Overexpression of ID1 mediates resistance to osimertinib in T790M positive non-small cell lung cancer through epithelial-mesenchymal transition

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Research Article

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Abstract

Objective

To analyzed the effect of ID1 overexpression on osimertinib resistance to T790M positive non-small cell lung cancer (NSCLC).

Methods

We established drug resistant cell line H1975/OR from osimertinib sensitive cell line H1975. Protein alterations of ID1 and Epithelial mesenchymal transition (EMT) were detected with western blot analysis. RT-PCR was used to evaluate the differences of gene mRNA. ID1 silencing and overexpression was used to investigate the effect of related gene on osimertinib resistance. Cell Counting Kit-8 (CCK8) was used to assess proliferation rate of ID1 differently expressed cells. Cell cycle and apoptosis was compared using flow cytometry.

Results

In our study, we found that in osimertinib resistant NSCLC cells, the expression level of EMT related protein E-cadherin was lower than that of sensitive cells, while the expression level of ID1 and vimentin was higher than that of sensitive cells. ID1 expression level was closely related to E-cadherin and vimentin both in osimertinib sensitive and resistant cells. Alteration of ID1 expression in H1975/OR cells could change the expression of E-cadherin. Downregulating ID1 expression of H1975/OR cells could promote the apoptosis induced by osimertinib and block cell cycle at G1/G0 stage. Our study indicated that ID1 may induce EMT in T790M positive NSCLC, which mediates drug resistance of osimertinib.

Conclusions

Our study reveal the mechanism of ID1 mediated resistance to osimertinib in T790M positive NSCLC through EMT, which may provide new ideas and methods for treatment of EGFR mutated NSCLC after osimertinib resistance.

1. Introduction

Lung cancer, one of the most common malignant tumors and also the primary cause of death in cancer patients worldwide, is classified into smallcell lung cancer and nonsmall cell lung cancer (NSCLC); the latter accounts for appropriate 80% of all cases of lung cancer [1, 2]. For patients with advanced NSCLC harboring epidermal growth factor receptor (EGFR) gene mutations, which has a ratio of 3050% of NSCLC cases in East Asia, the first generation of EGFR tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib

and erlotinib have superior therapeutic efficacy than traditional platinum-based chemotherapy[3,4] and the median progression free time (PFS) is 9–13 months. However, these patients will eventually suffered secondary drug resistance during the targeted therapy and most of them may harbor T790M mutation in exon 20 of EGFR gene[5]. For patients with T790M mutation, osimertinib still shows favorable therapeutic effect[6]. The median PFS of patients with NSCLC after failure of the first generation of EGFR-TKIs treatment is significantly higher than that of standard platinum-based chemotherapy[7]. However, osimertinib may also develop drug resistance.

Epithelial mesenchymal transition (EMT) may be one of the mechanisms that mediates osimertinib resistance. Previous studies have shown that resistance to gemcitabine in EMT producing pancreatic cancer cell lines is significantly increased[8]. Similarly, EMT can be found in fluorouracil resistant colon cancer cell lines[9], tamoxifen resistant breast cancer cell lines[10], cisplatin resistant cervical cancer cell lines[11], and gefitinib resistant lung cancer cell lines[12]. Rastogi I et al. Reported that in EGFR-TKIs resistant cell lines, ZEB1 was overexpressed and E-cadherin expression was inhibited, thus inducing EMT in lung cancer cells[13]. After down regulating ZEB1 with miR-200a or β-Catenin siRNA, EGFR-TKIs sensitivity could be restored. Recently, it has been found that Hedgehog signaling pathway is abnormally activated in EGFR-TKIs resistant cells[14]. Blocking hedgehog signaling pathway with sant-1 could restore the expression of E-cadherin and increase the sensitivity of EGFR-TKIs. In EGFR-TKIs sensitive cells, up-regulation of hedgehog signaling pathway can inhibit the expression of E-cadherin and increase the expression of snail and ABCG2, leading to drug resistance. These studies have shown that EMT plays an important role in mediating EGFR-TKIs resistance in lung cancer.

The process of EMT is very complex. There are many signal pathways involved in it. It is known that EMT can be induced by NF-κB/snail/PTEN loop, Notch-2 and MAPK/mitochondria. When EMT occurs in tumor cells, epithelial markers such as E-cadherin show low expression, while mesothelial markers such as vimentin show low expression and N-cadherin were highly expressed[15]. Therefore, inhibition of E-cadherin expression can induce EMT. The exogenous signal can inhibit the expression of E-cadherin and mediate EMT by forming complex with transcription factors such as snail, slug, ZEB1, SIP1 and twist and binding to the promoter of CDH1 gene[5]. In addition, many miRNAs can regulate the expression of E-cadherin and induce the production of EMT in tumor cells by regulating the transcription inhibitors ZEB1 and SIP1.

ID1 belongs to the bHLH transcription factor family, and is hardly expressed in adult tissues and cells[16]. However, it has been reported that ID1 is highly expressed in a variety of tumors and is involved in cell proliferation, cell cycle progression, differentiation inhibition and genomic instability. Studies have shown that ID1 can promote the growth of esophageal cancer cells through PI3K/Akt/NF-κB signaling pathway[17]. Inhibition of ID1 can lead to the inactivation of NF-κB, thus promoting the apoptosis of colorectal cancer cells[18]. Previous studies have also reported that ID1 is highly expressed in lung cancer cells. ID1 is associated with EGFR-TKIs resistance in lung cancer. The higher the expression of ID1, the stronger the resistance of cells to EGFR-TKIs[19]. The higher the expression of ID1, the worse the

prognosis of NSCLC. It is suggested that ID1 may inhibit the PI3K/AKT signaling pathway through negative feedback. As NF-κB is the downstream signal pathway of PI3K/Akt, the increase of ID1 expression may lead to EMT through NF-κB/snail/PTEN loop as illustrated above.

The aim of our study is to reveal the mechanism of ID1 mediated resistance to osimertinib in T790M positive non-small cell lung cancer through EMT, and provide new ideas and methods for clinical treatment of EGFR mutated NSCLC after osimertinib resistance.

2. Material And Methods

2.1. Chemicals and reagents

Osimertinib was obtained from Selleck Chemicals. RPMI-1640 was product of HyClone and Dulbecco's modifified Eagle's medium (DMEM) and fetal bovine serum (FBS) were products of Gibco BRL. Trizol was purchased from Takara Biomedical Technology. Cell Counting Kit-8 was obtained from Dojindo. Monoclonal antibodies against ID1 were products of Santa Cruz Biotechnology. Glyceraldehyde-3-phosphate de hydrogenase (GAPDH), E-cadherin and vimentin antibodies were purchased from Abcam.

2.2. Cell culture and ID1 silencing

The EGFR mutant human lung adenocarcinoma cell lines, H1975, were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. H1975/OR was obtained by gradually increasing the concentration of osimertinib into H1975 cell culture medium. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37●C in a humidifified incubator with 5% CO2. All cells were grown in culture medium that is free of drugs for more than 14 days before assay. ID1 silencing was achieved using siRNA (Synbio Technologies) against this gene and negative control siRNA diluted in Lipofectamine RNAiMAX (Thermo Fisher) and Opti-MEM (Thermo Fisher) used as described in the manufacturers specifications. Protein knockdown was assessed using western blot.

