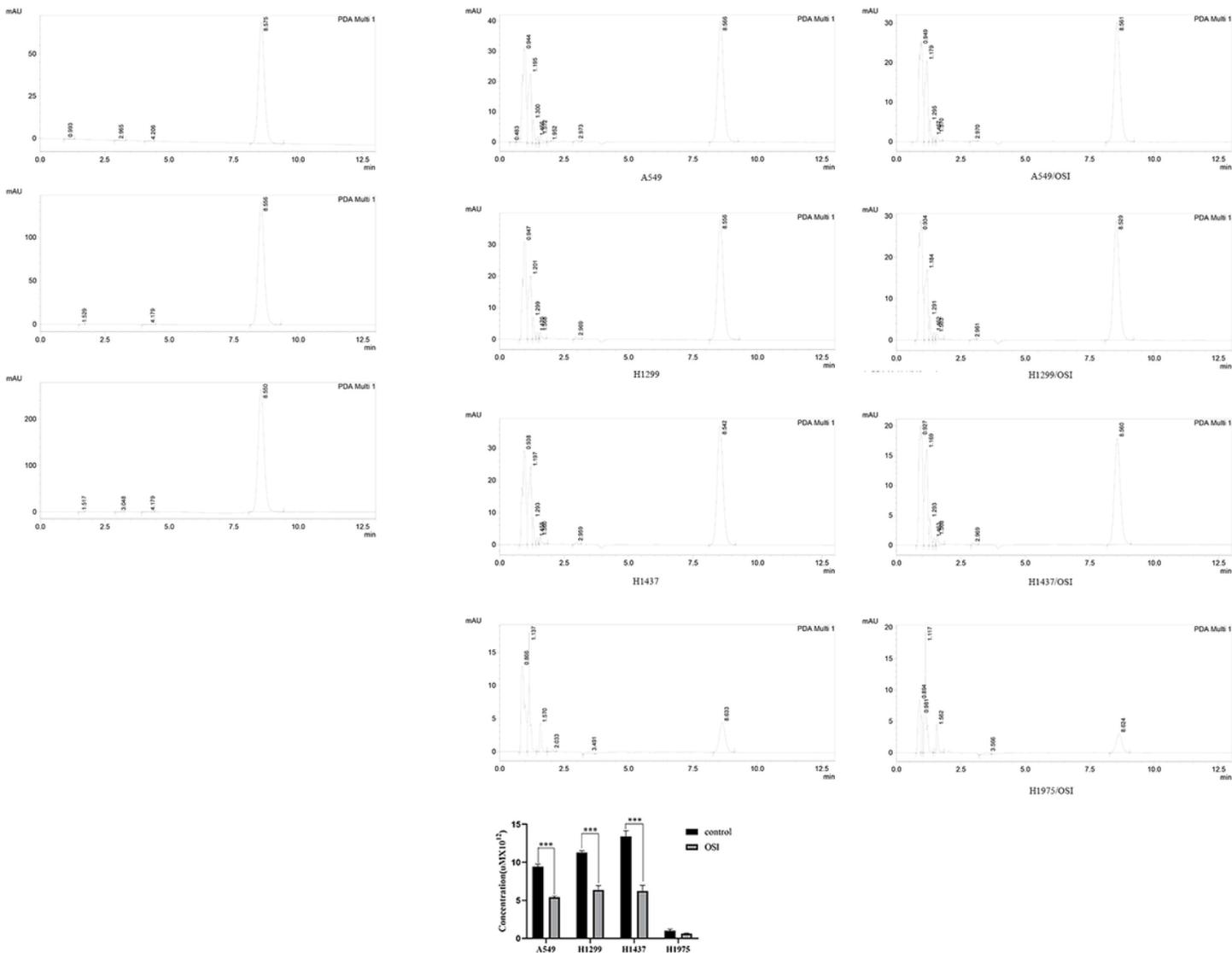


Figure 3

A. The concentrations of Osimertinib at 0.05 μ M, 0.1 μ M, and 0.2 μ M. The drug concentration was used as the abscissa and the area was used at the ordinate to derive a standard curve. The curve formula equates to $y=21458266.86x+69104.4$ with a correlation coefficient of 0.999. The peak time for Osimertinib was established as approximately 8.5s. B. The concentration of Osimertinib in cells was detected by high performance liquid chromatography. The error bars equate to the mean \pm S.E.M. (***) signifies an IC50 value for the drug resistant group that is significantly higher than the parental group at $p<0.005$.

