Genome-wide CRISPR screening identifies *CLDN1* as a central node in the anticancer mechanisms of berberine and as a therapeutic target in lung cancer

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Abstract. Berberine (BBR), a natural compound with diverse anticancer properties, exerts potent inhibitory effects on lung cancer cell proliferation. However, its particular molecular targets remain unknown. The present study aimed to identify the key genetic determinants that mediate the cellular response to BBR in lung cancer. To achieve this, genome-wide clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) knockout screening was conducted in A549 cells, followed by functional validation and mechanistic assays. In the study, the genome-wide CRISPR/Cas9 knockout screening in A549 lung cancer cells identified claudin-1 (*CLDN1*) as a critical modulator of BBR sensitivity. Notably, whilst *CLDN1* was enriched in the positive selection screen, its knockout markedly increased the sensitivity of A549 cells to BBR, thus leading to enhanced G1-phase arrest and reduced proliferation. These findings suggest that *CLDN1* could serve a dual role, promoting cellular resistance under selective pressure, while simultaneously demonstrating a therapeutic vulnerability when directly inhibited. Overall, the present study identified *CLDN1* as a key mediator of the anticancer effects of BBR, thus providing a foundation for its potential development as a therapeutic target to optimize lung cancer therapy.

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Abbreviations: BBR, berberine; *CLDN1*, claudin-1; CRISPR, clustered regularly interspaced short palindromic repeats; DARTS, drug affinity responsive target stability; sgRNA, single-guide RNA; KO, knockout; TCM, traditional Chinese medicine

Key words: BBR, *CLDN1*, lung cancer, lung adenocarcinoma, drug sensitivity and resistance

Introduction

Cancer remains a major health burden. Lung cancer ranks as the leading cause of cancer-related mortality worldwide, accounting for ~1.8 million new cases and ~1.6 million deaths annually (1). Non-small cell lung cancer, which accounts for ~85% of all lung canc er cases, is of particular concern as the majority of patients are diagnosed at advanced or metastatic stages (2). Although notable progress has been made in surgery, chemotherapy, radiotherapy, targeted therapy and immunotherapy, resistance to treatment and relapse remain major challenges, thus highlighting the need for novel therapeutic strategies (3-6).

Traditional Chinese Medicine (TCM) offers unique advantages due to its multi-target and multi-component nature, as well as its relative low toxicity. Emerging evidence has highlighted its role in cancer therapy, with research reporting improvements in patient quality of life and an increase in the efficacy and reduction of adverse effects of conventional therapies (7,8). TCM exerts its therapeutic effects via targeting several biomolecules, modulating downstream signaling pathways and disrupting disease-associated networks (9,10). Therefore, identifying the molecular targets of bioactive compounds in TCM is a pivotal step toward understanding its underlying therapeutic mechanisms and advancing their modern clinical applications (11).

Despite marked progress in drug discovery, elucidating the molecular targets of TCM compounds remains a major challenge due to the lack of effective tools for investigating

small molecule-protein interactions. Conventional methods include affinity chromatography (12), activity-based protein profiling (13) and label-free approaches such as drug affinity responsive target stability (DARTS) (14), oxidative rate-dependent protein stability analysis (15) and thermal proteome profiling (16-19). For example, oxidative rate-dependent protein stability leverages changes in protein oxidation kinetics upon ligand binding to identify potential targets (15). However, label-free methods commonly lack sufficient sensitivity, whilst label-based techniques frequently require structural modifications that can compromise compound activity (20). These limitations have hindered the comprehensive understanding of the mechanisms underlying TCM and slowed their broader integration into modern therapeutic applications.

The advent of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) genome-editing technology in 2013 has revolutionized functional genomics research (21). Genome-wide CRISPR libraries enable unbiased and high-throughput screening of functional genes, thus offering transformative potential in drug resistance (22), tumor biology (23), immune responses (24) and cancer immunotherapy (25). Previous applications of CRISPR-based screening in drug discovery have supported its potential in identifying molecular targets of bioactive compounds (26,27), thus providing novel insights into its adoption in TCM research.

Berberine (BBR), a natural alkaloid extracted from *Coptis chinensis* and related medicinal plants, exhibits several biological activities, including anti-inflammatory, antimicrobial and anticancer properties (28-30). Notably, a previous study reported that BBR could inhibit lung cancer cell proliferation (31). However, its particular molecular mechanisms remain largely unknown, thus limiting its therapeutic application. Recent studies have demonstrated the anticancer versatility of BBR. For instance, Li *et al* (32) reported that BBR induced apoptosis and cell-cycle arrest across different cancer cell lines. Additionally, Shen *et al* (33) reported that BBR delivered via polyethylene glycol-poly-lactide co-glycolide acid nanocarriers could enhance transcriptomic changes in colorectal cancer cells, thus modulating the Wnt, TGF-β, Hippo and mammalian target of rapamycin signaling pathways. Consistently, Sun *et al* (34) reported the multifaceted antitumor activities of BBR, including anti-inflammatory activity, inhibition of angiogenesis and reversal of drug resistance. In 2024, a review article further summarized the effects of BBR on nuclear factor κB, mitogen-activated protein kinase and phosphoinositide 3-kinase/protein kinase B signaling, as well as its synergistic effects with conventional therapies (35).