2.3. ID1 overexpression

According to the sequence of ID1 gene and vector pcDNA3.0, the primer was designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd.. The amplified fragment length was 468 bp. The sequence of primer was as follows: forward: 5'-GGGGTACCATGAAAGTCGCCAGTGGCAGC-3', reverse: 5'-CCCTCGAGTCAGCGACACAAGATGCGATCG-3'. ID1 overexpression was conducted as follows. We carried the experiment of RNA extraction, reverse transcription synthesis cDNA, PCR amplification of target gene, PCR recovery product and vector digestion, target fragment and vector connection, transformation of ligation product, PCR identification of bacterial liquid, plasmid extraction and recombinant plasmid enzyme digestion identification. Then, we did the blast analysis of sequencing results. The sequence was basically consistent with the known sequence on NCBI. ID1 gene was successfully cloned into pcDNA3.0 vector.

2.4. Cell proliferation assay

Cells were seeded at a density of 1.0 × 104 cells/well in a 96-well plate for 48 and 72 hours. Cell Counting Kit-8 ((CCK-8, Dojindo Molecular Technologies, Tokyo, Japan) solution was added to each well prior to the endpoint of incubation. The CCK-8 reagent was added into each well at a 1:10 (v/v) dilution per 100 μL and incubated for 2 h at 37 °C. We quantitated the results spectrophotometrically at a wavelength of 450 nm.

2.5. Western blot analysis

Cells were lysed after washing two times with ice-cold PBS. The protein concentration was quantified using the Bradford method. Equal amounts of protein were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. Chemoluminescence was used to detect the protein. Protein samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were blocked with fat-free milk combined with tris-buffered saline plus tween 20 for one hour at room temperature and then incubated with the appropriate primary antibody and horseradish peroxidase conjugated secondary antibodies.

2.6. Reverse transcription PCR

ID1 expression was assayed as described. Total RNA was isolated using the Trizol reagent RNA extraction kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. cDNA was synthesized using the BestarTM qPCR RT kit (DBI Bioscience), following the manufacturer's instructions. Quantitative real-time PCR analysis was performed with BestarTM qPCR MasterMix (DBI Bioscience). Each sample was run in triplicate for each gene. Transcript levels were normalized to the housekeeping gene phosphoglycerate kinase (PGK) and analyzed by the relative quantification 2-ΔΔCt method. The PCR primers were GAPDH: forward: 5'-TGTTCGTCATGGGTGTGAAC-3', reverse:5'-ATGGCATGGACTGTGGTCAT-3', Vimentin:forward:5'-AGTCCACTGAGTACCGGAGAC-3',reverse:5'-CATTTCACGCATCTGGCGTTC-3', E-cadherin: forward:5'-ATTTTTCCCTCGACACCCGAT-3',

reverse:5'-TCCCAGGCGTAGACCAAGA-3',ID1:forward:5'-CTGCTCTACGACATGAACGG-3',reverse:5'-GAAGGTCCCTGATGTAGTCGAT-3'. The products were resolved using gel electrophoresis (1.5% agarose gel).

2.7. Flow cytometry analysis of apoptosis

Cells were incubated with 0.25% trypsin, After that, the cells were observed under the microscope. When the cytoplasm retracted and the cells were no longer connected into pieces, the trypsin was removed, and 4 ml of complete culture medium was added, digestion was stopped, blowing, and single cell suspension was made; the single cell suspension was moved to flow tube, washed with PBS twice, centrifuged at 1000 rpm for 5 min; the supernatant was discarded and added with 200 μ l annexin V-FITC/PI staining solution was used to resuspend the cells; the cells were incubated in dark for 15 min and detected on the machine.

2.8. Flow cytometry analysis of cell cycle

To determine the effects of ID1 on cell cycle, 2×10^4 cells/well were seeded in six-well plates and incubated for 24 hours. Cells were taken out and made as single cell suspension. The single cell suspension was then moved to flow tube, washed with PBS twice, centrifuged at 1000 rpm for 5 minutes. the supernatant was completely removed as far as possible, and 1 ml was added. Cells were resuspended with 70% ethanol precooled at -20 ℃. The samples were stored at -20 ℃. The specimens were centrifuged at 1000 rpm for 5 min each time, washed with PBS for 1–2 times, and centrifuged at 1000 rpm for 5 min. 300 UL Annexin V-FITC/PI staining solution was added and stained for 15 min in dark, and then tested and analyzed.

2.9. Statistical analysis

All experiments were repeated at least three times and the differences were determined by using the Student's t-test. The significance was determined at $p < 0.05$.

3. Results

3.1. Generation of osimertinib resistance cell line H1975/OR

We selected NSCLC cell line H1975 in our study. H1975 harbors T790M mutation hence is sensitive to osimertinib. Its corresponding drug resistant cell line H1975/OR was induced by osimertinib. In detail, H1975 cells were exposed to the culture medium with increasing concentration of osimertinib. When was confirmed as resistant cell line, H1975/OR was screened by gene sequencing. We found that there were no secondary gene mutations occurred, such as C797S, amplifification of MET and loss of T790M. These cells were cultured in RPMI-1640 medium of 10% fetal bovine serum at 37℃ containing 5% CO2.

3.2. Different expression of ID1 and EMT related protein

In the study, we detected the expressions of ID1 and EMT related protein at mRNA and protein level using reverse transcription-PCR (RT-PCR) and Western blot analysis. At protein level, ID1 and vimentin appeared to be up-regulated in the H1975/OR cell line as compared to osimertinib sensitive cell line H1975, whereas the expression of E-cadherin was significantly decreased in H1975/OR (Fig. 1). The mRNA level of ID1, vimentin and E-cadherin were also changed accordingly. These results suggest that ID1 expression level was closely related to E-cadherin and vimentin both in osimertinib sensitive and resistant cells. Therefore, overexpression of ID1 may be one of the mechanisms of osimertinib resistance to T790M positive NSCLC cells through epithelial-mesenchymal transition.

3.3. The role of ID1 on expression of EMT related protein

To further understand and investigate overexpression of ID1 in relation to osimertinib resistance in H1957/OR, we conducted the experiment of gene silencing

using siRNA targeting ID1 and overexpression by cloning ID1 gene into pcDNA3.0 vector. Reverse transcription PCR and western Blot confirmed the effect of ID1 silencing and overexpression on drug-resistant cells. Down-regulation of ID1 expression in H1975/OR cells could promote the expression of E-cadherin, decrease the expression of vimentin (Fig. 2). Conversely, in the negative control cell line, both expression of E-cadherin and vimentin did not changed significantly as compared to that in the H1957/OR cell line. Our study indicated that overexpression of ID1 could induce EMT in T790M positive NSCLC, which mediates drug resistance of osimertinib.

