Am J Cancer Res 2023;13(5):2155-2171
www.ajcr.us /ISSN:2156-6976/ajcr0149407

Original Article

Ly6D facilitates chemoresistance in laryngeal squamous cell carcinoma through miR-509/β-catenin signaling pathway

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Received January 30, 2023; Accepted April 25, 2023; Epub May 15, 2023; Published May 30, 2023

Abstract: Chemotherapy resistance is a major limiting factor in the cure of patients with laryngeal squamous cell carcinoma (LSCC). Lymphocyte antigen 6 superfamily member D (Ly6D) is highly expressed in various tumors, but its role and underlying molecular mechanisms in chemoresistance of LSCC cells remains largely unclear. In this study, we reveal that overexpression of Ly6D facilitates LSCC cell chemoresistance, while Ly6D silencing abolishes this phenotype. Moreover, bioinformatics analysis, PCR array, and functional analysis confirmed that activation of the Wnt/β-catenin pathway contributes to Ly6D-mediated chemoresistance. The genetic and pharmacological inhibition of β-catenin compromises chemoresistance mediated by Ly6D overexpression. Mechanistically, Ly6D overexpression significantly attenuates the expression of miR-509-5p, thereby unleashing its target gene CTNNB1 to activate Wnt/β-catenin pathway and ultimately promote chemoresistance. In contrast, Ly6D augmenting β-catenin-mediated chemoresistance in LSCC cells were reversed by ectopic expression of miR-509-5p. Furthermore, ectopic expression of miR-509-5p markedly repressed the two other targets, MDM2 and FOXM1. Taken together, these data not only reveal the key role of Ly6D/miR-509-5p/β-catenin in chemotherapy resistance, but also provide a new strategy for the clinical treatment of refractory LSCC.

Keywords: Ly6D, miR-509, β-catenin, chemoresistance, laryngeal squamous cell carcinoma

Introduction

Laryngeal cancer is a common malignancy of the head and neck, accounting for approximately 5% of systemic malignancies, with more than 95% is laryngeal squamous cell carcinoma (LSCC) [1]. LSCC has a high mortality rate, which seriously endangering the life and health of patients. Despite great advances in surgical techniques and treatments such as radiotherapy and chemotherapy, the prognosis for advanced LSCC remains discouraging. It has been reported that about 60% of patients with laryngeal cancer are diagnosed with advanced stage (III or IV) [2]. For patients with advanced LSCC, chemotherapy remains the standard first-line regimen. Chemotherapy resistance is the main obstacle to prolonging the survival of LSCC patients [3]. Therefore, it is of great significance to elucidate the molecular mechanism of LSCC chemotherapy resistance to improve treatment effectiveness.

Lymphocyte antigen 6 superfamily member D (Ly6D), which belongs to member of LY6 family, is a membrane binding protein with glycosyl phosphatidylinositol (GPI) anchors on the cell surface [4]. Emerging data have shown that Ly6D is highly expressed in a variety of tumors, including ovarian cancer, breast cancer, lung cancer, head and neck cancer, and is closely related to poor prognosis [5]. Accumulating

findings have shown that Ly6D not only promotes tumor cell adhesion, but also regulates the critical interactions between endothelial cells and head and neck squamous cell carcinoma cells [6]. Studies have demonstrated that Ly6D, along with OLFM4 and S100A7, contribute to distant metastasis of breast cancer [7]. Radiotherapy induced Ly6D expression on the surface of breast cancer cells [8]. Through screening LSCC tumor stem cells, our previous work revealed that Ly6D is highly expressed in tumor stem cells and can serve as a marker for tumor stem cells [9]. Although many studies focused on Ly6D as a tumor surface marker, the role and underlying molecular mechanism of Ly6D in LSCC chemoresistance remained elusive.

MicroRNA (miRNA) are a kind of non-coding small RNA with a length of about 22 bp that participates in the complex malignant phenotype of cancer, such as stem cell maintenance, evasion of death, genomic instability, recurrence, and metastasis [10]. The expression of miRNAs is dysregulated in many tumors, indicating that they may function as tumor suppressors or oncogenes in context-dependent manner [11]. Compelling evidence have highlighted that miRNA plays an important role in regulating the response of tumor cells to chemotherapy drugs [12, 13]. Various studies have indicated that miR-509 acts as tumor suppressor, which fulfils a key role in suppressing tumor cell stemness, attenuating inflammation, inhibiting metastasis, and improving chemosensitivity [14, 15]. However, the relationship between miR-509 and chemotherapy resistance of LSCC cells is still unclear.

In this study, we present data aimed to clarify the function and mechanism of Ly6D on chemoresistance of LSCC cells. Our data revealed that Ly6D overexpression inhibits the expression of miR-509-5p to unleash its target gene CTNNB1 and facilitate Wnt/β-catenin signaling activation, and ultimately confers LSCC cells chemoresistance. This study reveals that targeting the Ly6D/miR-509-5p/β-catenin signaling axis may provide novel targets and strategies for improving chemosensitivity in LSCC.

Materials and methods

Antibodies and reagents

Paclitaxel, 5-FU, Cisplatin, and actinomycin D were obtained from Solarbio (Beijing, China). The antibodies against Myc, Bcl-2, flag, MDM2, FOXM1, and cyclin D1 were bought from Proteintech (Wuhan, China). The Ly6D antibody was purchased from Santa Cruz Biotechnology (CA, USA). Active β-catenin antibody was obtained from Millipore (Billerica, MA, USA). Antibodies for β-catenin and GAPDH were obtained from Abcam (Cambridge, MA, USA). ICG001 was purchased from Targetmol (Shanghai, China). The transfection reagent lipofectamine 3000 was obtained from Thermo Fisher Scientific (Waltham, MA, USA). The Cell-Light EdU Apollo567 In Vitro Kit was obtained from Ribobio (Guangzhou, China).

Cell culture

The human embryonic kidney 293T (HEK293T) and human LSCC cells Tu686 and CAL27 were purchased from ATCC (Manassas, VA, USA). The cells were cultured in DMEM medium containing 10% fetal bovine serum, streptomycin (100 μg/mL), and penicillin (100 U/mL). Then, cells were incubated at 37°C, 5% CO_2.

