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RESEARCH ARTICLE

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Ginsenoside Rg3 attenuates the osimertinib resistance by reducing the stemness of non-small cell lung cancer cells

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Abstract

In the present study, we found that Ginsenoside Rg3 attenuated the stemness of non-small cell lung cancer (NSCLC) cells, evident by decreasing spheroid formation ability, ALDH1 activity and stemness marker expression. Furthermore, osimertinib-resistant NSCLC cells displayed a stronger stemness than the parental cells. Ginsenoside Rg3 reduced the stemness and osimertinib resistance of osimertinib-resistant cells. RNA-sequencing revealed that Hippo signaling was shown on the top of the most upregulated pathways regulated by Ginsenoside Rg3 in NSCLC cells, and YAP/TAZ expression was suppressed by Ginsenoside Rg3. Notably, the inhibitor of Hippo signaling attenuated the effects of Ginsenoside Rg3 on the stemness of NSCLC cells. Therefore, Ginsenoside Rg3 attenuates the osimertinib resistance of NSCLC cells via suppressing the stemness, which is dependent on Hippo pathway.

KEYWORDS

Ginsenoside Rg3, osimertinib, non-small cell lung cancer, Hippo

1 | INTRODUCTION

Whether cancer incidence or mortality, lung cancer ranks the first, among which non-small cell lung cancer (NSCLC) accounts for about 80%-85% of lung cancer, and about 30%-40% of NSCLC patients are diagnosed as advanced.[1] Advanced NSCLC seriously endangers the lives and health of residents, emphasizing the urgent need to find novel markers for diagnosing and treating NSCLC patients.

Targeted therapeutic drugs, epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), have been used in NSCLC patients with EGFR gene mutations with strong inhibition of tumor growth and less side effects.[2] The first-generation EGFR-TKIs, including gefitinib, erlotinib, and ektinib have been confirmed in many large international multicenter phase III clinical studies and on the market as the first-line treatment of advanced NSCLC with EGFR gene mutation, however, after the treatment with the first generation of EGFR-TKIs, most of the patients with effective initial treatment develop again after 10 months of median remission.[3] Osimertinib, the third-generation of EGFR-TKI, is developed to effectively overcome the resistance of the first-generation of EGFR-TKIs with T790M mutation.[4] However, the resistance of osimertinib has been found in many patients, especially the patients with C797S mutation.[5] These results suggest that EGFR-TKIs might not enough for NSCLC patient treatment.

Cancer stem cells (CSCs) have been regarded as the root of tumor progression and drug resistance,[6] however, there is no effective drug targeting CSCs yet. Ginsenoside is an important active ingredient of ginseng and it has been used as an anticancer Chinese medicine in the treatment of malignant tumors such as melanoma and prostate cancer.[7,8] Ginsenoside Rg3, the main component of Ginsenoside, has been shown to inhibit tumor progression and chemoresistance, for example, Ginsenoside Rg3 attenuates temozolomide resistance and epithelial-mesenchymal transition (EMT) progression in glioblastoma[9]; Ginsenoside Rg3 inhibits prostate cancer cell proliferation through inducing cell cycle arrest.[10] Recent studies showed that Ginsenoside Rg3 could target CSCs in colorectal cancer[11] and Ginsenoside Rg3 could enhance cisplatin sensitivity via blocking EMT process and stemness in lung cancer cells.[12] However, it is unclear whether Ginsenoside Rg3 could target CSCs and reverse osimertinib resistance in NSCLC.