3.4. Impact of ID1 on proliferation of osimertinib sensitive and resistant cells

We then investigate whether the alteration of ID1 expression could change the sensitivity to osimertinib in H1975/OR cells. As is shown in Fig. 3, we detected that downregulating ID1 could restore drug sensitivity in resistant cells, while overexpression of ID1 could further escalate osimertinib resistance. In negative control cells, the drug sensitivity of osimertinib did not change significantly as compared to that of H1975/OR cells.

3.5. Impact of ID1 on apoptosis and cell cycle of T790M positive cell line

In our study, we found that alteration of ID1 expression of H1975/OR cells could change apoptosis induced by osimertinib. The apoptosis rate in H1975/OR cells was significantly decreased as compared with that in osimertinib sensitive cells. After ID1 silencing, the apoptosis rate was increased as compared to H1975/OR cells, whereas in ID1 overexpressed cells, the apoptosis rate was decreased dramatically. (Fig. 4). This result is in accordance with that of the sensitive cells, which indicated that by inhibiting the expression of ID1, the drug resistance of osimertinib may be reversed in H1975/OR cells. To confirm this phenomenon, we checked the result in the negative control group. The rate of cell apoptosis did not significantly changed as compared to that in H1975/OR cells. We further conduct experiment to investigate the impact of ID1 on cell cycle of osimertinib sensitive and resistant cells. As compared to H1975/OR cells, cell cycle of H1975 were blocked at G1/G0 stage (Fig. 5). By down-regulating ID1, this result was reversed accordingly, which is almost similar to that in the osimertinib sensitive H1975 cells.

4. Discussion

Currently, first-line treatment for patients with EGFR mutated advanced NSCLC could prescribe single targeted agents such as osimertinib, gefitinib and erlotinib. For lung cancer patients with T790M mutation, osimertinib as first-line treatment or as subsequent treatment after initial targeted therapy should be given priority[7]. However, osimertinib may still face the problem of drug resistance after a period of therapeutic time. The mechanism of osimertinib resistance is very complex. The most important mechanism is C797S mutation of EGFR gene[20]. C797S mutation is located in the EGFR tyrosine kinase

region, which can inhibit the effect of osimertinib and mediate the drug resistance of lung cancer cells. For C797S mutation, EAI045 has been developed to overcome osimertinib resistance[21].

For patients without C797S mutation, it has been reported that HER2 or c-Met amplification pathways are involved in osimertinib resistance[22]. Abnormal activation of HER2 can activate downstream pathways such as PI3K/Akt and MEK/MAPK through the formation of homologous or heterologous dimer, which leads to abnormal proliferation of tumor cells and the production of osimertinib resistance; c-met amplification bypasses EGFR activation of downstream PI3K/Akt mediated signal pathway, leading to resistance to osimertinib. The activation of RAS, a downstream signaling pathway, can also lead to osimertinib resistance[23]. In addition to these reasons, there are still many unknown mechanisms of osimertinib resistance, especially in the front-line settings. Therefore, illustrating the resistance mechanism of osimertinib and overcoming the resistance problem of the third generation EGFR-TKIs have become one of the research focuses for NSCLC.

Till date, there were several resistance mechanisms in previous studies such as FLAURA and AURA3. However, it was still not clear whether other mechanisms of acquired resistance of osimertinib existed when used as front line treatment. As were showed in previous studies, EMT can be resistant to traditional anticancer drugs and EGFR-TKIs. EMT can promote the growth, migration and metastasis of tumor cells. It has been found that EMT tumor cells own the abilities of self-renewal, unlimited proliferation and anti-apoptosis, and highly express CD133, CD44 + and ABCG2, which is very similar to the characteristics of cancer stem cells, which may be the potential mechanism of osimertinib resistance[24].

In our study, we found that in osimertinib resistant NSCLC cells, the expression level of EMT related protein E-cadherin was lower than that of sensitive cells, while the expression level of ID1 and vimentin was higher than that of sensitive cells. In the study, ID1 expression level was closely related to E-cadherin and vimentin both in osimertinib sensitive and resistant cells. Alteration of ID1 expression in H1975/OR cells could change the expression of E-cadherin. Downregulating ID1 expression of H1975/OR cells could promote the apoptosis induced by osimertinib and block cell cycle at G1/G0 stage. Our study indicated that ID1 may induce EMT in T790M positive NSCLC, which mediates drug resistance of osimertinib. These results may compensate the pre-existing osimertinib resistance mechanisms.

In previous studies, we know that ID1 can participate in liver metastasis of lung cancer cells through EMT and knockout of ID1 may lead to decreased expression of vimentin, TGF-β, Snail[25]. Down-regulation of ID1 expression can not only inhibit EMT formation, but also induce tumor apoptosis. It has been reported that ID1 knockout in ovarian cancer cells can significantly inhibit the growth and invasion of tumor cells, and promote the apoptosis of tumor cells[26]. In colon cancer HCT116 cells, ID1 inhibited apoptosis induced by chemotherapy drugs and ultraviolet light[27]. In small cell lung cancer, it has been reported that the high expression of ID1 can significantly inhibit the apoptosis of tumor cells[28]. Our study is highly in line with results of these former studies.

In head and neck cancer cells, snail-induced EMT enables cancer cells to maintain their tumor stem cell-like properties, thus increasing resistance of chemotherapy and invasiveness[29]. When tumor cells produce EMT, they often secrete more cytokines such as CXCL9 and CXCL10, which can inhibit NK cells' killing function on tumor stem cells, thus promoting tumor immune escape[30]. Our study found that in T790M mutant NSCLC, increased ID1 expression can mediate EMT formation, inhibit cell apoptosis and promote cell proliferation.Down-regulation of ID1 expression can block cell cycle at G1/G0 stage. After ID1 expression level changes, does the proportion of tumor stem cells change? At present, there is no report that ID1-mediated EMT promotes the formation of tumor stem cells to regulate the immune escape of T790M mutated lung cancer. We will conduct more studies to investigated these mechanisms.

However, our study may also have several deficiencies. Firstly, the role of ID1 in osimertinib resistance was only explored in H1975 and H1975/OR cells. It is hard to confirm whether ID1 is definitely related to drug resistance of osimertinib in other cell lines. Therefore, further studies is warranted to include more NSCLC cell lines harboring T790M mutation. Secondly, due to shortage of time and funding, several experiments were not investigated in the current study such as the correlation of ID1, EMT downstream signaling pathway and immune escape after osimertinib resistance. Furthermore, our study was only conducted in vitro, but in vivo experiments such as nude mouse xenograft model were not performed. Hence, the role of ID1 need to be carefully verified in animal experiments. Last but not the least, our study did not involved in human specimens, which made the experiment results a little pale as compared to other similar researches. As a consequence, further exploration of ID1 in T790M positive lung cells is needed.

In conclusion, our study reveal the mechanism of ID1 mediated resistance to osimertinib in T790M positive non-small cell lung cancer through EMT, which may provide new ideas and methods for clinical treatment of EGFR mutated NSCLC after osimertinib resistance. Further studies are needed to illustrate the deep mechanism of osimertinib resistance as first-line targeted therapy.