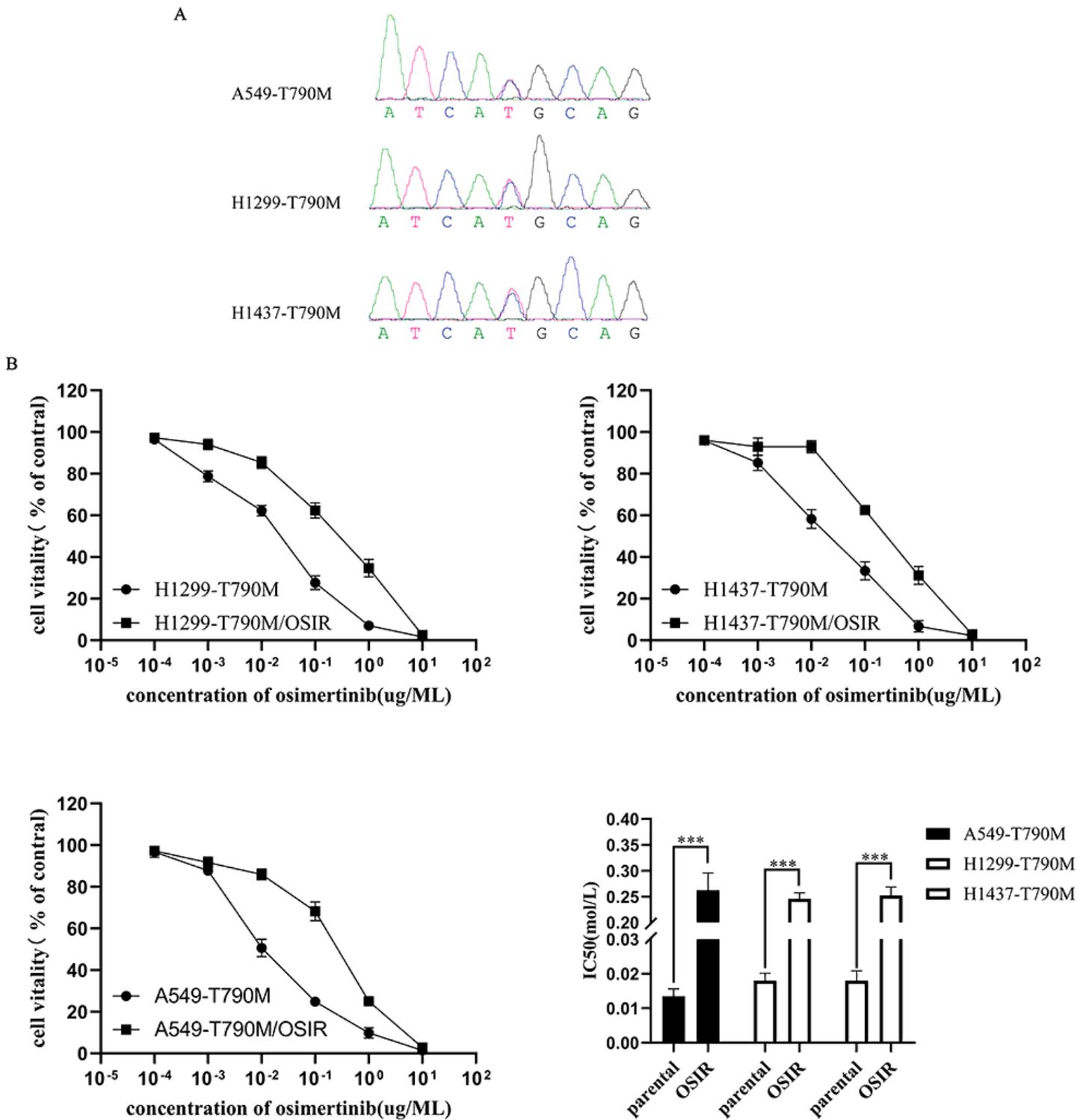


Figure 4

Transfection of lentivirus generates cells harboring the T790M mutation. The IC₅₀ (proliferation) in drug-resistant and parental cells. A. Gene sequencing to detect mutation sites. B. The IC₅₀ was detected by CCK-8. The error bars equate to the mean \pm S.E.M. (***) signifies an IC₅₀ value for the drug resistant group that is significantly higher than the parental group at $p < 0.005$.