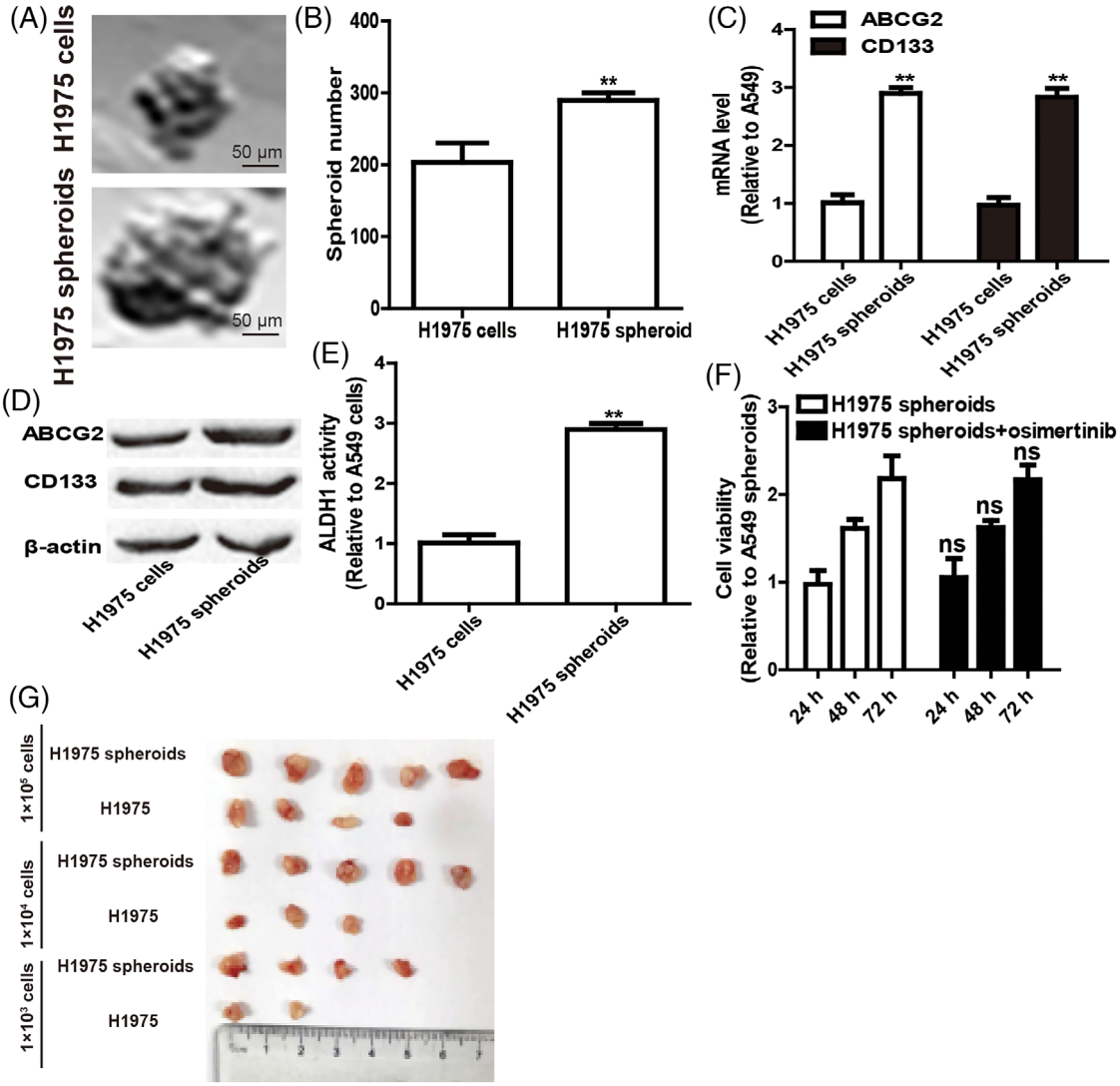

FIGURE 1 Non-adherent H1975 spheroids exhibits a higher stemness and resistance to osimertinib. A and B, The spheroid formation ability was evaluated in H1975 cells and spheroids via measuring the spheroid size, A, and number, B. C and D, The expression of lung cancer stemness markers (ABCG2 and CD133) was examined in H1975 cells and spheroids. E, ALDH1 activity was detected in H1975 cells and spheroids. F, H1975 spheroids were treated with or without osimertinib and followed by detecting cell viability via CCK8 assay. G, The tumorigenic rate of H1975 cells and H1975 spheroids was evaluated via the subcutaneous tumorigenesis experiment. Data were presented as the mean ± SD, $^{**}P < .01$ vs H1975 cells or control [Color figure can be viewed at wileyonlinelibrary.com]

In the present study, we collected non-adherent spheroids formed by NSCLCs and constructed osimertinib-resistant NSCLC cells. Functional experiments showed that Ginsenoside Rg3 attenuated the stemness of NSCLC cells, spheroids and osimertinib-resistant NSCLC cells. Importantly, Ginsenoside Rg3 reversed osimertinib resistance of NSCLC cells. RNA-sequencing analysis revealed that Ginsenoside Rg3 activated Hippo pathway and the expression of YAP/TAZ, the key executors of Hippo signaling, was suppressed by Ginsenoside Rg3. Therefore, our results demonstrate that Ginsenoside Rg3 attenuates the osimertinib resistance of NSCLC cells via suppressing the stemness, which might be involved in Hippo signaling.

2 | MATERIAL AND METHODS

2.1 | Cell culture and reagents

Human NSCLC cell line H1975 (L858R/T790M) cells were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Osimertinib-resistant H1975 cells (H1975-OR) were constructed by culturing with 15 nM osimertinib (Selleck Chemicals, Houston, Texas) for 2 weeks and then cultured with 50 nM osimertinib for another month by referring to the previous study with some subtle changes.[13] The remaining clones were selected and expanded with 1 nM osimertinib for the long time. The resistance index was confirmed