Declarations

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

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

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Authors' contributions

KJL, NXF and LGW conducted the experiment and participated in drafting and revising the manuscript. SYC did the statistical work. LMC and JJ designed and supervised the process of whole study. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work presented in the manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

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Figures

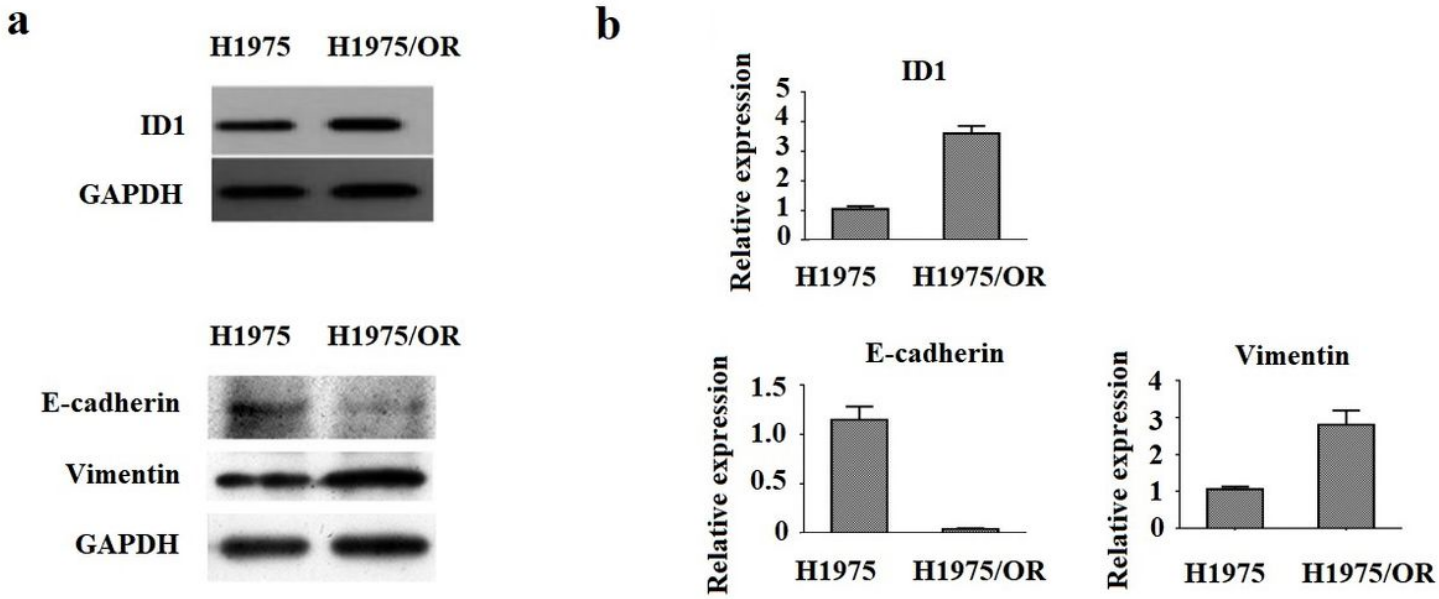
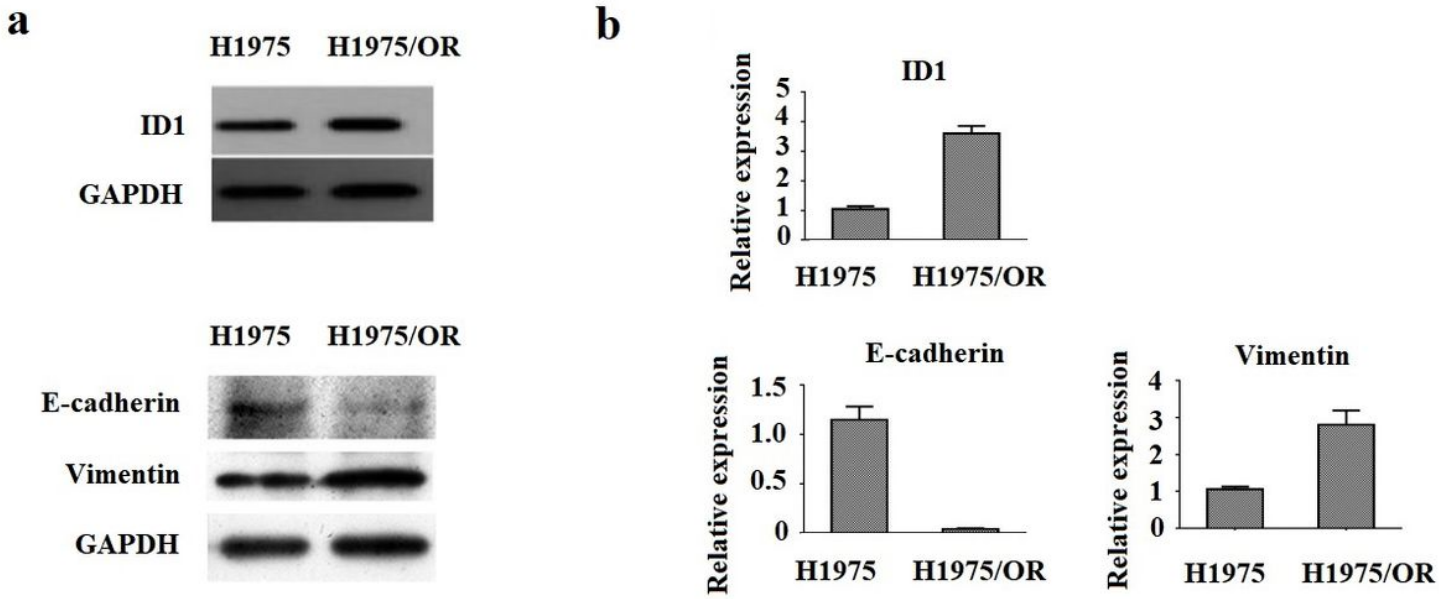


Figure 1

Western blot (a) and realtime PCR (b) were used to detect the expression of ID1, PD-L1, E-cadherin and vimentin in H1975 and H1975/OR cells. The expression levels of PD-L1 and E-cadherin were lower than those of sensitive cells, while the expression levels of ID1 and vimentin were higher than those of sensitive cells. A representative result is shown from at least three independent experiments.