Therefore, the present study aimed to systematically identify genes responsive to BBR in A549 lung cancer cells via employing a genome-wide CRISPR/Cas9 screening library. This approach could provide a powerful platform for identifying molecular targets of TCM, thus contributing to a deeper understanding of their mechanisms of action and accelerating their integration into modern oncological practice.

Materials and methods

Reagents and instruments. BBR (purity ≥98%; cat. no. 2086-83-1) was purchased from MedChemExpress. F-12K medium, fetal bovine serum (FBS) and trypsin were purchased from Gibco (Thermo Fisher Scientific, Inc.). RIPA lysis buffer, protease inhibitor cocktail, western blot reagents, Cell Counting Kit 8 (CCK-8) assay and BCA protein assay kits were purchased from Beyotime Biotechnology. The cell culture plates and microplate reader were purchased from Falcon (Corning Life Sciences) and Thermo Fisher Scientific, Inc., respectively.

Cell culture. The A549 human lung adenocarcinoma cell line was purchased from Shanghai Hanyu Biotechnology Co., Ltd. Cells were maintained in F-12K medium supplemented with 10% FBS at 37˚C in a humidified incubator with 5% CO_2. Cells cultured at a maximum of 5-20 passages were used for all experiments. Routine mycoplasma testing was performed and confirmed negative. Culture medium was replaced every 2-3 days. The incubator was maintained at ~95% relative humidity to ensure optimal growth conditions. All experiments, including CCK-8 assays, CRISPR screening, western blotting and flow cytometry, were performed in at least three independent biological replicates, each using separately cultured cell batches. For all assays, cells in the logarithmic growth phase were digested, counted and seeded as single-cell suspensions at a density of 2,000 cells/well in 12-well plates, to ensure uniform distribution. Edge wells were filled with sterile water to minimize evaporation.

Cell viability assay. The effects of BBR on cell viability were assessed using a CCK-8 assay. Briefly, cells were treated with 1-60 μM BBR or with the corresponding control reagent (DMSO). Following incubation for 72 h, each well was supplemented with 10 μl CCK-8 reagent, followed by incubation for an additional 2 h. Finally, to quantify cell viability, the absorbance in each well was measured at a wavelength of 450 nm using a microplate reader.

CRISPR/Cas9 genome-wide knockout screening. A genome-wide CRISPR knockout library was generated using the GeCKO v2.0 platform (36), encompassing 123,411 single-guide (sg)RNAs targeting 19,050 human genes. The sgRNA plasmid library (Addgene plasmid #1000000048) used in the present study may be obtained through Addgene, Inc. (https://www.addgene.org/pooled-library/zhang-human-gecko-v2/), and its use and construction have been previously described (36). Each gene was targeted by six independent sgRNAs distributed across constitutive exons to maximize knockout efficiency.

To achieve comprehensive genome coverage, ~1.6×10^8 A549 cells were infected with lentiviral particles at a multiplicity of infection of 0.3-0.5. Cells were seeded in the presence of 8 μg/ml polybrene and subjected to spin infection (450 x g at 37˚C for 2 h), followed by replacement with fresh medium. Cells were then selected with puromycin (1 μg/ml) for 14 days. To confirm stable expression, the expression levels of Cas9 in A549 cells were assessed using western blot analysis. The screening employed a single-vector lentiviral system (lentiCRISPR v2; cat. no. 52961; Addgene, Inc.), in which both Cas9 and sgRNA expression cassettes are integrated within the same backbone; therefore, no additional Cas9 transfection was required.

Library quality was evaluated using next-generation sequencing (NGS), which verified uniform sgRNA

representation, with >200-fold coverage per sgRNA. Genomic DNA was amplified using the NEBNext High-Fidelity 2X PCR Master Mix (cat. no. M0541; New England Biolabs, Inc.) and purified using the QIAquick Gel Extraction Kit (cat. no. 28704; Qiagen GmbH). The integrity of the 200- to 300-bp amplified fragments was verified by 1.5% agarose gel electrophoresis prior to sequencing. Sequencing was performed on an Illumina NovaSeq 6000 platform using the NovaSeq 6000 S4 Reagent Kit v1.5 (cat. no. 20028312; Illumina, Inc.), generating paired-end 150 bp (PE150) reads at an average depth exceeding 300 times. The final pooled library was loaded at a concentration of 1.457 μg/μl (2.18×10^{-9} mol/μl), quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). To maintain library complexity, a total of 4×10^7 cells were collected every 3 days and stored at -80˚C. For NGS, genomic DNA was extracted on day 14, and candidate genes responsive to BBR were identified using MAGeCKFlute (version 0.5) analysis (37). Genes were ranked based on the changes in their sgRNA abundances between treatment and control groups. Those significantly enriched or reduced ($P<0.05$) were subsequently subjected to Gene Ontology (GO) classification and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis using the Omicsbean proteomics toolkit (www.omicsbean.cn).