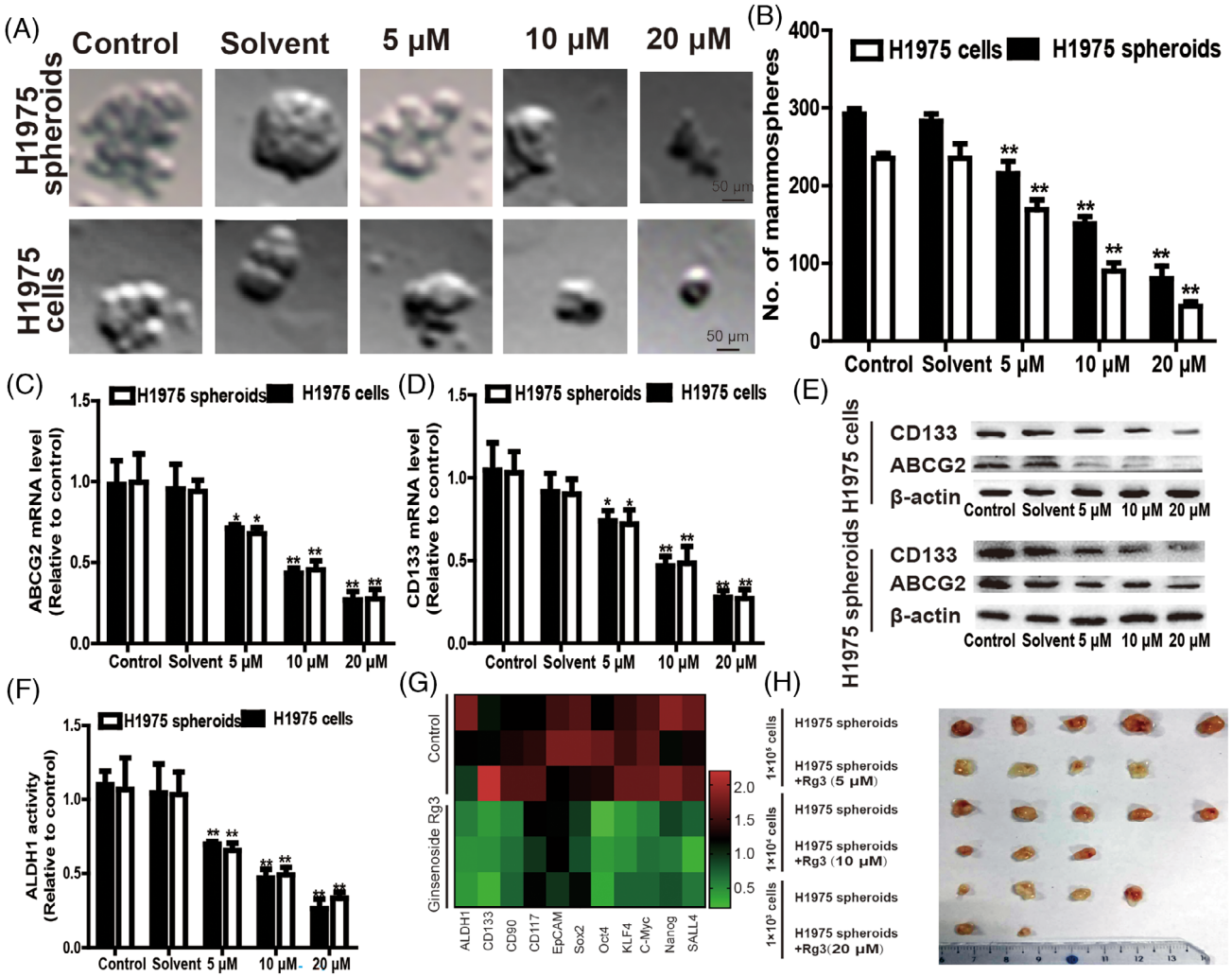

FIGURE 2 Ginsenoside Rg3 attenuates the stemness of H1975 spheroids and cells. A and B, The spheroid formation ability was determined in H1975 cells and spheroids treated with different concentration of Ginsenoside Rg3. C and D, The mRNA levels of CD133 and ABCG2 were measured in the cells and spheroids described in, A. E, The protein levels of CD133 and ABCG2 were examined in the cells and spheroids depicted in, (A). F, ALDH1 activity was detected in the cells and spheroids described in, A. G, The expression of lung cancer stemness-related markers was determined in H1975 cells with or without Ginsenoside Rg3 treatment via RNA-sequencing analysis, denoted as heatmap. H, The tumorigenic rate of H1975 spheroids with or without Ginsenoside Rg3 treatment was determined. Data were presented as the mean ± SD, $^{*}P < .05$, $^{**}P < .01$ vs control [Color figure can be viewed at wileyonlinelibrary.com]

before using. All the above-mentioned cell lines were cultured in 1640 medium (Thermo Fisher Scientific, Waltham, Massachusetts), supplemented with 10% FBS (fetal bovine serum, Thermo Fisher Scientific) under humidified atmosphere with 5% CO_2 at 37°C. XMU-MP-1, the inhibitor of mammalian STE20-like protein kinase 1/2 (MST1/2), was purchased from MedChem Express (Monmouth Junction, New Jersey). (20R)Ginsenoside Rg3 (Cat # 9021) was purchased from Selleck.cn.

2.2 | Quantitative real-time PCR

Total RNA was extracted using TRIeasy total RNA extraction reagent (YEASEN, Shanghai, China). Then cDNA was reversely synthesized and quantitative real-time PCR (qRT-PCR) was performed using Hifair III One Step RT-qPCR Probe Kit (YEASEN) according to the standard procedure. qRT-PCR was performed on the StepOne Plus PCR system and GAPDH was served as an internal reference. $2^{-\triangle\triangle ct}$ method was performed to analyze the relative expression levels of transcripts.

2.3 | Western blot

Cells were lysed and whole protein was extracted using RIPA lysis buffer (YEASEN). BCA Protein Quantification Kit (YEASEN) was used to measure the protein concentration. Then 30 μg of protein was separated by SDS-PAGE and transferred onto PVDF membranes (Bio-Rad, Hercules, California) which were followed by incubating with nonfat milk at room temperature for 1.5 hours. Then the membranes were incubated with the corresponding primary antibodies overnight