CCK8 assay and colony formation assay

The cell viability was evaluated by CCK8 assay using Enhanced Cell Counting Kit-8 (Beyotime, Beijing, China) as previously described [16]. Briefly, the cells of each group were seeded into 96-well plates with a density of 5000 cells/well, and then treated with different concentrations of drugs. After treatments, 10 μl of CCK8 reagent was added to each well and cells were further cultured at 37°C for 2 h. The optical density value (OD450) was determined by a microplate reader (BioTek, VT, USA).

The colony formation assay was performed as previously described [17]. In brief, cells were plated in 6-well plates at 1000 cells per well. Cells were treated with indicated drugs and cell medium was changed every 3 d for 2 weeks. Then, colonies were washed with PBS and fixed with 4% paraformaldehyde and stained with crystal violet. The colonies were analyzed using ImageJ software.

EdU incorporation assay

The cells were plated in 96-well plates. After drug treatment for 24 h, EdU staining were carried out following the manufacturer's instructions (Ribobio, Guangzhou). The proportion of cells incorporating EdU were analyzed by fluorescence microscopy (Zeiss, Oberkochen, German).

Quantitative real-time PCR (qPCR)

Total RNA was extracted from cells using Trizol reagent (Takara, Dalian, China) for miRNA or mRNA analysis as previously described [18]. Briefly, the cDNA of mRNA and miRNA were synthesized with PrimeScript™ RT kit (Takara, Dalian, China) and All-in-One miRNA qRT-PCR Detection Kit (GeneCopoeia, Rockville, USA), respectively. The mRNA or mature miRNA expression was performed using 2×SG Fast qPCR Master Mix (Sangon Biotech, Shanghai, China). U6 RNA and GAPDH were used as internal controls for miRNA and mRNA, respectively. The sequences of the primers were listed in Table S1.

Gene expression array analysis

The Cancer Drug Resistance PCR Array (GeneCopoeia, MD, USA) was used to analyze the effect of overexpression of Ly6D on chemotherapy resistance of LSCC cells. The Scan Mir miRNA PCR array for CTNNB1 (Wcgene Biotech, Shanghai, China) was performed to profile Ly6D-mediated expression of the miRNA following manufacturer's instructions.

Western blotting

Total cell lysate was extracted, and the protein concentration was measured by BCA Protein Assay Kit (Beyotime, Beijing, China). 20 μg of each protein sample was subjected to 10% SDS-PAGE and transferred to a PVDF membrane. After blocking with 5% skim milk powder for 1 h, the membrane was incubated overnight at 4°C with the primary antibody. Subsequently, the corresponding secondary antibody was incubated and immunoblots were developed using an enhanced chemiluminescence detection kit (Seven Biotech, Beijing, China).

Chromatin immunoprecipitation (ChIP) assays

The ChIP experiments were performed with an EZ-ChIP™ chromatin immunoprecipitation kit (Millipore, MA, USA) as previously reported [19]. Briefly, the indicated cells were prepared and fixed with 1% formaldehyde for 10 min. After washed with ice-cold PBS and harvested by centrifugation, the cell pellets were lysed and sonicated to an average length of 200-1000 bp. After centrifugation, the supernatant was collected and immunoprecipitation with anti-β-catenin antibody or rabbit IgG (negative control). The purified DNA was used for ChIP-qPCR analysis using the primers in Table S1.

Plasmid construction, cell transfection, and generation of stable cells

The gene expression plasmids of Ly6D or β-catenin were amplified using DLD1 cDNA and pLVX-AcGFP1-N1-β-catenin plasmid respectively and cloned into psin-flag mammalian expression plasmid using seamless cloning master mix (Sangon Biotech, Shanghai, China). The primers used to construct the plasmid were as follows: pSIN-Ly6D-F: 5'-ACAAGGATGACGACGATAAGGAATTCATGAGGACAGCATTGCTGCTCC-3'; pSIN-Ly6D-R: TGGCCGCCCTAGATGCATGCGGATCCTCACAGGCTGGGGGCTAAGATG. pSIN-β-catenin-F: 5'-ACAAGGATGACGACGATAAGGAATTCATGGCTACTCAAGCTGATTTGATGG-3'; pSIN-β-catenin-R: 5'-TGGCCGCCCTAGATGCATGCGGATCCTTACAGGTCAGTATCAAACCAGGCC-3'.

Two sets of short hairpin RNA targeting human Ly6D gene are shown in Table S1. After annealing, the fragment was digested with *EcoR*I and *Age*I and cloned into pLKO.1 vector. All plasmids were verified by sequencing. Cell transfections were conducted using lipofectamine 3000 as per manufacturer's recommendations.

The stable cell lines were generated following previous method [20]. Briefly, the plasmid, psPAX2 packaging plasmid, and pMD2.G envelope plasmid was co-transfected into 293T cells for 48 h and then lentivirus was infected into Tu686 and CAL27 cells. The stable cell clones were selected by 2 μg/ml puromycin (Solarbio, Beijing, China).

Luciferase reporter assay

miR-509 mimics and inhibitors were synthesized from Sangon Biotech (Shanghai, China). The 3'-UTR sequence of CTNNB1 that potentially interacts with miR-424 was cloned into pGL4.23 vector. The 3'-UTR of mutant CTNNB1 is obtained using the Easy Mutagenesis System (Transgen Biotech, Beijing, China). The primers used for construction were as follows. CTNNB1 3'-UTR-WT: 5'-AGGTAAGAAGTTTTAAAAAGCCA-3'; 5'-TGAATTAAAAGTTTAATTCTGAA-3'. The luciferase reporter assay was performed as previously reported [21].

TOP/FOP flash reporter assay

The cells were seeded in 96-well plate and were transiently cotransfected with TOP-Flash reporter or FOP-Flash reporter with pTK-RL plasmid (internal control) using lipofectamine 3000. Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) following the manufacturer's instructions and was normalized to pTK-RL luciferase signals.

Statistical analysis

Statistics were conducted using GraphPad Prism 8 software (San Diego, CA, USA). The data is displayed as mean ± S.D. Significant differences between two groups were analyzed by student's *t*-test. One-way analysis of variance (ANOVA) test, followed by Tukey's post test was used for analyzing more than two experimental groups. Results were considered statistically significant at $P < 0.05$.