Treatment of genome-wide edited cell libraries with BBR. A549 cells harboring the CRISPR knockout library were expanded and divided into the following three groups: i) Untreated (control); ii) BBR-treated; and iii) vehicle-treated (DMSO) groups. Cells in the experimental group were exposed to 10 μM BBR at 37˚C in a humidified 5% CO_2 incubator for 3 days, whilst those in the control group were cultured under routine conditions with regular medium changes and passaging. When cell viability in the BBR-treated group reached ~10% of that of the untreated group, genomic DNA was extracted for NGS to identify sgRNA enrichment patterns.

Claudin (CLDN)-1 knockout and lentiviral transduction. A second-generation lentiviral packaging system was used for transduction. The lentiviral vector encompassing sgRNA sequences targeting *CLDN1* was constructed after cloning oligonucleotide sequences (sgRNA-Z9, 5'-AGCGAGTCATGG CCAACGCG-3'; sgRNA-Z10, 5'-GGCGCCGATCCATCC CAGGA-3') into the lentiCRISPR v2 plasmid (cat. no. 52961; Addgene, Inc.). Prior to transduction, the successful construction of plasmids was verified by Sanger sequencing, which was performed by Sangon Biotech Co., Ltd. (Shanghai, China) using an Applied Biosystems (Thermo Fisher Scientific) platform. Lentiviral particles were produced in 293T cells (ATCC CRL-3216) by co-transfecting the transfer plasmid (lentiCRISPR v2) with the packaging plasmids psPAX2 (cat. no. 12260; Addgene, Inc.) and the envelope plasmid pMD2.G (cat. no. 12259; Addgene, Inc.) at a mass ratio of 2:1:1, using polyethylenimine as the transfection reagent. Transfection was performed at 37˚C in a humidified incubator with 5% CO_2. The viral supernatant was collected 48 and 72 h post-transfection, pooled, filtered through a 0.45-μm filter and concentrated by ultracentrifugation. A549 cells were seeded in 6-well plates at ~70% confluency and were then infected with lentiviral supernatant at a multiplicity of infection of 5-10, containing 8 μg/ml polybrene. Following incubation for 12 h at 37˚C, the medium was replaced with fresh F-12K supplemented with 10% FBS. Transduced cells were subsequently selected with 2 μg/ml puromycin for 7 days to establish stable *CLDN1* knockout cell lines. A puromycin concentration of 1 μg/ml was used for the maintenance of selected cells. Following a 7-day recovery period after puromycin selection, the cells were used for subsequent experimentation. Genomic DNA from puromycin-resistant A549 cells was extracted using a genomic DNA purification kit (Tiangen Biotech, Co., Ltd.), and the *CLDN1* target region encompassing the Z10 site was amplified using PCR performed with PrimeSTAR HS DNA Polymerase (Takara Bio Inc.) and the following primers: Forward, 5'-GCAACCGCAGCTTCTAGTATC-3'; and reverse, 5'-TGCACACTTGAGAAGTTACC-3'. The following thermocycling conditions were applied: Initial denaturation at 98˚C for 2 min; 35 cycles of 98˚C for 10 sec, 58˚C for 10 sec and 72˚C for 15 sec; with a final extension at 72˚C for 5 min. PCR products were separated on a 1.2% agarose gel stained with GoldView (Takara Bio Inc.) and visualized using a GelDoc XR+ imaging system (Bio-Rad Laboratories, Inc.). PCR products were purified and subjected to Sanger sequencing by Sangon Biotech Co., Ltd. (Shanghai, China) to verify the successful genome editing of *CLDN1*.

Western blot analysis. Protein lysates were prepared using NP-40 lysis buffer supplemented with protease inhibitors. The protein concentration was measured using a BCA kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein extract (20 μg per lane) were first separated using 10% SDS-PAGE and were then transferred to PVDF membranes. Following blocking with 3% bovine serum albumin (Beyotime Biotechnology) at room temperature for 2 h, the membranes were incubated overnight at 4˚C with the following primary antibodies: Anti-CLDN1 (1:1,000 dilution; cat. no. 28674-1-AP; Proteintech Group, Inc.), anti-cyclin D1 (1:1,000 dilution; cat. no. 26939-1-AP; Proteintech Group, Inc.), anti-Cas9 (1:1,000 dilution; cat. no. 26758-1-AP; Proteintech Group, Inc.), anti-GAPDH (1:5,000 dilution; cat. no. 10494-1-AP; Proteintech Group, Inc.) and anti-β-actin (1:5,000 dilution; cat. no. 66009-1-Ig; Proteintech Group, Inc.). Subsequently, the membranes were incubated with corresponding HRP-conjugated secondary antibodies (goat anti-rabbit IgG; 1:5,000 dilution; cat. no. SA00001-2; Proteintech Group, Inc.) for 1 h at room temperature, and the immunoreactive bands were visualized using enhanced chemiluminescence (BeyoECL Plus; cat. no. P0018S; Beyotime Biotechnology).