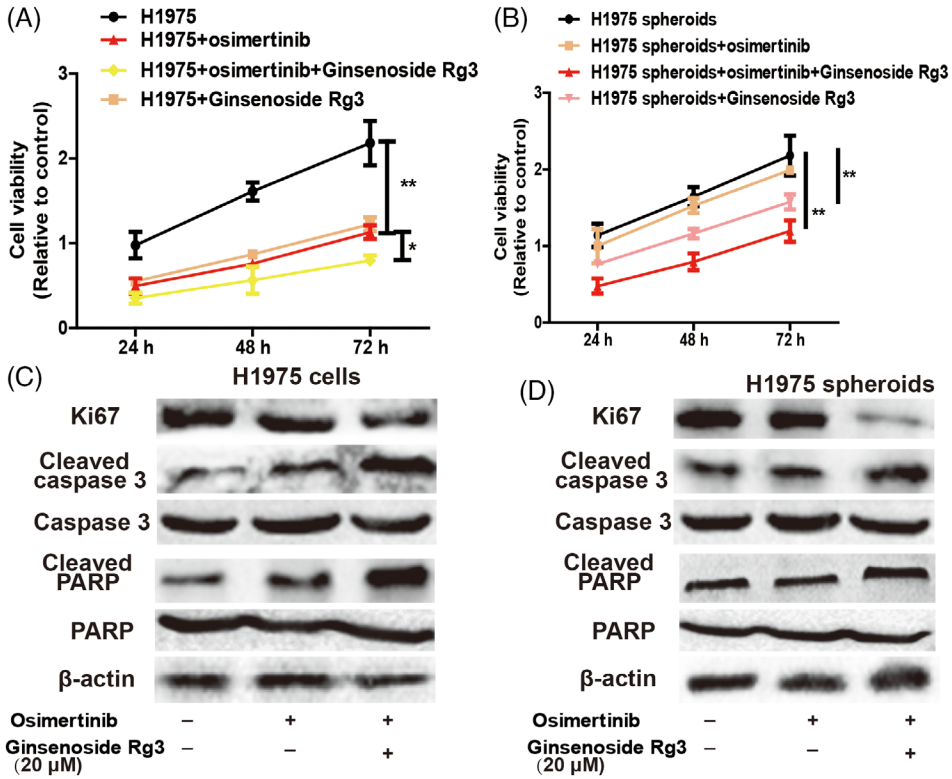

FIGURE 3 Ginsenoside Rg3 attenuates osimertinib resistance of H1975 spheroids and enhances osimertinib sensitivity of H1975 cells. A, H1975 spheroids were treated with osimertinib as well as Ginsenoside Rg3 or not, and followed by detecting cell viability. B, H1975 cells were treated with osimertinib as well as Ginsenoside Rg3 or not, and followed by detecting cell viability. C and D, The expression of proliferation marker (Ki67) and apoptotic executors (Cleaved caspase3 and cleaved PARP) was examined in the cells and spheroids described in, A and B. Data were presented as the mean ± SD, $^{*}P < .05$, $^{**}P < .01$ vs control [Color figure can be viewed at wileyonlinelibrary.com]

at 4°C and followed by incubating with the secondary antibodies for 1 hour at room temperature. SuperSignal SuperDura Extended Duration Substrate (YEASEN) was used to detect the signal on Tanon 5200 machine (Tanon, Shanghai, China). The primary antibodies against ABCG2 (Cat # 27286-1-AP, 1:1000), CD133 (Cat # 66666-1-Ig, 1:1000) and β-actin (Cat # 60008-1-Ig, 1:3000) were purchased from Proteintech (Wuhan, China). The primary antibodies against Ki67 (Cat # ab16667, 1:5000), YAP (ab52771, 1:3000), p-YAP (ER1675Y, 1:1000), Cleaved caspase 3 (Cat # ab2302, 1:2000), caspase 3 (Cat # ab197202, 1:3000), Cleaved PARP (Cat # ab32064, 1:2000) and PARP (Cat # ab74290, 1:3000) were purchased from Abcam (Cambridge, Massachusetts).

2.4 | RNA sequencing and data analysis

RNA sequencing and related data analysis were performed by Novogene (Beijing, China).

2.5 | Spheroid formation assay

H1975 and H1975-OR cells were cultured in ultra-low attachment 24-well plates (Corning, Union City, California) at 1000 cells/well with DMEM/F12 medium supplemented with 1 × B27 (Sigma, St. Louis, Missouri), 20 ng/mL bFGF (Sigma), 20 ng/mL EGF (Sigma) and antibiotics at 37°C under a 5% humidified CO_2 atmosphere. After 10 days, the number and size of spheroid were evaluated and quantified under a microscope. For analysis on H1975 cell spheroids, the spheroids were digested and re-seeded into the plates, followed by Ginsenoside Rg3 treatment and performing the functional experiments.

2.6 | ALDH1 activity assay

ALDH1 activity was determined using ALDEFLUOR Kit (Cat # KA3742, Stemcell Technologies, Vancouver, BC, Canada) following the manufacturer's recommendation.

2.7 | CCK8 assay

Tumor resistance to drug was assessed by Cell Counting Kit-8 (CCK8) (Gilson, Middleton, Wisconsin) assay. Cells were plated in 96-well plate at the density of 3000 cells/well, and incubated with osimertinib, Ginsenoside Rg3, osimertinib, and Ginsenoside Rg3. The number of viable cells was evaluated by CCK8, and 600 nm absorbance was measured using a Microplate Reader (BIO-TEK).

2.8 | In vivo tumor initiation assays

All animal experiments were performed with the approval of Ethics Committee for Animal Experimentation of Dongguan People's