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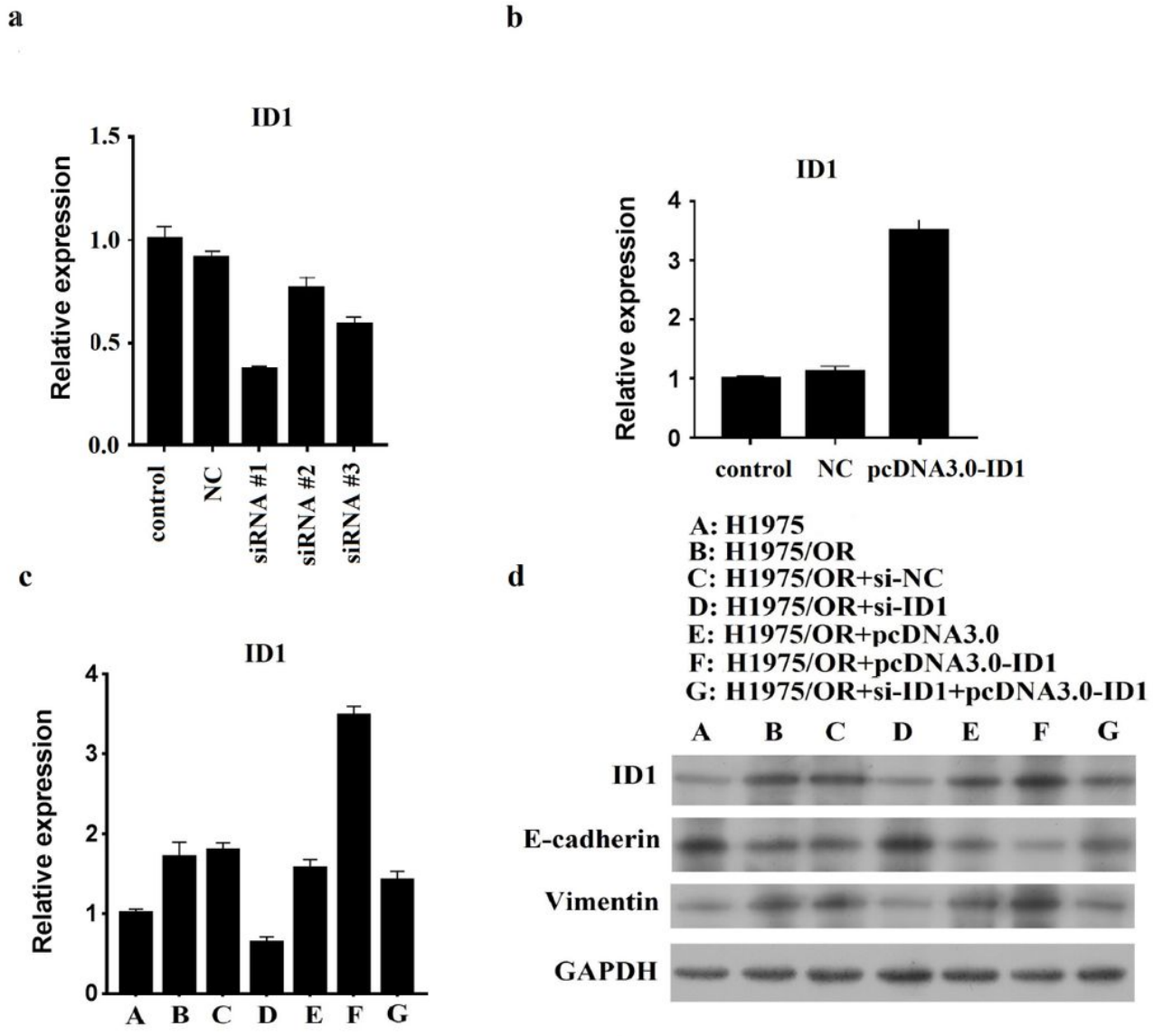
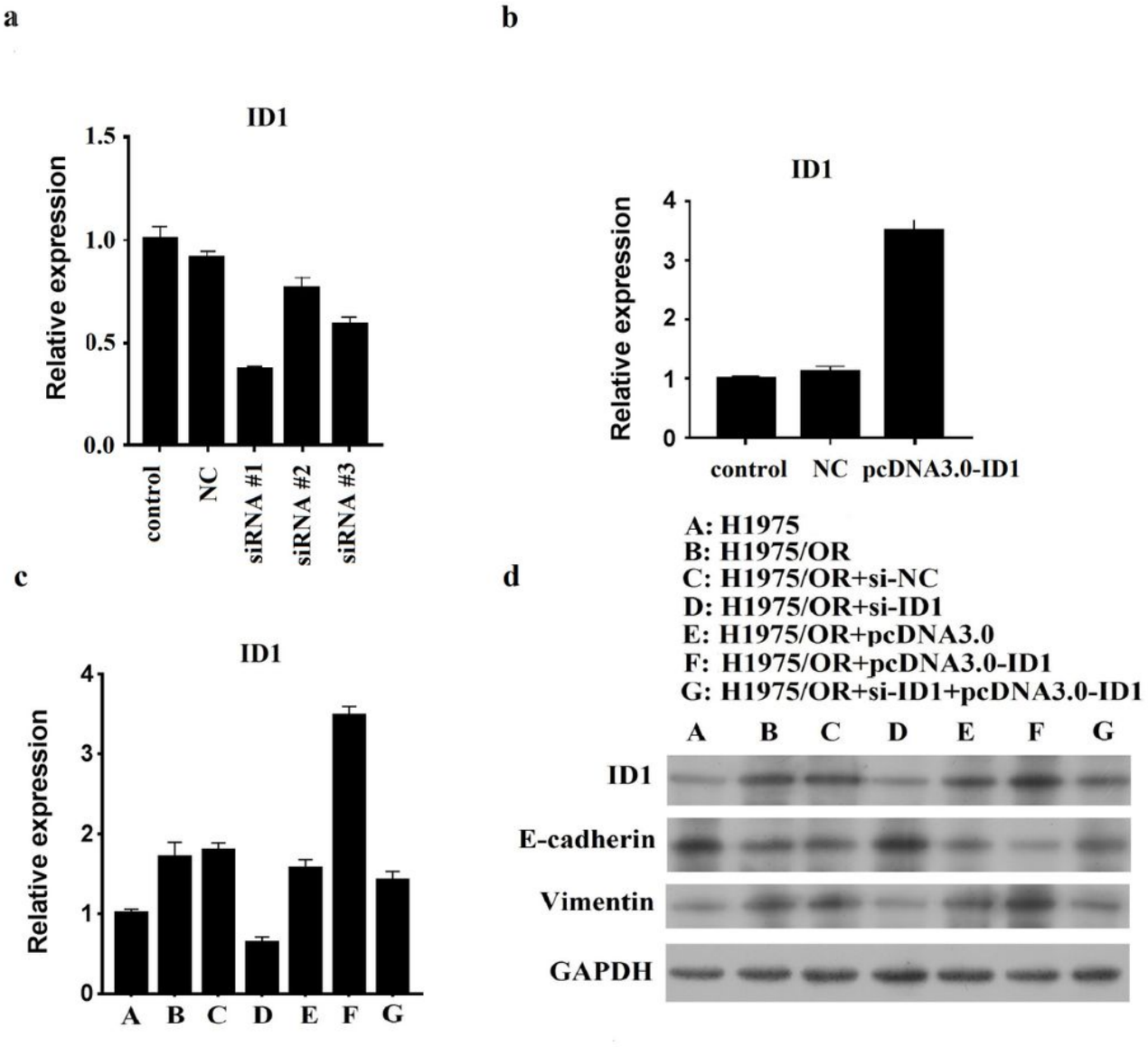


Figure 2

Gene silencing using siRNA targeting ID1 (a) and overexpression of ID1 by cloning the gene into pcDNA3.0 vector (b). Reverse transcription PCR and western Blot were used to confirmed the effect of ID1 silencing and overexpression on osimertinib resistant cells (c and d). Results showed that the expression of ID1 was closely related to E-cadherin and vimentin. Up and Down regulation of ID1 expression in H1975/OR cells could inhibit and promote the expression E-cadherin, respectively. Each point represents the mean ± SD for three independent determinations.