Flow cytometry analysis. To assess the effects of BBR on cell cycle progression, A549 and *CLDN1*-knockout A549 cells were treated with 2.5 μM BBR, a sublethal dose slightly below the calculated half-maximal inhibitory concentration (IC_{50}; 2.856 μM). This concentration was selected to minimize cytotoxicity whilst allowing the detection of sensitization effects associated with *CLDN1* knockout. This was consistent with standard pharmacological studies aiming to evaluate differential cellular responses using sub-lethal or slightly sub-IC_{50} concentrations (38,39). Following incubation for 72 h, cells were fixed with 70% ethanol, washed with PBS and stained with propidium iodide. Samples were incubated at 37˚C in the

dark for 30 min prior analysis on a flow cytometer (CytoFLEX; Beckman Coulter, Inc.) equipped with a 488-nm laser. Data analysis was performed using FlowJo software (version 8.0; FlowJo LLC).

Statistical analysis. The experimental data were statistically analyzed using GraphPad Prism 5.0 software (Dotmatics). Groups were compared using an unpaired two-tailed Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of BBR on A549 cell proliferation. Previous studies have reported that BBR can inhibit the growth of lung cancer cells, including that of A549 cells, in a dose-dependent manner. However, effective concentrations vary among different cell lines (40-42). Therefore, to determine the optimal concentration of BBR in A549 cells, the cells were treated with increasing concentrations of BBR (1, 2.5, 5, 10, 20, 40 and 60 μM) and cell viability was then assessed using a CCK-8 assay. The results revealed that BBR significantly reduced cell viability in a dose-dependent manner, with only 21.5% of cells remaining viable at a concentration of 60 μM (Fig. 1). Analysis using GraphPad Prism software revealed that the IC_{50} was 2.856 μM. These findings indicated that BBR could effectively suppresses A549 cell proliferation, thus providing a foundation for subsequent mechanistic experiments.

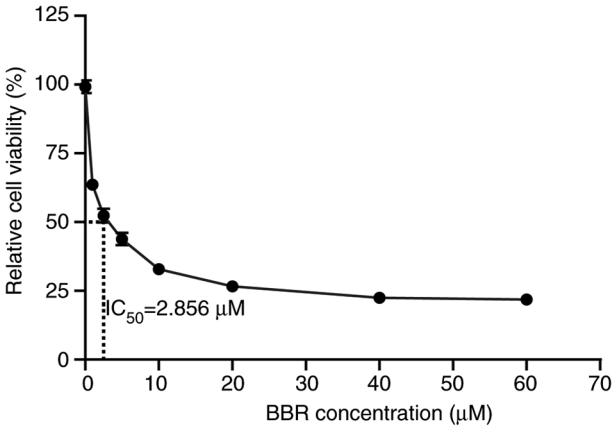

Figure 1. Effect of BBR on A549 cell proliferation. A549 cells were treated with different concentrations of BBR (1, 2.5, 5, 10, 20, 40 and 60 μM) for 72 h, and cell viability was assessed using a Cell Counting Kit-8 assay. The data are expressed as the mean ± standard deviation. BBR, berberine.

Construction of A549 genome-wide knockout library and BBR target screening. To comprehensively identify genes responsive to BBR treatment, a genome-wide CRISPR/Cas9 knockout library was constructed in A549 cells (Fig. 2A). Library generation followed established protocols (36), with lentiviral transduction enabling stable expression of *Streptococcus pyogenes* (*sp*)Cas9 in A549 cells. Western blot analysis was performed to confirm the successful expression of spCas9 protein at the expected molecular weight of 140-160 kDa (Fig. 2B). The resulting knockout library was designated as P0. To assess the capacity of the library in identifying BBR targets, P0 cells were treated with BBR until cell viability reduced to ~10% of the untreated cell population. Subsequently, genomic DNA was extracted from surviving cells, and sgRNA sequence representation was determined using NGS. Candidate genes were ranked based on positive (poslscore) or negative (neglscore) selection values using the MAGeCK algorithm, with a significance threshold of $P<0.05$. A total of 3,873 genes exhibited significant differential selection, including 749 positively selected genes (associated with resistance) and 3,124 negatively selected ones (associated with sensitivity) (Tables SI and SII).

Enrichment analysis of resistance and sensitivity genes. To elucidate the biological pathways underlying BBR response, KEGG pathway and GO enrichment analyses were performed on the differentially selected genes. KEGG analysis revealed that the selected genes were mainly enriched in the 'aminoacyl-tRNA biosynthesis', 'homologous recombination', 'DNA replication' and 'non-homologous end-joining' pathways (Fig. 3A). Notably, several DNA damage repair-related pathways were highlighted, thus suggesting that BBR could be associated with genomic stability and cell cycle regulation. GO analysis further revealed that the selected genes were enriched into the following molecular functions: 'ribosome binding', 'beta-tubulin binding' and 'NADH dehydrogenase activity'; cellular components: 'mitochondrial nucleoid', 'Cajal body' and 'polysomal ribosome'; and biological processes: 'mitochondrial electron transport', 'mRNA polyadenylation' and 'transmembrane transporter activity' (Fig. 3B). These findings indicate that BBR could potentially disrupt cellular energy metabolism and fundamental biological processes. Among the top-ranked candidate genes (Fig. 3C), slit guidance ligand 3, DNAJ heat shock protein family (*Hsp40*) member B1, ubiquitin conjugating enzyme E2 N and *CLDN1* were of particular interest due to their established roles in lung cancer cell proliferation, invasion and drug resistance (43-50). Notably, *CLDN1*, a member of the CLDN family involved in tight junction integrity, has been associated with tumor progression and chemoresistance in A549 cells (50), highlighting its potential as a compelling target for further investigation.