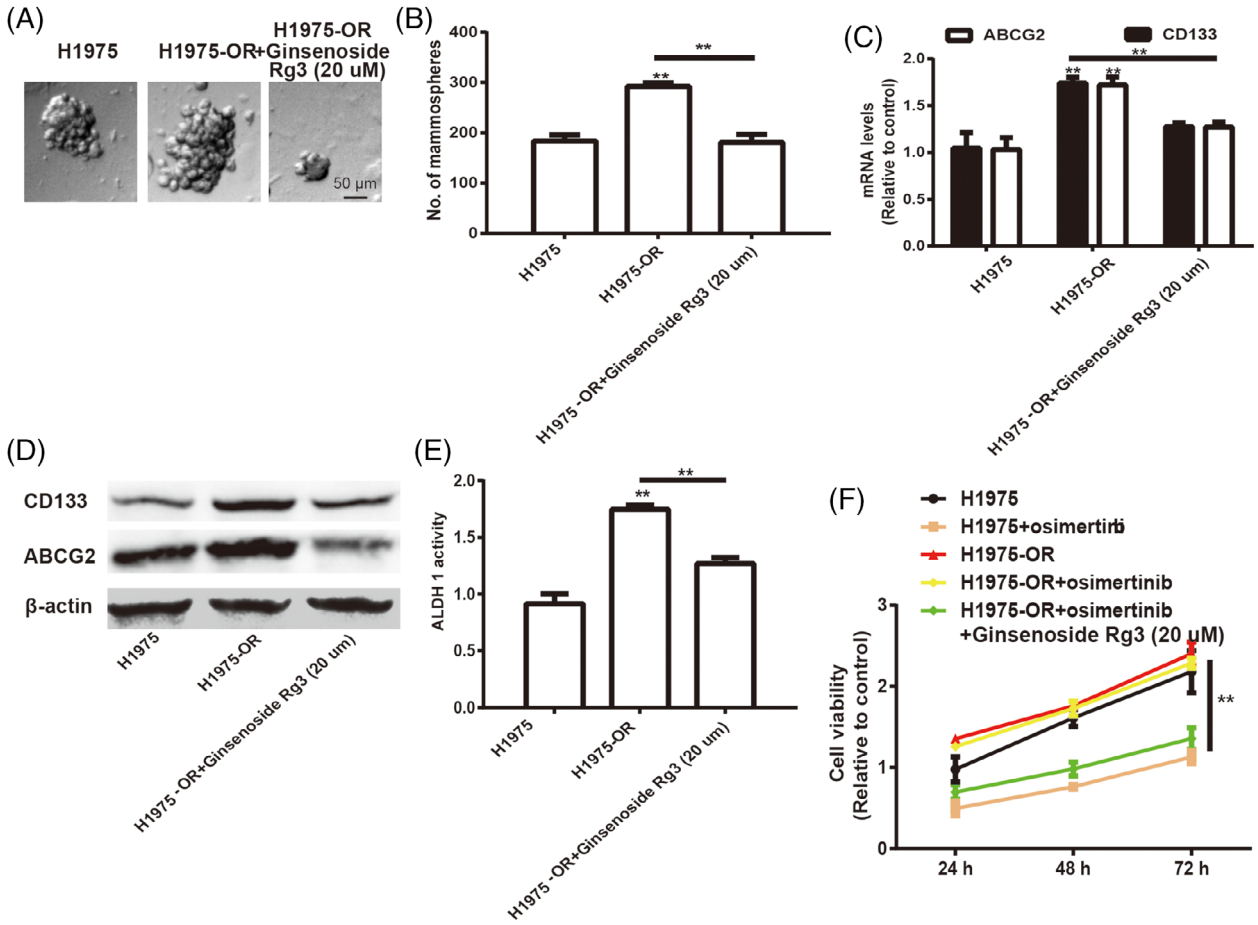

FIGURE 4 Ginsenoside Rg3 attenuates the stemness and resistance of osimertinib-resistant NSCLC cells. A and B, The spheroid formation ability was evaluated in H1975 cells and H1975-OR cells with or without Ginsenoside Rg3 treatment. C and D, The expression of CD133 and ABCG2 was examined in the cells described in, A. E, ALDH1 activity was determined in the cells depicted in, A. F, Cell viability was detected in the cells with different treatment as indicated. Data were presented as the mean ± SD, $^{**}P < .01$ vs control. NSCLC, non-small cell lung cancer [Color figure can be viewed at wileyonlinelibrary.com]

Hospital. Six weeks of athymic BALB/c nude mice were purchased from the Gempharmatech (Nanjing, China). For tumor-limiting dilution assays, tumor cells were mixed 1:1 with Matrigel matrix (BD Biosciences) and subcutaneously implanted in mice. After 12 days, all mice were killed and tumor tissues were collected and weighed. For analyzing of Ginsenoside Rg3 on the tumorigenic ability of H1975 spheroids, H1975 spheroids were co-cultured with Ginsenoside Rg3 for 72 hours before implanting in mice, the following process was the same with the above-mentioned process.

2.9 | Statistical analysis

All results were denoted as Mean ± SD and analyzed using Graphpad Prism (Version X; La Jolla, California). Student's *t* test was used to assess the differences between groups. $P < .05$ or less was considered statistically significant.

3 | RESULTS

3.1 | Non-adherent H1975 spheroids exhibits a higher stemness and resistance to osimertinib

We firstly collected the non-adherent H1975 cell spheroids, which were confirmed to hold CSC-related characteristics.[14] We found that H1975 cell spheroids indeed exhibited a higher stemness compared with the adherent H1975 cells, evident by the increased spheroid formation ability (Figure 1A,B), lung cancer stemness markers expression (Figure 1C,D) and ALDH1 activity (Figure 1E). Notably, H1975 spheroids displayed an osimertinib-resistant trait (Figure 1F). Consistently, the in vivo experiments showed that the tumor-initiating ability of H1975 spheroids was stronger than H1975 cells, which was evident by the increase of tumorigenic rate (5/5 vs 4/5 at the high density, 5/5 vs 3/5 at the medium density, and 4/5 vs 2/5 at the low density) (Figure 1G). Our results suggest that H1975 spheroids could be used to as a CSCs model of NSCLC cells.