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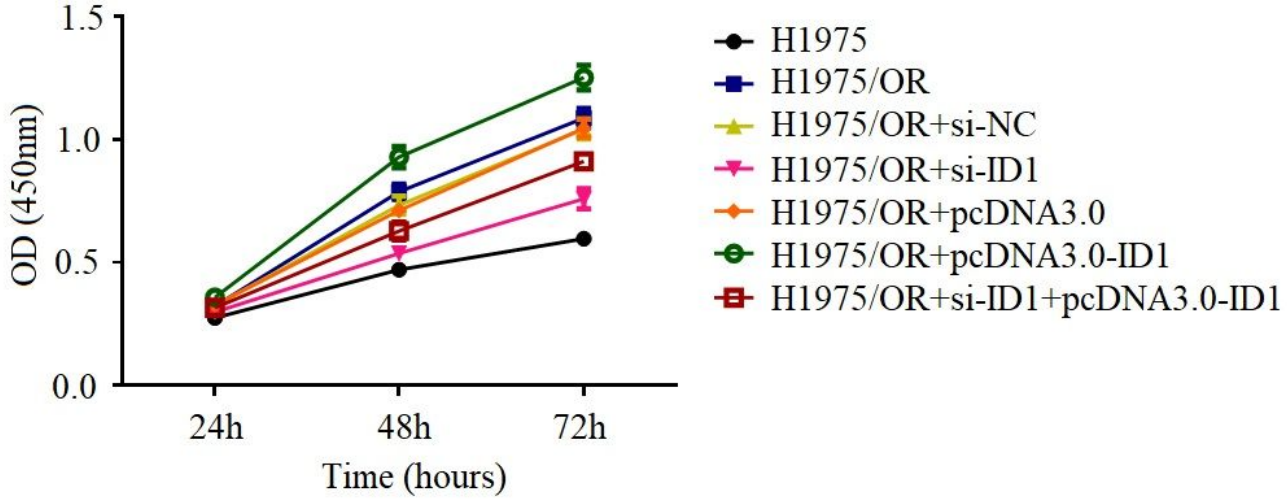
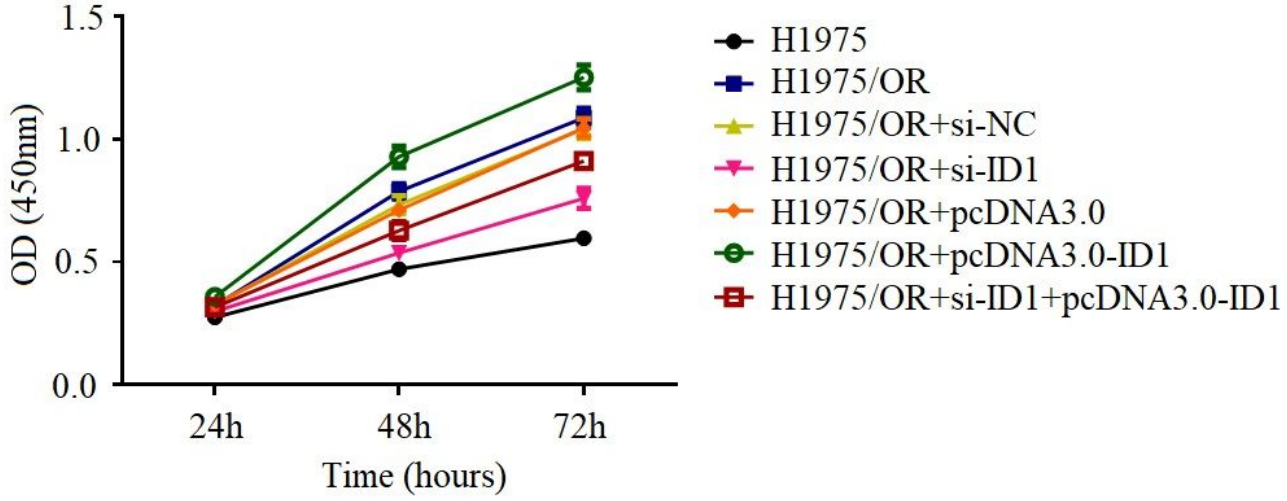


Figure 3

Cell proliferation assay was conducted to investigate the effect of ID1 on osimertinib sensitive and resistant cells. Results showed that downregulating ID1 could restore drug sensitivity in resistant cells, while overexpression of ID1 could further escalate osimertinib resistance. The values represent mean ± SD from three independent experiments.