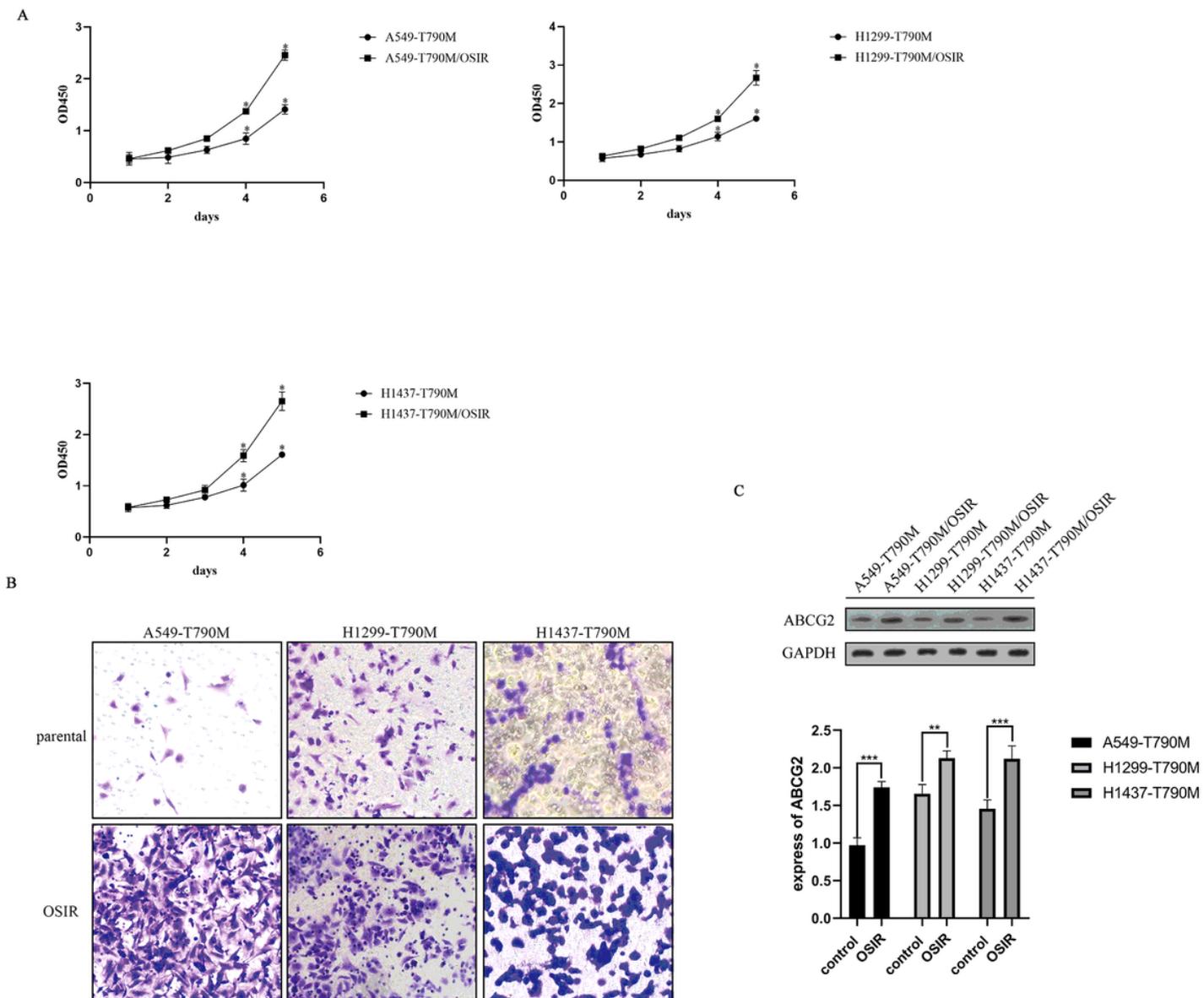


Figure 5

The detection of cell proliferation, invasion and protein expression. A. Cell proliferation detection. B. Transwell assay detection of the invasion of parental cells and drug-resistant cells. C. The intracellular ABCG2 expression. The error bars equate to the mean \pm S.E.M. (***) signifies an IC₅₀ value for the drug resistant group that is significantly higher than the parental group at $p < 0.005$.