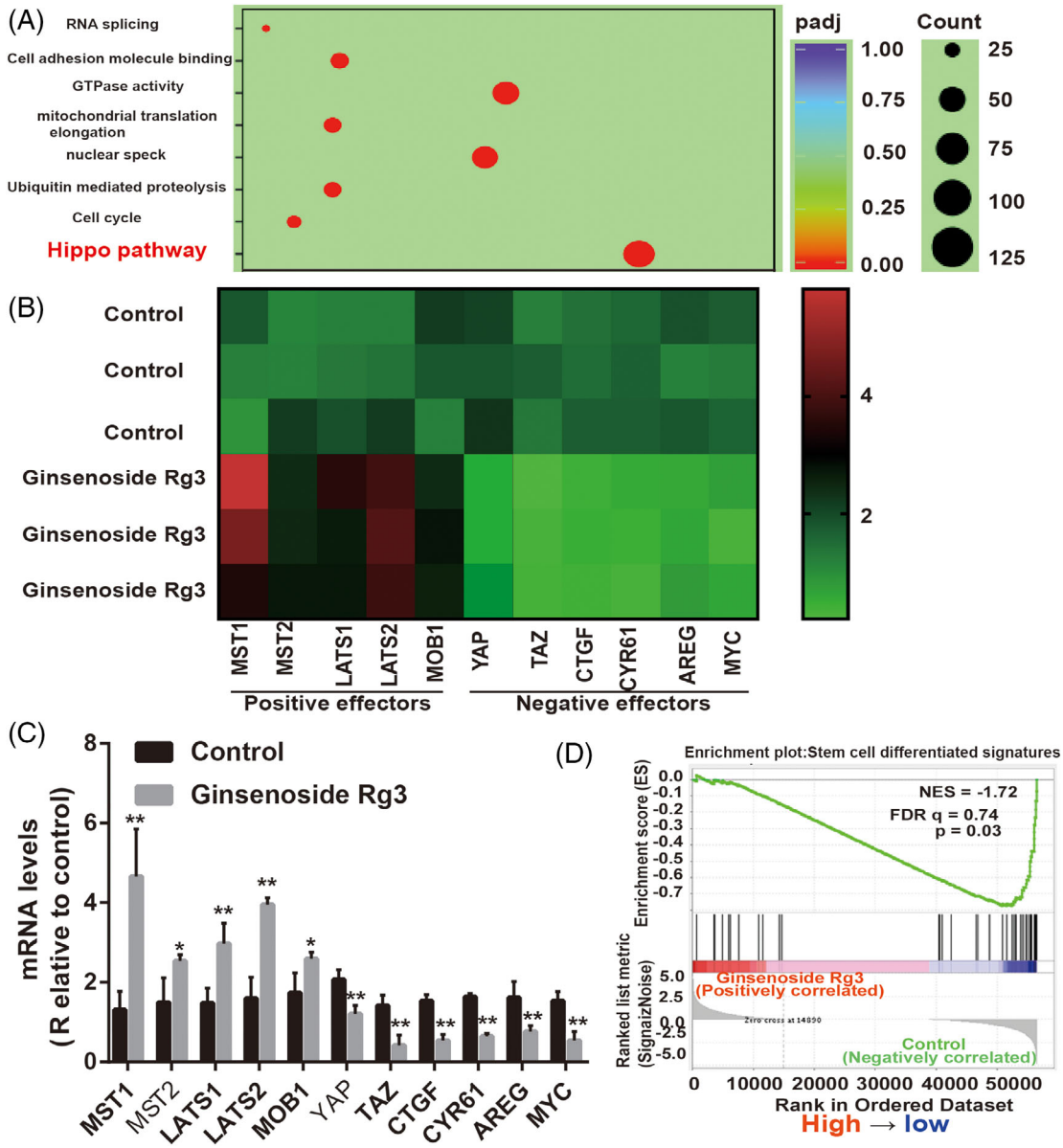

FIGURE 5 Ginsenoside Rg3 activates Hippo signaling in H1975 cells. A, KEGG pathway enrichment was performed in H1975 cells with or without Ginsenoside Rg3 treatment based on RNA-sequencing data. B, The expression of Hippo components was examined in H1975 cells with or without Ginsenoside Rg3 treatment based on RNA-sequencing data, shown as heatmap. C, The mRNA levels of Hippo components were determined in H1975 cells with or without Ginsenoside Rg3 treatment via qRT-PCR assay. D, GSEA were constructed in with or without Ginsenoside Rg3 treatment based on RNA-sequencing data. Data were presented as the mean ± SD, **P* < .05, ***P* < .01 vs control. GSEA, gene set enrichment analysis [Color figure can be viewed at wileyonlinelibrary.com]

3.2 | Ginsenoside Rg3 attenuates the stemness of H1975 spheroids and cells

Then we detected the effects of Ginsenoside Rg3 on the stemness of H1975 spheroids and cells. As expected, Ginsenoside Rg3 reduced the stemness of H1975 spheroids and cells in a concentration-dependent manner, characterized as the decreased spheroid formation capacity (Figure 2A,B), NSCLC stemness marker expression (Figure 2C-E) and ALDH1 activity (Figure 2F). Consistently, RNA-sequencing analysis showed that Ginsenoside Rg3 treatment decreased the expression of most of the lung cancer type-specific stemness-related markers (ALDH1, CD133, CD90, CD117, EpCAM, Sox2, Oct4, KLF4, C-Myc, Nanog, and SALL4) (Figure 2G). Importantly, the in vivo tumorigenic assay revealed that Ginsenoside Rg3 significantly attenuated the tumorigenic of H1975 spheroids, characterized the decrease of tumorigenic rate of cells (5/5 vs 4/5, 5/5 vs 3/5, and 4/5 vs 2/5) (Figure 2H).