Functional validation of CLDN1 in BBR response. To assess the role of *CLDN1* in mediating response to BBR, two sgRNAs targeting the *CLDN1* coding region (sgRNA-Z9, 5'-AGCGAG TCATGGCCAACGCG-3'; sgRNA-Z10, 5'-GGCGCCGAT CCATCCCAGGA-3') were designed, and *CLDN1*-knockout A549 cells were established via lentiviral transduction. Sanger sequencing confirmed a single-nucleotide insertion at the Z10 target site, introducing a premature stop codon at amino acid position 68 (Fig. 4A). Additionally, western blot analysis demonstrated the complete absence of CLDN1 protein expression in knockout cells (A549-KO cells; Fig. 4B). Furthermore, A549-KO cell treatment with BBR significantly reduced cell viability compared with that in wildtype A549 cells, with notable differences observed at 48 and 72 h, thus supporting the dose- and time-dependent sensitization effects of BBR (Fig. 4C). Additionally, flow cytometry revealed a marked reduction in the S-phase population and a concomitant increase in the G1-phase population in A549-KO cells following BBR

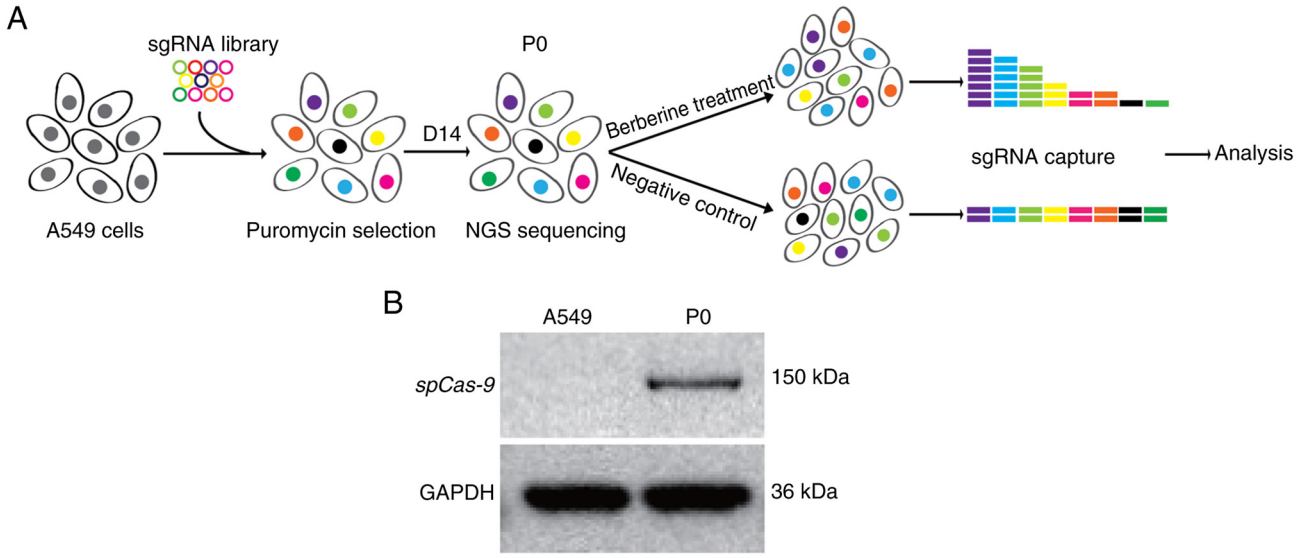

Figure 2. Genome-wide CRISPR/Cas9 screening in A549 cells. (A) Schematic workflow of the CRISPR/Cas9 screening strategy. (B) Western blot analysis of spCas9 protein levels in A549 and P0 cells. CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein 9; spCas9, *Streptococcus pyogenes* CRISPR-associated protein 9; sgRNA, single-guide RNA; NGS, next-generation sequencing.