Results

Knockdown of Ly6D sensitizes LSCC cells to conventional chemotherapy

Numerous studies have demonstrated that the high expression of Ly6D in various tumor tissues is closely related to poor clinical prognosis [5]. To ascertain the role of Ly6D in chemotherapy resistance of LSCC cells, we used two sets of shRNA to obtain stable cells of Ly6D silencing, named shLy6D-1 and shLy6D-2, respectively. The results of western blot and qPCR analysis demonstrated that the protein and mRNA levels of Ly6D were substantially decreased in both stable cell lines, and shLy6D-2 was more effective than shLy6D-1 (**Figure 1A**, **1B**). Subsequently, the sensitivity of the Ly6D silenced stable cell lines to various chemotherapeutic drugs (5-FU, cisplatin, paclitaxel) was detected using CCK-8 assay. The results showed that targeting Ly6D with two different shRNAs noticeably sensitized LSCC cells to various chemotherapy drugs (**Figure 1C**, **1D**). Since the superior effect of shLy6D-2 on Ly6D depletion and chemosensitivity compared to shLy6D-1, the following experiments were conducted using shLy6D-2 cells. EdU assay further confirmed that knockdown of Ly6D obviously improved the killing effect of cisplatin on LSCC cells (**Figure 1E**). Colonic formation assay was used to further mimic the long-term clinical treatment. Consistent with the above results, we found that Ly6D silencing in LSCC cells significantly enhanced chemotherapeutic effects (**Figure 1F**). These results collectively showed that depletion of Ly6D obviously thwarts chemoresistance.

Overexpression of Ly6D promotes chemoresistance of LSCC cells

To test the functional correlation of Ly6D with chemoresistance, Tu686 and CAL27 cells were stably transfected with psin-flag or psin-flag-Ly6D plasmids. The stable cell lines with stably overexpressing Ly6D were established and confirmed by western blot and qPCR analysis (**Figure 2A** and **2B**). The measurement of cell responses to a variety of chemotherapeutic drugs showed that overexpression of Ly6D in LSCC cells significantly facilitated cell proliferation and chemoresistance, indicating a key role of Ly6D in chemotherapy resistance (**Figure 2C** and **2D**). The EdU assay and clonogenic results further revealed that overexpression of Ly6D conferred LSCC cells chemoresistance and blunted the killing effect of chemotherapy drugs towards LSCC cells (**Figure 2E** and **2F**). Collectively, these data demonstrated that overexpression of Ly6D confers LSCC cells more intractable to chemotherapy.

Ly6D-mediated chemoresistance-related genes expression in LSCC cells

To further gain insight into the mechanism of Ly6D regulating chemoresistance in LSCC cells, the Cancer Drug Resistance PCR Array was conducted to determine the impact of Ly6D overexpression on chemoresistance related genes. As shown of a volcano plot in **Figure 3A**, six upregulated genes were identified in Tu686 cells with stably overexpression of Ly6D, including CCNE1, CCND1, ABCC1, ABCB1, BCL2, and MYC. Accumulating evidences have shown that cell cycle related genes CCNE1 and CCND1, drug efflux proteins ABCC1 and ABCB1, anti-apoptotic protein BCL2, and oncogene MYC closely associated with drug resistance [22]. Next, we used qPCR to detect the expression of these genes in CAL27 cells stably overexpressing Ly6D, and found that overexpressing Ly6D promoted the expression of ABCB1, ABCC1, BCL2, MYC and CCND1, and caused no appreciable change of CCNE1 (**Figure 3B**). On the