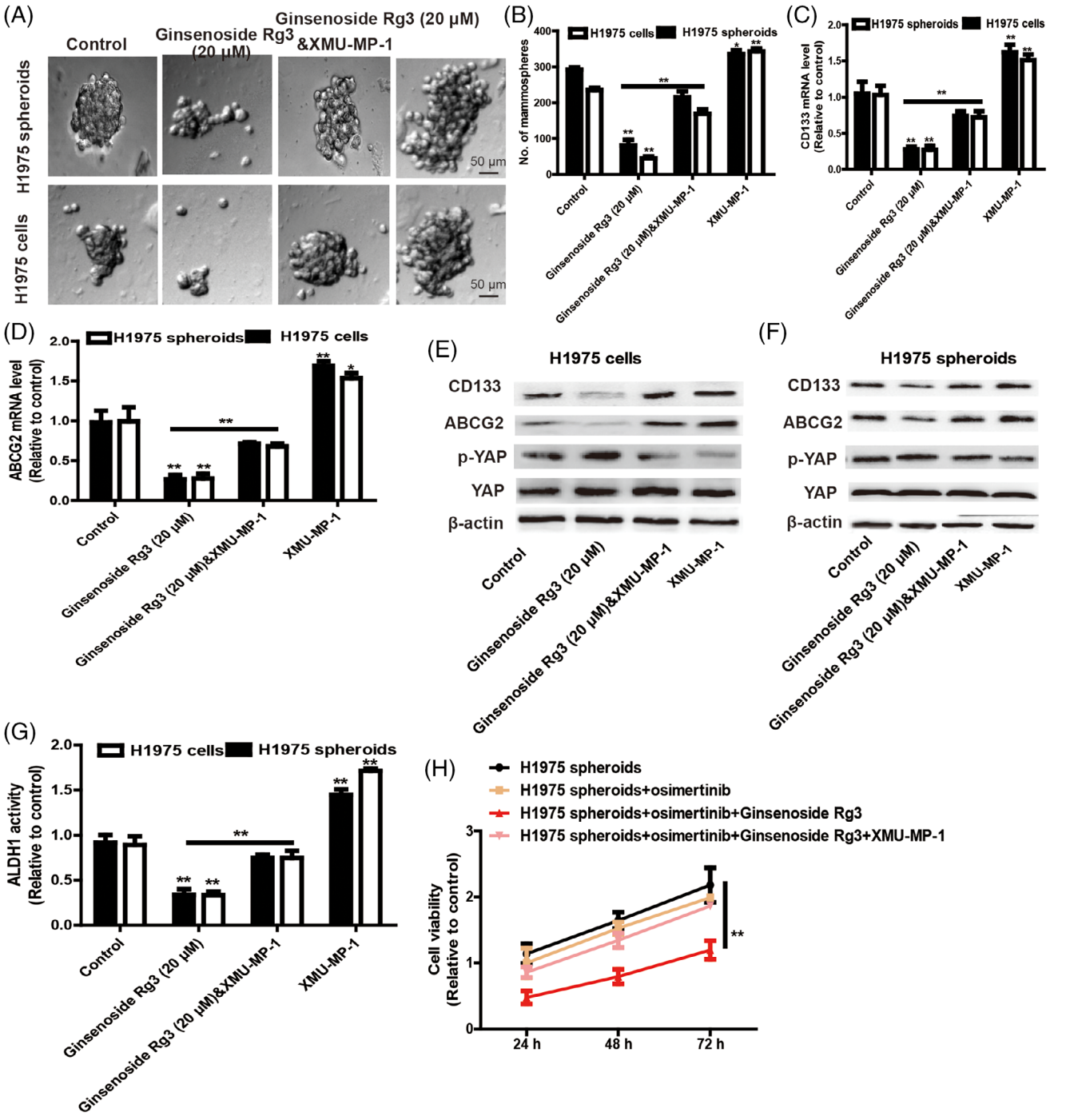

FIGURE 6 Ginsenoside Rg3 attenuates the stemness and osimertinib resistance of H1975 spheroids dependent on Hippo signaling. A and B, The spheroid formation capacity was evaluated in H1975 cells and spheroids with or without Ginsenoside Rg3 treatment plus XMU-MP-1 or not. C and D, The mRNA levels of CD133 and ABCG2 were examined in the cells and spheroids described in, A and B. E and F, The protein levels of CD133 and ABCG2 were detected in the cells and spheroids depicted in, A and B. G, ALDH1 activity was determined in the cells and spheroids described in, A and B. H, H1975 spheroids were treated with osimertinib as well as Ginsenoside Rg3 plus XMU-MP-1 or not, and followed by detecting cell viability. Data were presented as the mean ± SD, $^{**}P < .01$ vs control [Color figure can be viewed at wileyonlinelibrary.com]

3.3 | Ginsenoside Rg3 attenuates osimertinib resistance of H1975 spheroids and enhances osimertinib sensitivity of H1975 cells

As CSCs contributed to drug resistance, we further detected the effects of Ginsenoside Rg3 on the osimertinib sensitivity of H1975 spheroids and cells. Cell viability analysis showed that Ginsenoside Rg3 attenuated osimertinib resistance of H1975 spheroids (Figure 3A) and enhanced osimertinib sensitivity of H1975 cells (Figure 3B). Additionally, the expression of proliferation marker (Ki67) and apoptotic executors (Cleaved caspase 3 and cleaved PARP) were examined. As shown in Figure 3C,D, Ki67 expression was decreased in H1975

spheroids and cells by Ginsenoside Rg3 treatment, and the expression of cleaved caspase 3 and cleaved PARP was increased. Collectively, these results suggest that Ginsenoside Rg3 attenuates the stemness and thus the osimertinib resistance of H1975 cells.