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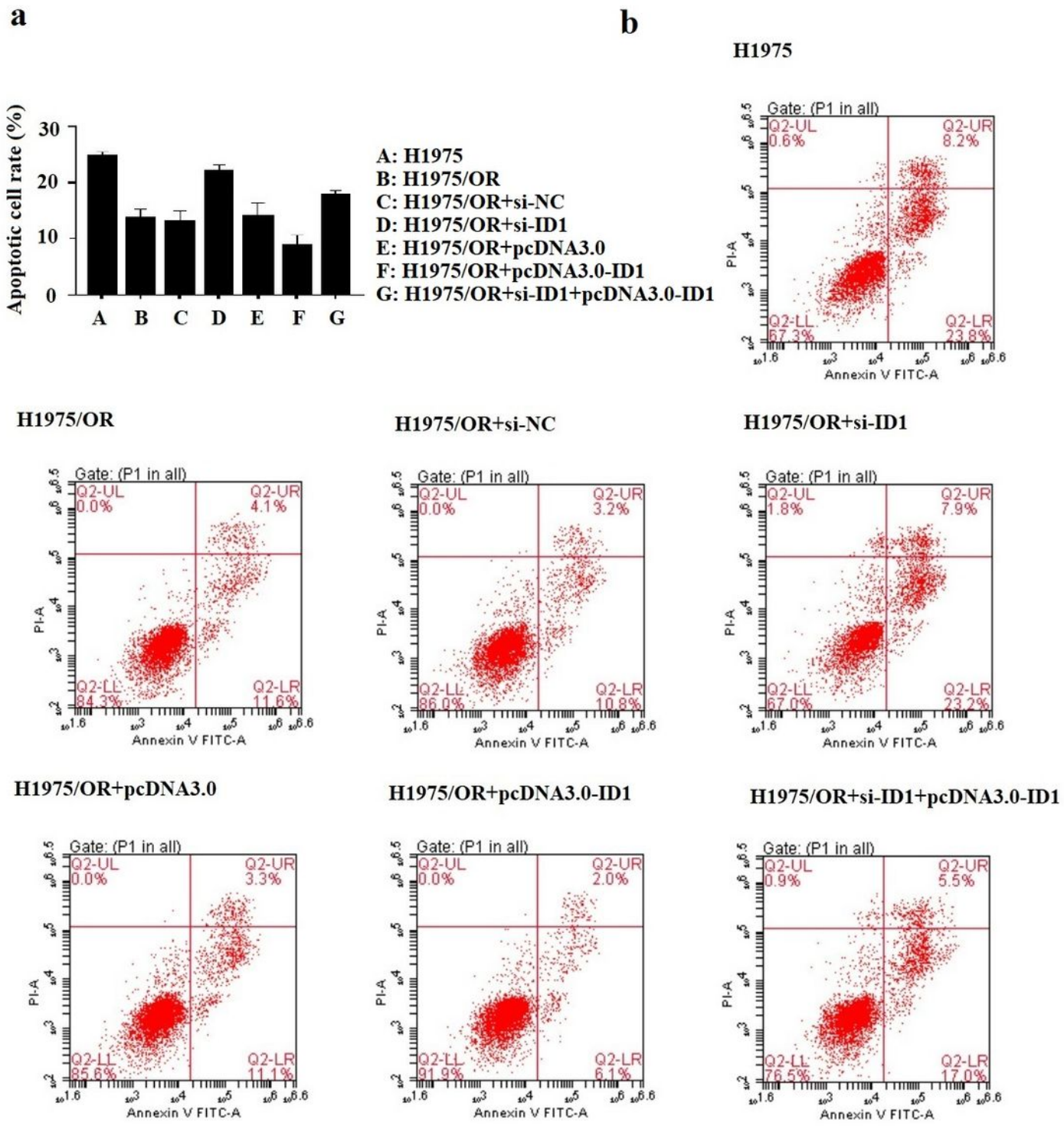

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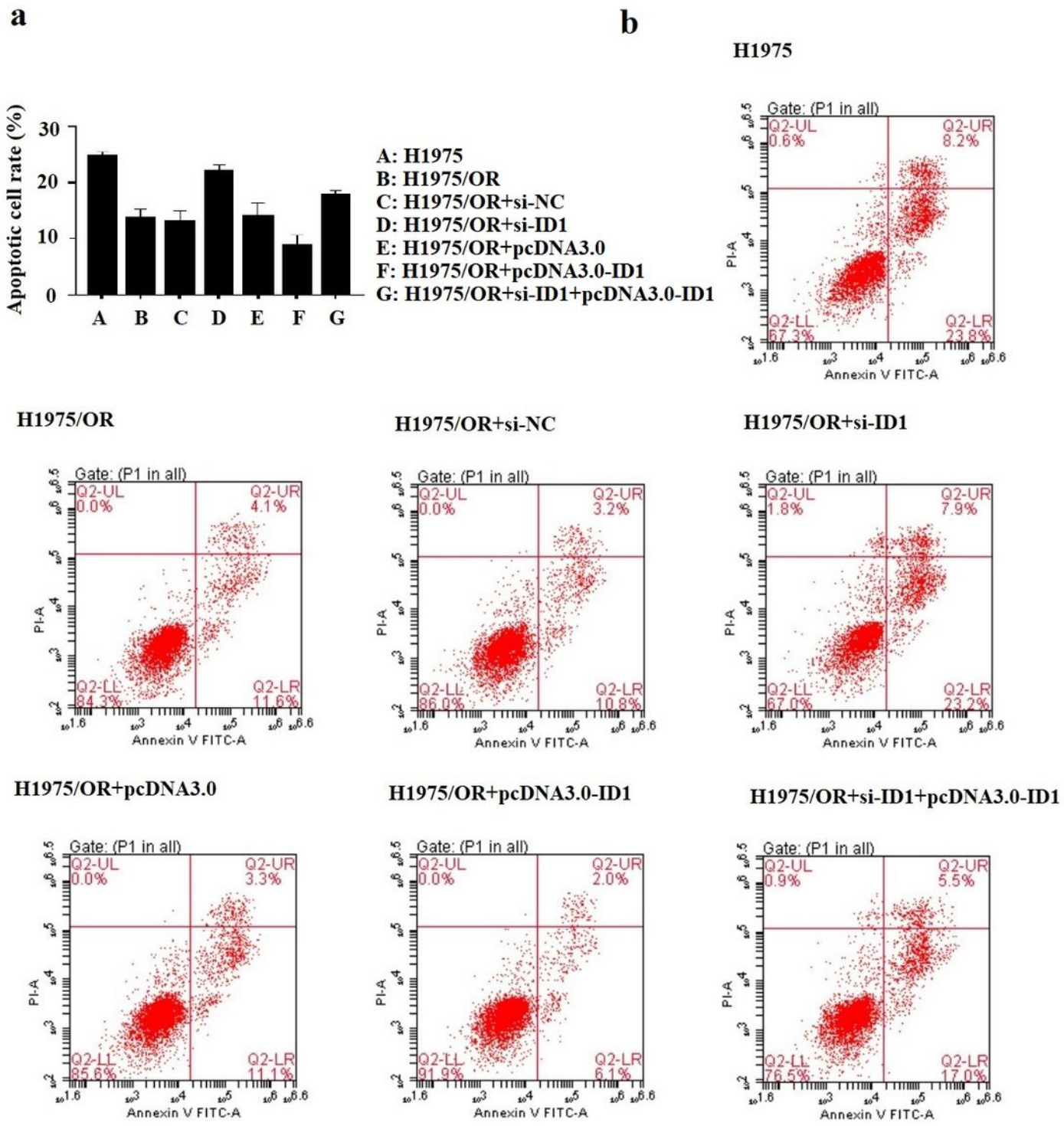
Flow cytometry analysis was used to detect apoptosis of osimertinib sensitive and resistant cells. The apoptosis rate in H1975/OR cells was significantly decreased as compared with that in osimertinib sensitive cells. After ID1 silencing, the apoptosis rate was increased as compared to H1975/OR cells, whereas in ID1 overexpressed cells, the apoptosis rate was decreased. A representative result is shown from at least three independent experiments.