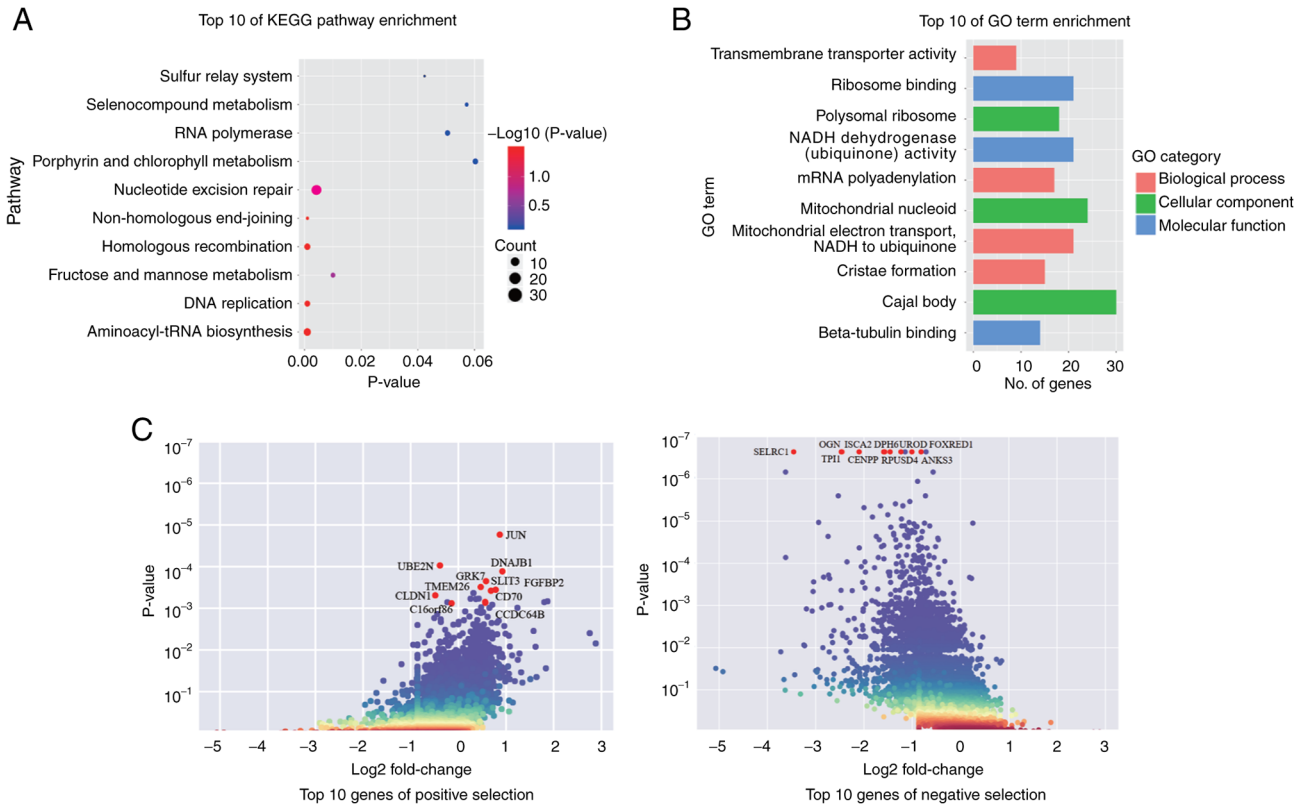

Figure 3. Enrichment analyses. (A) KEGG pathway and (B) GO term enrichment analyses. (C) Top 10 differentially expressed genes of positive and negative selection. KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology.

treatment, indicative of G1-phase cell cycle arrest (Fig. 4E). Consistent with the aforementioned findings, cyclin D1, a key regulator of G1/S transition (51), was notably downregulated in BBR-treated A549-KO cells compared with that in wild-type cells (Fig. 4D). These results indicate that *CLDN1* could be involved in BBR-induced cell cycle arrest and growth suppression in A549 cells.

Discussion

Lung cancer, accounting for ~1.8 million new cases and ~1.6 million deaths annually, remains a global health challenge due to its low 5-year survival rate, ranging from 4-17% depending on the stage of diagnosis and geographical region (52,53). Despite advances in chemotherapy,