3.4 | Ginsenoside Rg3 attenuates the stemness and resistance of osimertinib-resistant NSCLC cells

We constructed osimertinib-resistant H1975 cells (H1975-OR) and found that H1975-OR cells indeed exhibited a stronger stemness than the parental H1975 cells (Figure 4A-E). Furthermore, Ginsenoside Rg3 attenuated the stemness of H1975-OR cells, evident by decreasing the spheroid formation ability, stemness marker expression and ALDH1 activity (Figure 4A-E). Importantly, Ginsenoside Rg3 reduced the osimertinib resistance of H1975-OR cells (Figure 4F).

3.5 | Ginsenoside Rg3 activates Hippo signaling in H1975 cells

Then we continued to explore the mechanism by which Ginsenoside Rg3 regulates the stemness of H1975 cells. RNA-sequencing data revealed that Hippo pathway was shown on the top of the activated pathways (Figure 5A). Additionally, the expression of the activating components of Hippo pathway, such as MST1/2 and LATS1/2, was increased by Ginsenoside Rg3 treatment, while the negative executor or effector expression was decreased (Figure 5B). qRT-PCR analysis exhibited a consistent result (Figure 5C). Further gene set enrichment analysis (GSEA) of this data set revealed that positive enrichment of stem cell differentiated signatures was observed in H1975 cells with Ginsenoside Rg3 treatment (Figure 5D).

3.6 | Ginsenoside Rg3 attenuates the stemness and osimertinib resistance of H1975 spheroids dependent on Hippo signaling

Finally, we investigated whether Ginsenoside Rg3 attenuated the stemness through activating Hippo pathway. XMU-MP-1, the inhibitor of mammalian STE20-like protein kinase 1/2 (MST1/2), was used to inhibit Hippo pathway. As expected, XMU-MP-1 attenuated the inhibitory effects of Ginsenoside Rg3 on H1975 spheroid stemness, evident as the increased spheroid formation ability (Figure 6A,B), expression of stemness markers (Figure 6C-F) and ALDH1 activity (Figure 6G). The attenuative effects on osimertinib resistance of H1975 spheroids were rescued by XMU-MP-1 (Figure 6H). Indeed, XMU-MP-1 decreased p-YAP level, confirming its inhibition efficiency (Figure 6E,F). Therefore, our results demonstrate that Ginsenoside Rg3 attenuates the stemness and osimertinib resistance of NSCLC cells by activating Hippo signaling.

4 | DISCUSSION

Osimertinib, as the third representative of skin growth factor receptor (EGFR) TKI, has been widely used in the treatment of advanced NSCLC with positive EGFR-T790M mutation after first or second generation of EGFR-TKI resistance.[15] However, it is the same as the first or second generation of EGFR-TKI with inevitable drug resistance. The mechanism of drug resistance is complex, including the EGFR-dependent drug resistance, such as C797S mutation; and the EGFR-independent drug resistance, such as activation of various bypass pathways, transformation of small cell lung cancer (SCLC), and epithelial-mesenchymal transformation (EMT).[16] As CSCs have been regarded as the root of drug resistance, we focused on finding potential drugs that could reverse osimertinib resistance through targeting CSCs. Here, we found that Ginsenoside Rg3 could attenuate osimertinib resistance through activating Hippo pathway based on RNA-sequencing analysis combined with functional experiments. To our knowledge, this is the first study revealing the role of Ginsenoside Rg3 in NSCLC cells stemness.

Ginsenoside Rg3 has been shown to enhance chemotherapeutic sensitivity, for example, Ginsenoside Rg3 can specifically and efficiently reverse drug resistance in P388 leukemia cells and KBV20C cells[17]; Combined treatment with Ginsenoside Rg3 and docetaxel reduces the drug resistance of prostate cancer cells[18]; Ginsenoside Rg3 enhances TRAIL sensitivity via increasing TRAIL receptor DR5 expression in HepG2 cells.[19] However, the combined using of Ginsenoside Rg3 and osimertinib is never been reported. In the present work, we collected NSCLC cell spheroids, which had been shown to hold CSCs-related traits and found that these spheroids were resistant to osimertinib. Importantly, we found that Ginsenoside Rg3 attenuated resistance of H1975 spheroids. Additionally, Ginsenoside Rg3 attenuated the stemness of H1975 cells and spheroids, evident by decreasing the spheroid formation ability, stemness marker expression, and ALDH1 activity. Moreover, although H1975 cells do not hold C797S mutation, they could also become resistant to osimertinib with long-term culture, this indicates that the EGFR-independent drug resistance contribute to osimertinib resistance in H1975 cells. Notably, H1975-OR cells indeed exhibited a stronger stemness than the parental cells and a previous study has shown that tumor heterogeneity in NSCLC contributes to TKI resistance,[20] and the tumor heterogeneity could be led by CSCs. These results suggest that Ginsenoside Rg3 might target NSCLC CSCs. However, further in vivo experiments should be performed to confirm our conclusion. Additionally, because there are two kinds of isomers for Rg3 and (20R)Ginsenoside Rg3 was used in this research, the other isomer of Rg3, (20S)Ginsenoside Rg3, should be investigated in the future work.

Collectively, this work demonstrates that Ginsenoside Rg3 could be used as a potential osimertinib sensitizer in NSCLC patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

G.J. and Q.T. designed research; Q.T., S.L., Y.Z., and M.Y. analyzed data; Q.T., S.L., Y.Z., and K.L. performed experiments; H.Y. and C.L. contributed new reagents or analytic tools.

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