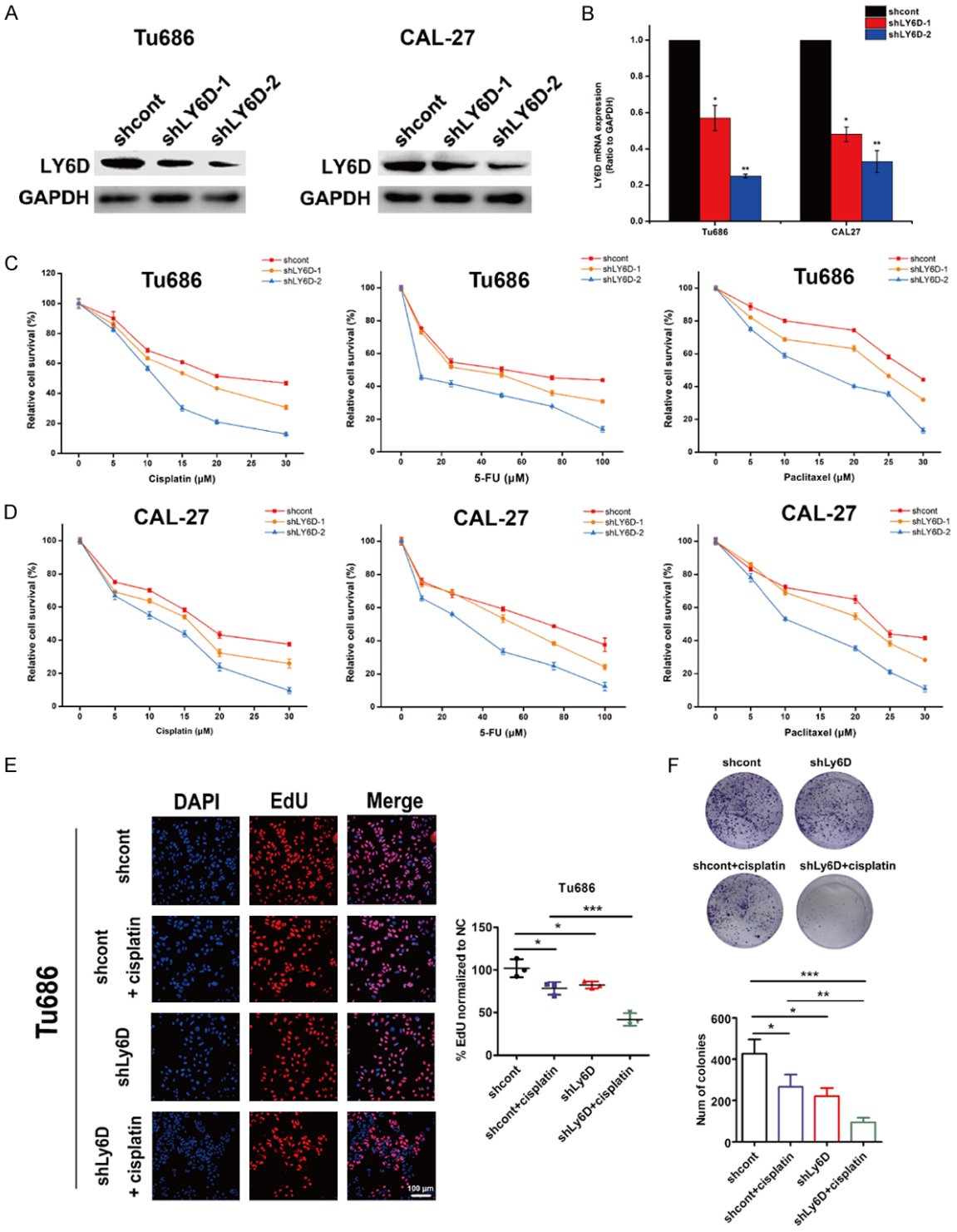

Figure 1. Loss of Ly6D confers chemosensitivity of LSCC cells. (A, B) The protein and mRNA levels of Ly6D were determined by western blot (A) and qPCR analysis (B) in indicated cells which were stably depleted of Ly6D. (C, D) Tu686 (C) and CAL27 cells (D) were stably expressing non-targeting shRNA (shcont) or shRNA against Ly6D (shLy6D-1 and shLy6D-2) and treated with various concentrations of chemotherapy drugs for 24 h. Then, the relative cell survival was detected by CCK8. (E) Tu686 cells with stable knockdown of Ly6D were treated with cisplatin (20 μM) for 24 h and subsequently evaluated by EdU assay. (F) Colony formation assay was performed for Tu686 cells with stable knockdown of Ly6D and cisplatin (20 μM) treatment.

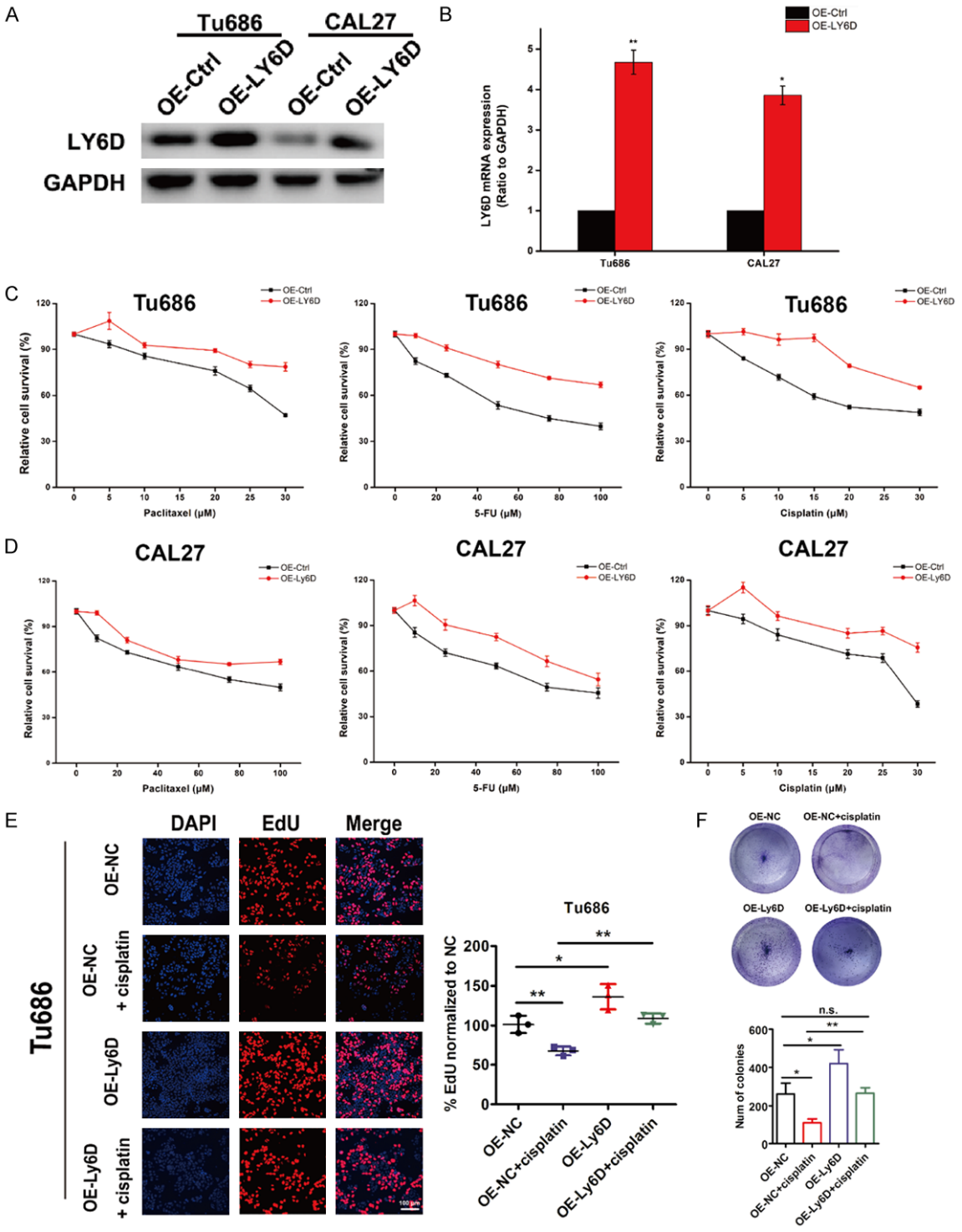

Figure 2. Overexpression of Ly6D favors chemoresistance in LSCC cells. (A, B) Tu686 and CAL27 cells were stably transduction of psin-flag or psin-flag-Ly6D plasmid and then the levels of Ly6D were analyzed by western blot (A) and qPCR analysis (B). (C, D) Tu686 (C) and CAL27 cells (D) were stably expressing flag-Ly6D in the presence of various concentrations of chemotherapy drugs for 24 h. Relative cell survival was detected by CCK8. (E) Tu686 cells with stable overexpression of Ly6D were exposed to cisplatin (20 μM) for 24 h. Then cell viability was measured by EdU assay. (F) Colony formation assay of Tu686 cells with stable overexpression of Ly6D in the presence of cisplatin (20 μM).