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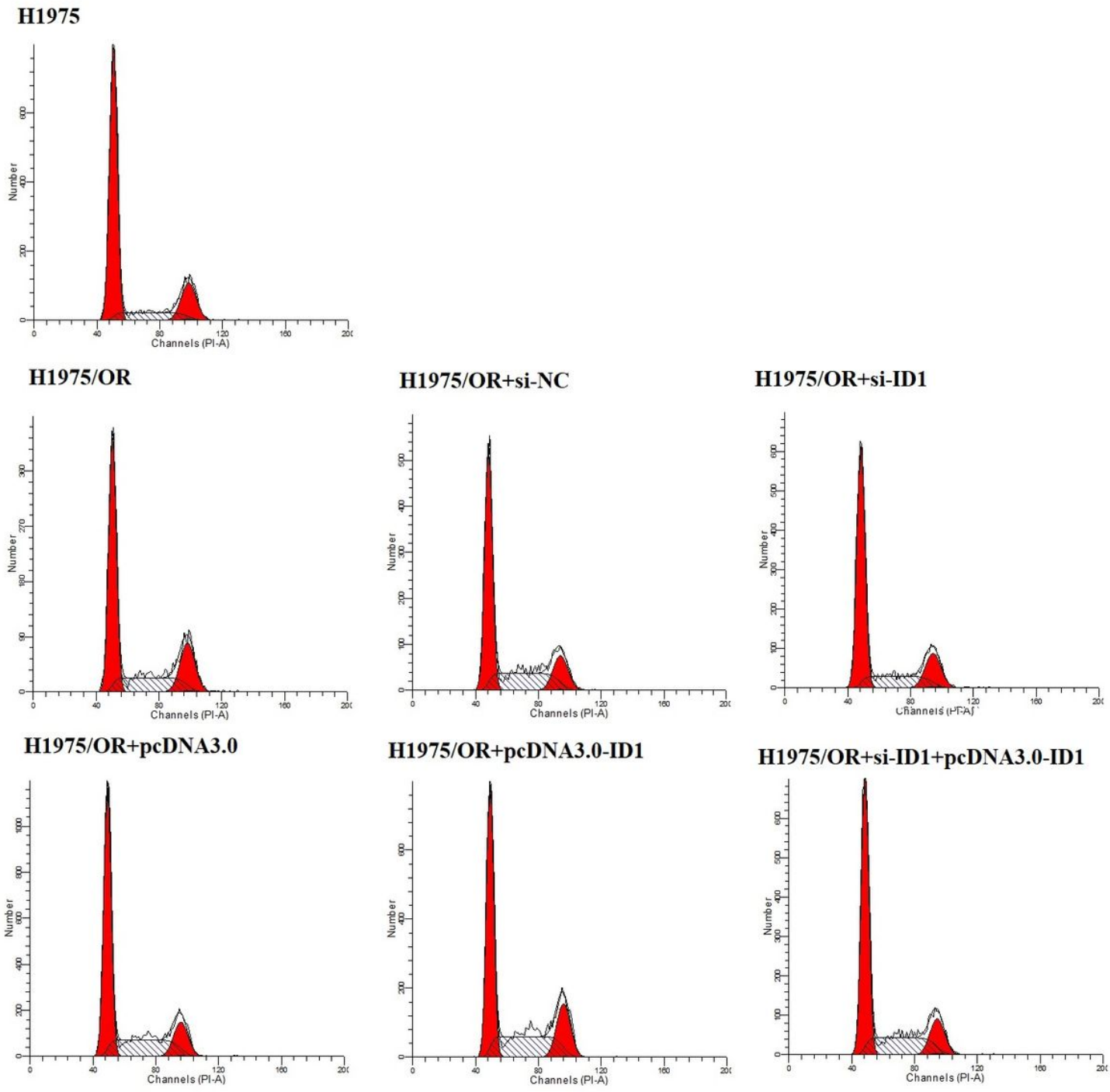

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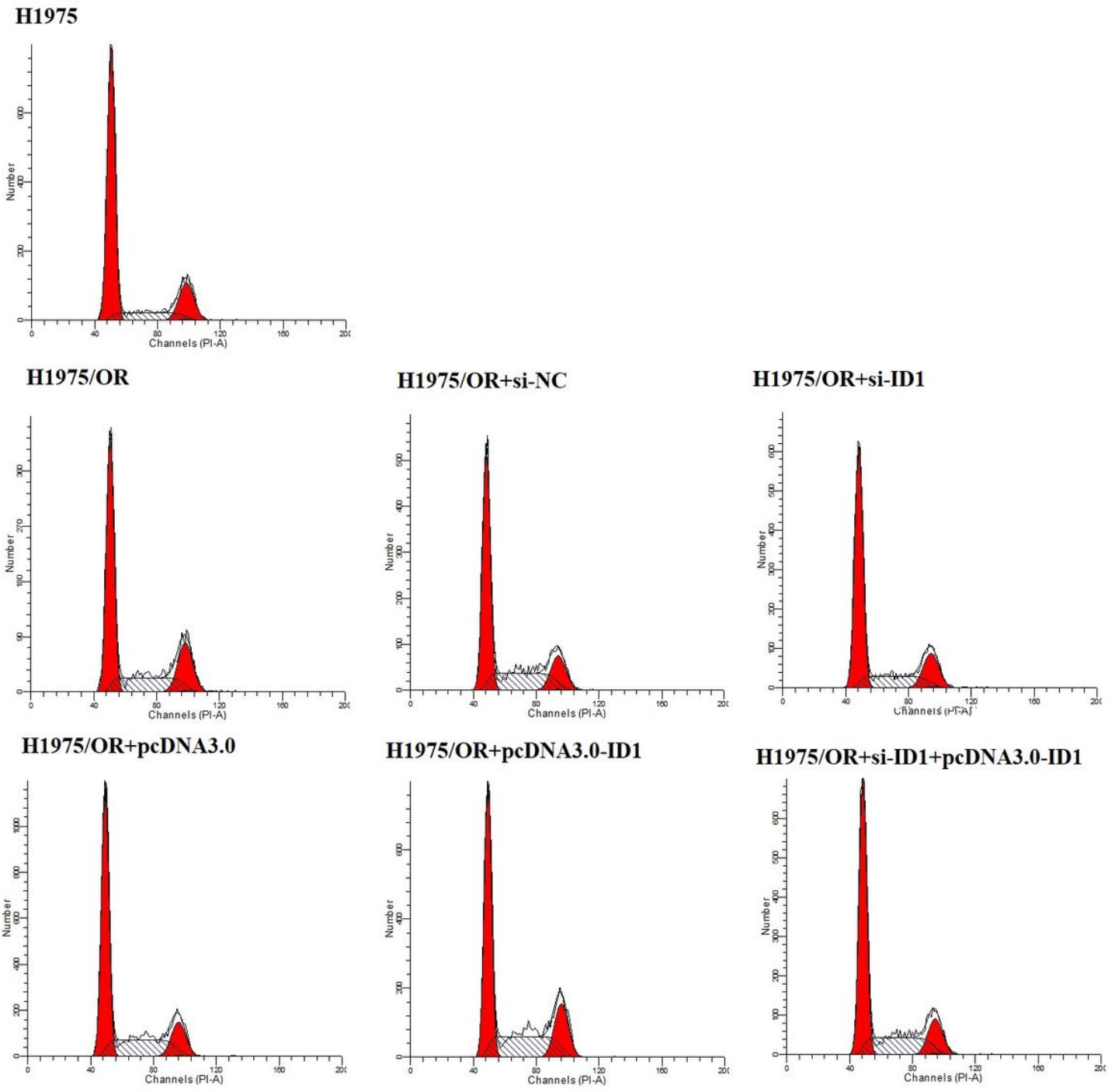

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