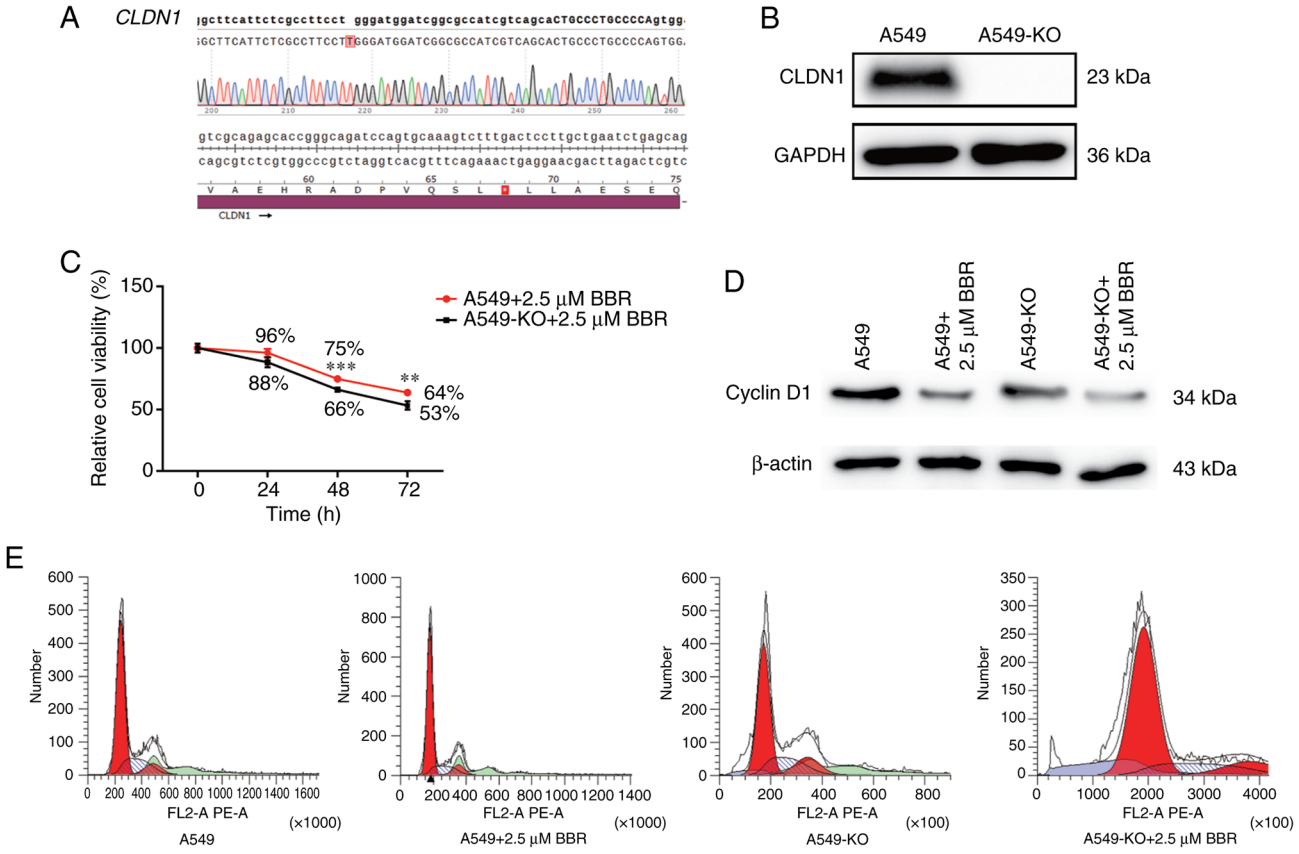

Figure 4. Effect of *CLDN1* knockout on the response of A549 cells to BBR. (A) CRISPR/CRISPR-associated protein 9-mediated CLDN1 knockout in A549 cells. (B) Loss of *CLDN1* protein expression assessed in A549-KO cells using western blot analysis. (C) Cell viability of wild type A549 and A549-KO cells treated with 2.5 μM BBR for 24, 48 and 72 h. Data are expressed as the mean ± standard deviation (n=3). **P<0.01; ***P<0.001. (D) Western blot analysis of cyclin D1 expression in A549 and A549-KO cells following treatment with BBR. (E) Flow cytometry analysis of cell cycle distribution highlighting the differences in the G1 and S phases between A549 and A549-KO cells. BBR, berberine; *CLDN1*, claudin-1; CRISPR, clustered regularly interspaced short palindromic repeats; A549-KO, *CLDN1*-depleted A549 cells; KO, knockout.

radiotherapy, targeted therapies and immunotherapies, drug resistance still poses a critical barrier, notably limiting long-term efficacy (54). In this context, TCM offers a promising complementary approach, owning to its multi-target and multi-component characteristics. Among TCM-derived compounds, BBR has shown potent anticancer properties via modulating key cellular processes, such as proliferation, apoptosis, autophagy and immune modulation within the tumor microenvironment (55). However, the particular molecular targets and pathways underlying its anticancer effects remain insufficiently characterized.

The identification of molecular targets of bioactive compounds is essential for the modernization of TCM and the development of novel cancer therapeutics (9,11). Although label-based approaches, such as chemical probe-assisted affinity capture, have successfully identified key targets, their application is limited due to chemical modification-induced activity loss and structural constraints (56,57). By contrast, label-free methods such as DARTS, cellular thermal shift assay and thermal proteome profiling offer non-invasive alternatives. However, their use is commonly hindered by limited sensitivity to weak or low-abundance protein interactions (14,15,17,58). These limitations highlight the urgent need for scalable and precise strategies to enable systematic target identification.

In the present study, a CRISPR/Cas9 genome-wide knockout library was constructed in A549 lung adenocarcinoma cells to systematically investigate the molecular targets and resistance mechanisms associated with BBR treatment. The unbiased screening identified a total of 3,873 genes with differential selection under BBR exposure, including 749 positive-selection genes associated with resistance and 3,124 negative-selection genes essential for cellular viability. Among the resistance-related genes, CLDN1, a tight junction protein of the CLDN family, could serve as a pivotal mediator of BBR resistance. In a previous study, *CLDN1* overexpression was associated with poor prognosis and resistance to cisplatin in patients with lung cancer (50). Consistently, the results of the present study demonstrated that *CLDN1* knockout enhanced the sensitivity of A549 cells to BBR, characterized by reduced cell proliferation, decreased S-phase entry and G1-phase arrest. The CRISPR-based identification of *CLDN1* as a mediator of the anticancer effects of BBR aligned with broader advances in CRISPR therapeutic strategies. Previous comprehensive reviews summarized the potential of CRISPR in targeting oncogenes, precise gene modulation and overcoming drug resistance (59). Notably, an *in vivo* study reported that CRISPR-lipid nanoparticle platforms could eliminate ≤50% of tumors in mouse models (60). Furthermore, BBR could inhibit tumor growth in lung cancer models via several pathways,