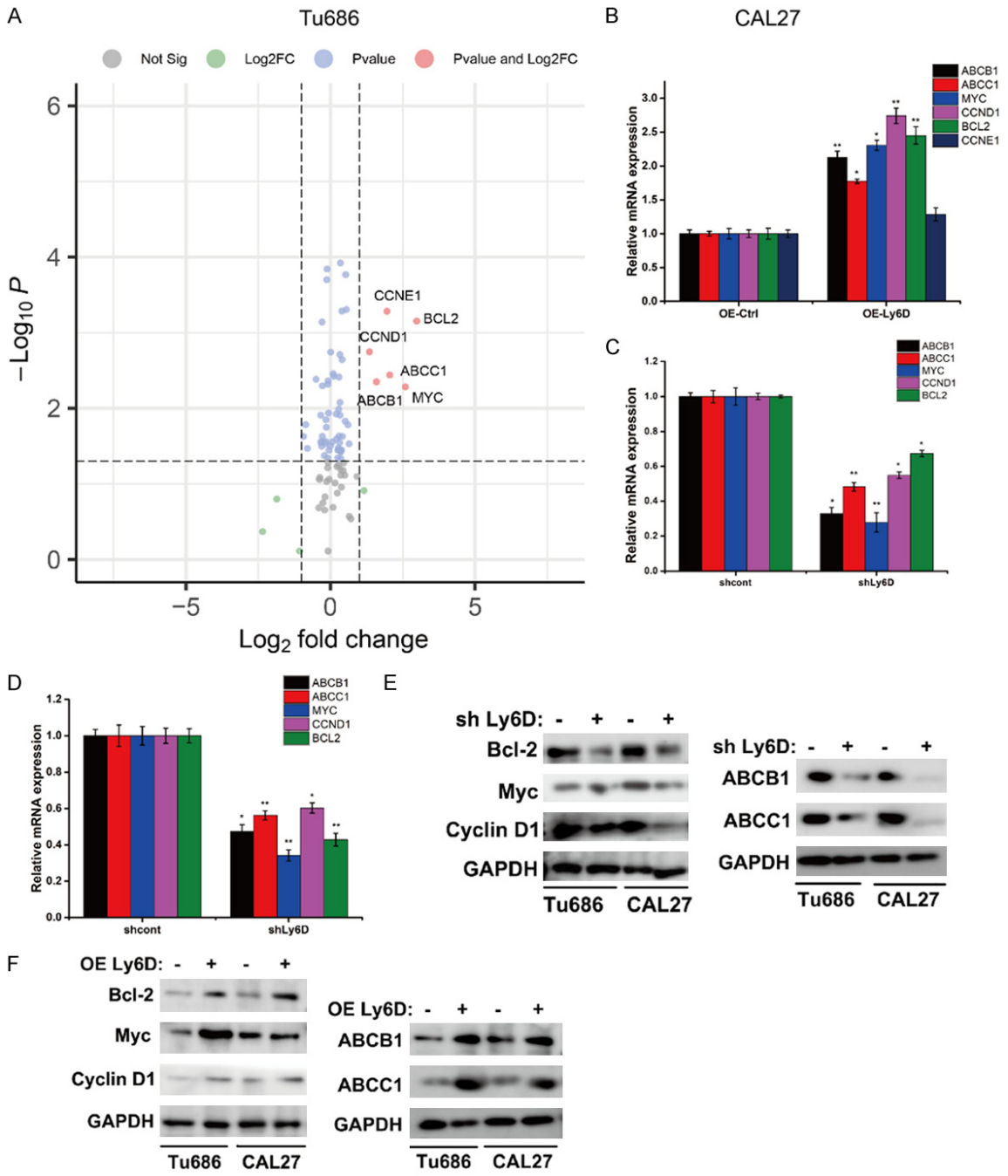

Figure 3. Effects of Ly6D on chemoresistance-related genes in LSCC cells. (A) Volcano plot displayed the genes significantly up-regulated in Tu686 cells stably expressing Ly6D from qPCR array analysis. The fold changes > 2-fold change (log_2) and P < 0.05 ($-log_{10}$) were the selected genes and labeled with light red color. (B, C) The indicated mRNA expressions was analyzed by qPCR in CAL27 cells stably expressing Ly6D (B) or Ly6D-targeting shRNA (C). (D) The mRNA levels of ABCB1, ABCC1, MYC, CCND1, and BCL2 was determined by qPCR analysis in LSCC cells expressing shcont or shLy6D-2. (E) Relative expression of the indicated proteins was detected by western blot in stable Ly6D knockdown cells. (F) Levels of indicated proteins were detected by western blot in LSCC cells with stably overexpressing Ly6D.

contrary, depletion of Ly6D in both Tu686 and CAL27 cells significantly attenuated the expression of these drug resistance related genes (**Figure 3C** and **3D**). Western blot results further revealed that the expression of ABCB1, ABCC1, BCL2, MYC, and CCND1 was signifi-

cantly upregulated and downregulated by Ly6D overexpression or Ly6D silencing, respectively (**Figure 3E** and **3F**). Taken together, these data indicated that Ly6D promotes chemotherapy resistance in LSCC cells by upregulating the expression of ABCB1, ABCC1, BCL2, MYC and CCND1.