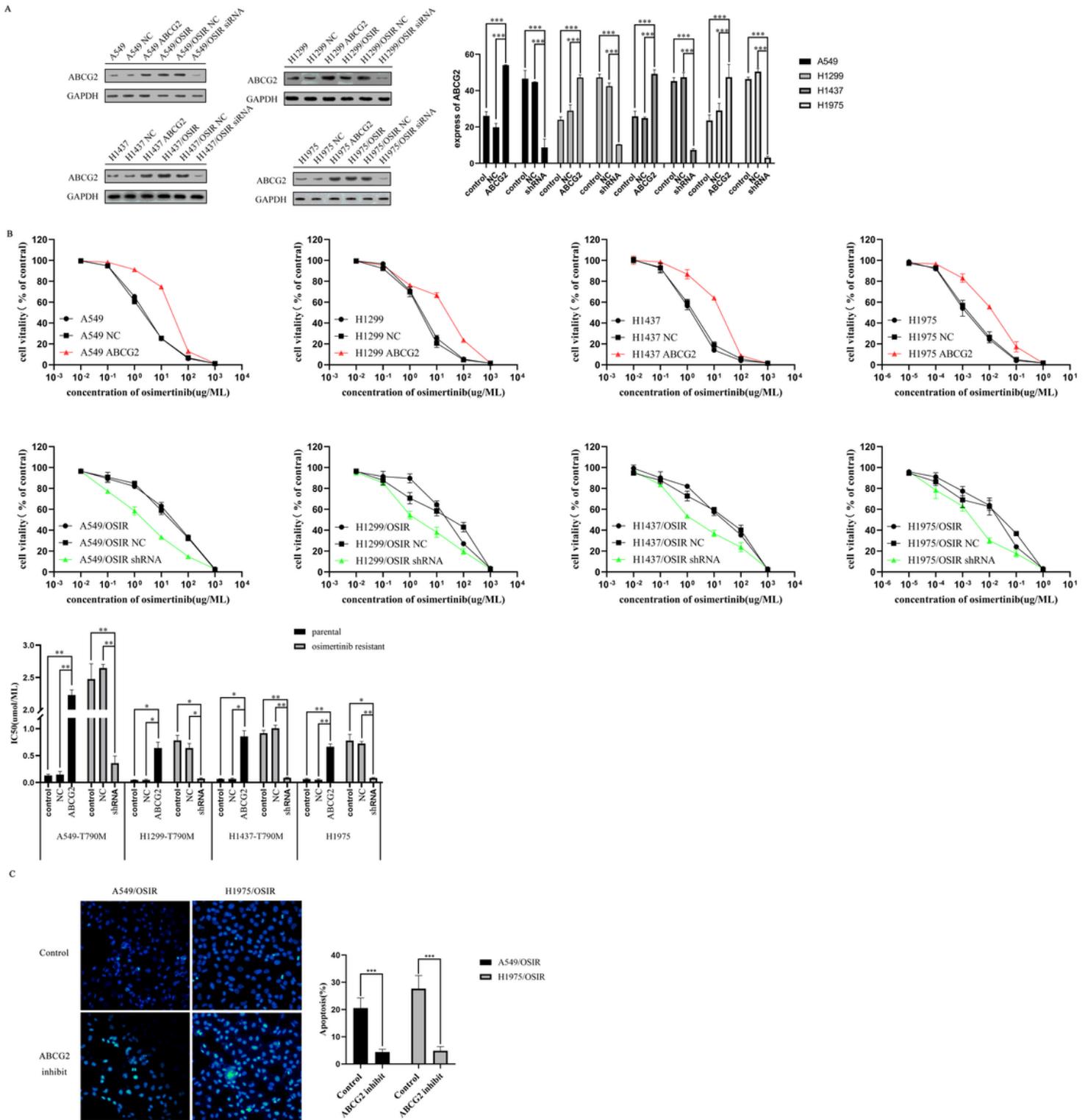


Figure 6

A. The protein expression of cells following the transfection of the plasmid and the regulation of ABCG2 expression in the cell. The intracellular protein expression increases or decrease following transfection with the plasmid. B. The IC₅₀ of the transfected cells exhibiting regulation of ABCG2 expression in the cells. The cell's IC₅₀ significantly changed after up-regulation and down-regulation of ABCG2. C. Detection of apoptosis levels of NSCLC cells with or without ABCG2 inhibition using the Hoechst 33258

stain. The error bars equate to the mean \pm S.E.M. (***) signifies that the ABCG2 regulation group is significantly different from the control group at $p < 0.005$.