including epigenetic and DNA-repair modulation (61). Taken together, the results of the present study could extend current therapeutic paradigms by positioning *CLDN1* as a critical regulator of BBR responsiveness. Notably, the functional assays in the present study were performed at 2.5 μM BBR, slightly below the IC_{50} value (2.856 μM), thus ensuring that the observed differences were due to genetic manipulation rather than cytotoxicity. However, future studies are needed to refine this analysis using a concentration gradient (such as 2.0, 2.5 and 3.0 μM) to further validate the robustness of the findings. Nevertheless, the results strongly suggest that *CLDN1* could serve a key role in mediating resistance to BBR via modulating cell cycle progression.

Beyond *CLDN1*, other CLDN family members have also been involved in cancer progression and therapeutic targeting. For example, a study reported that *CLDN4* could act as a molecular target in epithelial cancers, thus being involved in epithelial-to-mesenchymal transition (EMT) and YAP-mediated proliferation, with ongoing preclinical efforts exploring CLDN4-directed antibody therapies (62). Additionally, CLDN18.2 could serve as a clinically actionable target in gastric and pancreatic cancers, with monoclonal antibody approaches, such as zolbetuximab, demonstrating promising antitumor activity (63). Collectively, these findings could frame *CLDN1* within a broader CLDN-triggered regulatory network and highlight the potential of multi-CLDN targeting approaches in cancer therapy.

Beyond its role in cell cycle regulation, *CLDN1* has also been involved in additional signaling pathways associated with cancer biology. A previous study reported that *CLDN1* overexpression could activate the TGF-β1/EMT signaling pathway, thus mitigating the sensitivity of lung cancer cells to doxorubicin (64). These findings indicated that *CLDN1* could act as a multifunctional regulator linking junctional integrity to oncogenic signaling. Whilst the present study primarily established *CLDN1* as a mediator of BBR sensitivity, future research is needed to further investigate EMT- and TGF-β1-associated mechanisms downstream of *CLDN1* to provide a more comprehensive mechanistic framework. Notably, the identification of *CLDN1* as a BBR-resistance gene underscored the broader utility of the current CRISPR/Cas9 screening platform. Compared with conventional methods, this genome-wide approach could enable unbiased and high-throughput target identification, thus providing a powerful framework for elucidating the molecular mechanisms of TCM-derived compounds. The discovery of *CLDN1* could not only deepen the understanding of the pharmacological effects of BBR, but also highlight its potential as a therapeutic target for overcoming drug resistance in lung cancer.

Moving forward, the development of specific *CLDN1* inhibitors could provide novel opportunities for combination therapies, enhancing the efficacy of BBR and that of other anticancer agents. In addition, the present study established a proof-of-concept for applying CRISPR-based screening in TCM research, thus offering novel insights into the systematic exploration of bioactive compounds and their molecular targets. This integrative approach could not only support the modernization and global acceptance of TCM, but it could be also involved into the broader advancement of precision oncology.

While the present study successfully identified *CLDN1* as a pivotal mediator of berberine (BBR) resistance through a genome-wide CRISPR/Cas9 screening in A549 cells, several aspects merit further investigation. The findings were derived from a single lung adenocarcinoma model, and validation in additional lung cancer subtypes or *in vivo* systems would strengthen the generalizability of the results. Moreover, this study focused on gene-level knockout screening; integration with transcriptomic or proteomic data could provide a broader mechanistic perspective on the BBR-CLDN1 network. In addition, although a sub-IC_{50} concentration of BBR was used to highlight genetic effects rather than cytotoxicity, evaluation across a concentration gradient would better characterize dose-dependent responses. Addressing these aspects in future work will refine the mechanistic insights and enhance the translational relevance of the findings.

In conclusion, the present study identifies *CLDN1* as a critical determinant of berberine responsiveness in lung cancer cells and establishes a CRISPR/Cas9-based framework for target discovery in traditional Chinese medicine. These findings provide mechanistic insight into BBR resistance and offer a promising direction for the development of *CLDN1*-targeted combination therapies.

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Availability of data and materials

The NGS data generated in the present study may be found in the NCBI BioProject database under accession number PRJNA1345357 or at the following URL: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1345357. All other data generated in the present study may be requested from the corresponding author.

Authors' contributions

XGW and XT contributed to the design of CRISPR/Cas9 screening strategies. YDW contributed to the design and optimization of the functional validation experiments following the primary CRISPR/Cas9 screen. XGW, BZM and YDW were involved in the literature search and review. XJW, BZM, YJX and FL performed the experiments. JFL and JYH performed the data analysis. BZM and YDW provided key reagents, cell lines and instrumental resources. XGW, YJX and YDZ wrote the manuscript. YDZ conceived and coordinated the study,

integrated the overall data analysis and manuscript preparation, and was responsible for communication and revision with the journal, with YDW assisting in correspondence and resource support. XGW and YDZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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