Ly6D overexpression facilitates LSCC cells chemoresistance via the activation of Wnt/β-catenin pathway

To explore how Ly6D regulates the expression of drug resistance related proteins in LSCC cells, we used TCGA database to analyze the differential genes between patients with high Ly6D expression and low Ly6D expression. Gene Ontology (GO) analysis showed that Ly6D-mediated differential genes were mainly enriched in Wnt signaling pathway, cell cycle, negative regulation of apoptosis, etc. (**Figure 4A**). The results of volcano plot further showed that there were 126 upregulated genes and 4 downregulated genes in LSCC patients with Ly6D overexpression with fold changes were more than twice, among which S100A8, S100A7, and cyclin D1 were significantly upregulated (**Figure 4B**). Numerous studies have demonstrated that S100A8, S100A7, and cyclin D1 are the target genes of Wnt/β-catenin contribute to chemoresistance [23, 24]. Subsequently, qPCR analysis was performed to detect the expression of target genes of Wnt/β-catenin. As shown in **Figure 4C**, Ly6D silencing and Ly6D overexpression significantly suppressed or raised the expression of β-catenin target genes, such as AXIN2, JAG1, LEF1, and S100A8. The levels of S100A7 had no appreciable change upon Ly6D silencing or overexpression. The ratio of TOP-Flash to FOP-Flash in cells provides a measurement method for detecting β-catenin-mediated transcriptional activity [25]. The results showed that Ly6D silencing inhibited the TOP/FOP reporter activity, while overexpression of Ly6D activated the TOP/FOP fluorescence activity in LSCC cells, indicating that Ly6D could regulate the transcriptional activity of β-catenin (**Figure 4D**). Western blot results revealed that a dramatic inhibitory or activation effect of β-catenin expression was observed upon Ly6D silencing or overexpression, respectively (**Figure 4E**). Furthermore, the down-regulation of ABCB1, MYC, CCND1, S100A8, and AXIN2 mediated by Ly6D knockdown could be compromised by β-catenin overexpression (**Figure 4F**). In addition, ChIP-qPCR results showed that knockdown of Ly6D indeed disrupted the recruitment of β-catenin to the promoter region of AXIN2, CCND1, and ABCB1 (**Figure 4G**). Together, these results indicated that Ly6D promotes the expression of drug resistance-related proteins via the activation of Wnt/β-catenin pathway.

Suppression of Wnt/β-catenin pathway abrogates chemoresistance mediated by overexpression of Ly6D

To determine whether targeting β-catenin affects Ly6D-mediated chemoresistance in LSCC cells, CCK8 assay and colony formation assays were carried out to evaluate the effect of β-catenin on relative cell survival and long-term treatment in Ly6D knockdown cells with cisplatin treatment. We found that Ly6D silencing rendered LSCC cells more sensitive to chemotherapy which was reverted by β-catenin overexpression (**Figure 5A** and **5B**). Moreover, the effect of β-catenin on chemosensitivity in LSCC cells with Ly6D overexpression were evaluated by CCK8 and clonogenic assays. The results showed that the killing effects of cisplatin on LSCC cells with overexpressing Ly6D was significantly inhibited and depletion of β-catenin elicited a significant increase in chemosensitivity in Ly6D overexpression cells (**Figure 5C** and **5D**). The LSCC cells with Ly6D overexpression were further treated with ICG-001, an inhibitor of Wnt/β-catenin pathway, and then measured sensitivity to cisplatin using CCK8 and clonogenic assays. As indicated, Ly6D overexpression conferred chemo-refractory nature of LSCC cells was diminished upon ICG001 treatment (**Figure 5E** and **5F**). In summary, we showed that inhibition of β-catenin is sufficient for abolishing Ly6D-conferred chemoresistance.

Ly6D attenuates miR-509 expression to derepress β-catenin in LSCC cells

To investigate the effect of Ly6D on β-catenin gene (CTNNB1), we detected the expression level of CTNNB1 in LSCC cells with stable knockdown of Ly6D or overexpression of Ly6D. The results showed that the mRNA levels of CTNNB1 were obviously reduced or increased in Ly6D silenced or overexpressed cells, respectively (**Figure 6A**). In view of both the protein