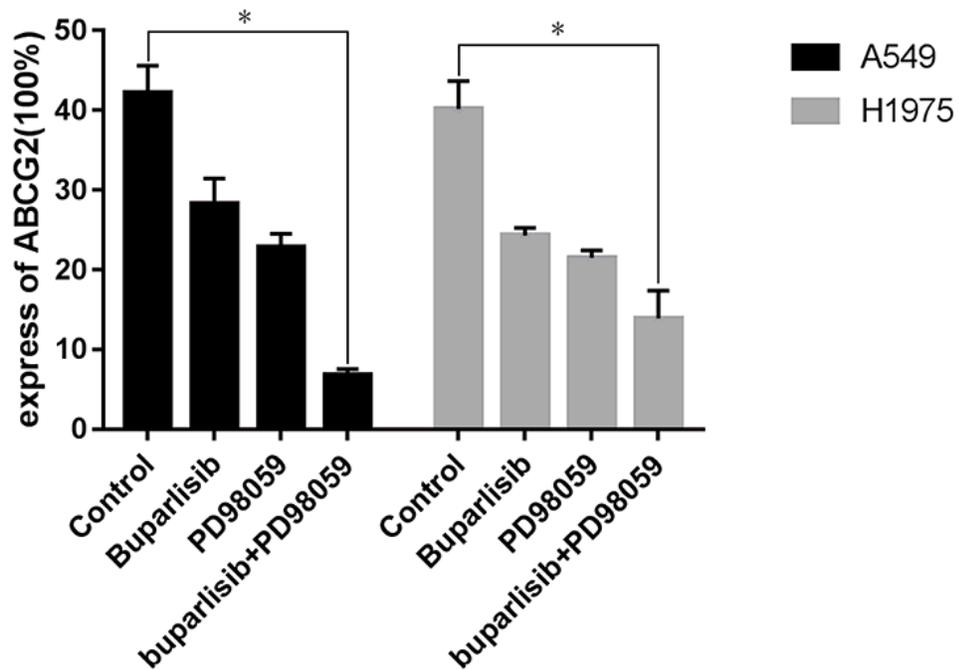
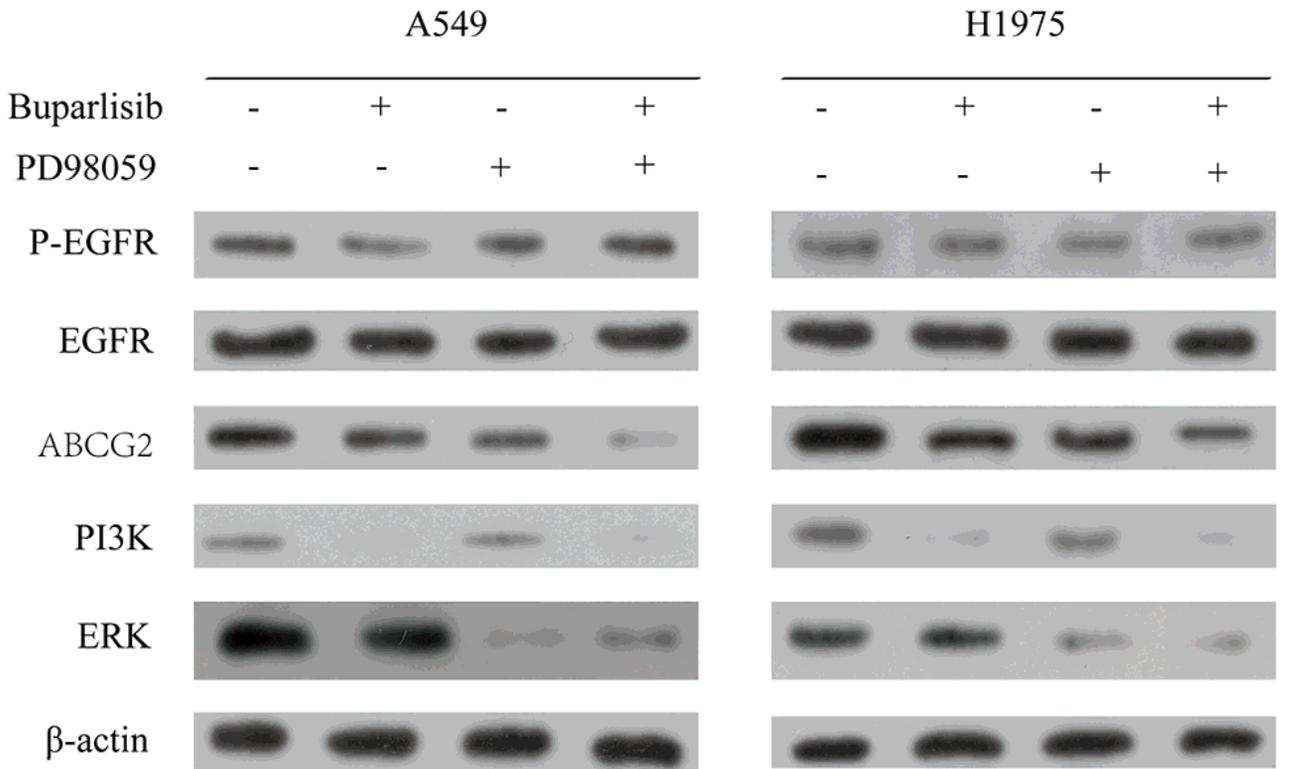


Figure 7

The expression of related proteins in cells following treatment with inhibitors. The error bars equate to the mean \pm S.E.M. (*) signifies an IC50 value for the dual-drug group that is significantly different than that of the control group at $p < 0.05$.