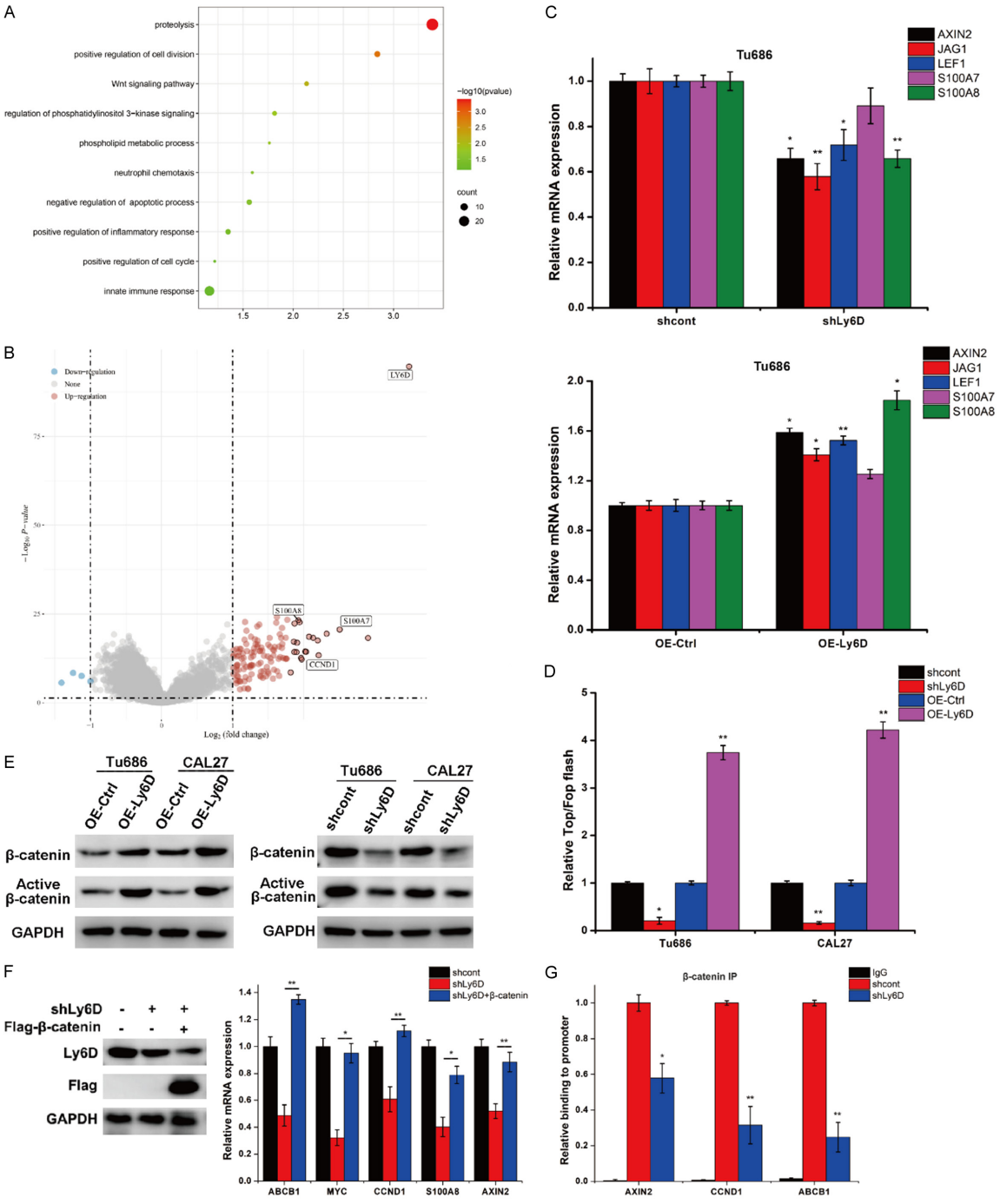

Figure 4. Wnt/β-catenin pathway is indispensable for Ly6D-mediated the expression of chemoresistance-related genes in LSCC. A. Gene Ontology (GO) analysis showed Ly6D-related pathways in LSCC tissue of TCGA database based on high expression group or low expression group of Ly6D. B. Volcano plot displayed that the differential genes between patients with high and low expression of Ly6D in LSCC. C. The mRNA levels of AXIN2, JAG1, LEF1, S100A7, and S100A8 of Tu686 cells with Ly6D overexpression or Ly6D silencing were determined by qPCR analysis. D. TOP/FOP flash reporter assay of LSCC cells with Ly6D overexpression or Ly6D silencing. The data were normalized to Renilla luciferase (internal control). E. Protein levels of β-catenin and active-β-catenin were detected by western blot in LSCC cells with Ly6D overexpression (left panel) or Ly6D silencing (right panel). F. Western blot analysis for the Tu686 cells with Ly6D knockdown were transient transfection of psin-flag-β-catenin plasmids (left panel). Then, the relative expression of indicated mRNAs was determined by qPCR analysis. G. The shcont or shLy6D cells were subjected to ChIP-qPCR analysis to determine the binding of β-catenin to the promoter regions of AXIN2, CCND1, and ABCB1. IgG was served as negative control.

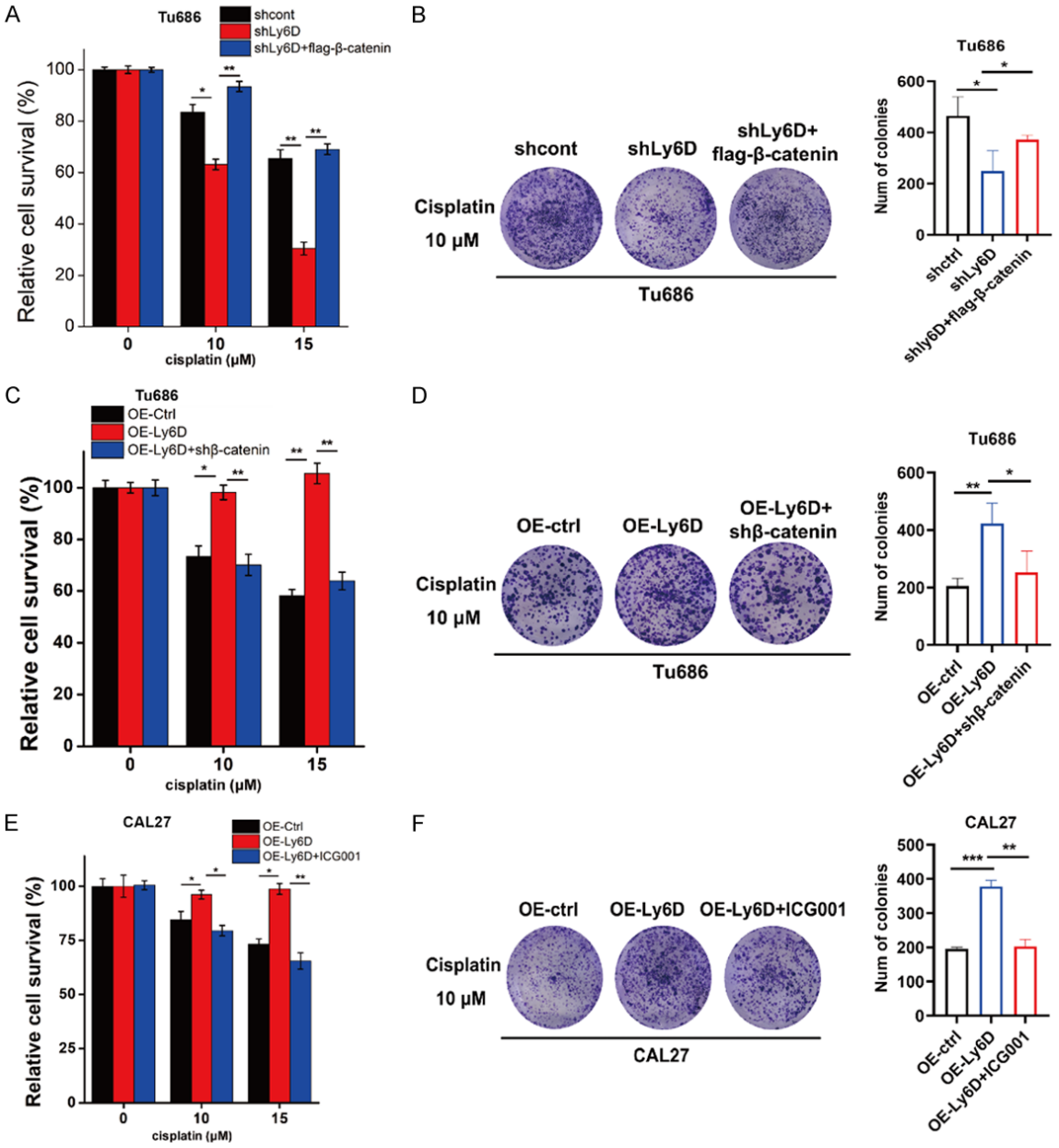

Figure 5. Inhibition of β-catenin disables Ly6D-mediated chemoresistance in LSCC cells. (A, B) Tu686 cells with Ly6D knockdown were transfection of psin-flag-β-catenin plasmids in the presence of various concentrations of cisplatin for 24 h and then analyzed by CCK8 (A) and clonogenic assay (B). (C, D) Tu686 cells with Ly6D overexpression were transfected of sh-β-catenin plasmids in the presence of various concentrations of cisplatin for 24 h and then analyzed by CCK8 (C) and clonogenic assay (D). (E, F) Tu686 cells with Ly6D overexpression were treated with 2 μM ICG001 in the presence of various concentrations of cisplatin for 24 h and then analyzed by CCK8 (E) and clonogenic assay (F).

and mRNA levels of β-catenin were induced by Ly6D overexpression, Actinomycin D assay was performed to examine the effect of Ly6D on the half-life of CTNNB1 mRNA. We found that knockdown of Ly6D significantly exacerbated the decay of CTNNB1 mRNA (**Figure 6B**). There is increasing evidence that miRNA plays a key role in mediating the stability of mRNA [18]. To clarify how Ly6D regulates the stability of CTNNB1 mRNA through miRNA, the Scan Mir miRNA PCR array for CTNNB1 was conducted by qPCR analysis. As shown in **Figure 6C**, the levels of miR-509-5p, miR-4776-5p, and let-7f-1-3p were upregulated, and the levels of miR-

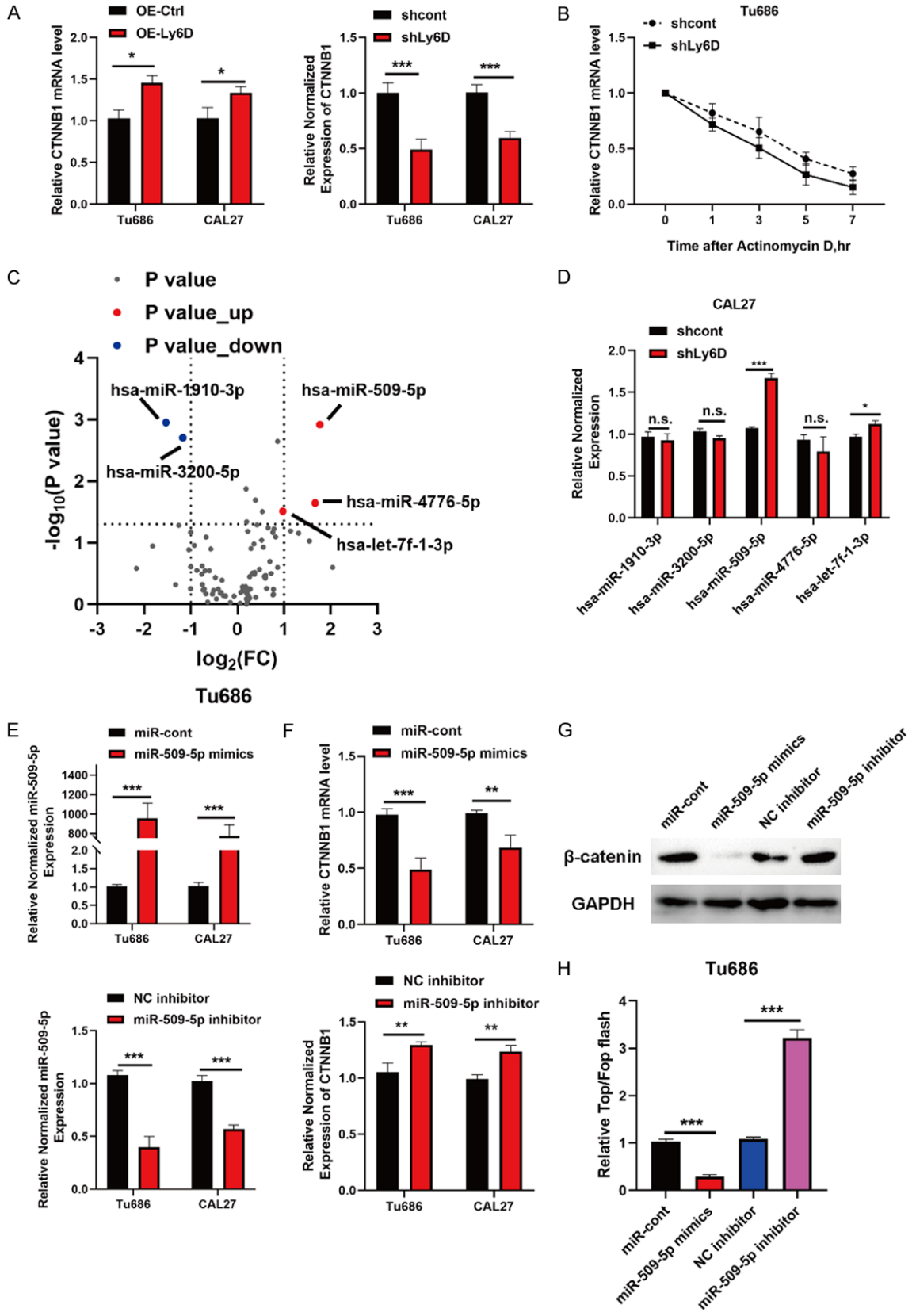

Figure 6. Ly6D promotes β-catenin expression via suppression of miR-509-5p. (A) The mRNA levels of CTNNB1 in LSCC cells with Ly6D silencing or Ly6D overexpression was detected by qPCR analysis. (B) qPCR analysis was per-

formed to determine the mRNA half-life time of CTNNB1 in LSCC cells with Ly6D silencing in the presence of actinomycin D (5 μg/ml) for 0 to 7 h. (C) Volcano plot showed the differential miRNAs which regulated by Ly6D in Tu686 cells with Ly6D silencing from the Scan Mir miRNA PCR array for CTNNB1. The fold changes > 2-fold change (log_2) and $P < 0.05$ ($-log_{10}$) were selected. The up-regulated miRNAs are marked red, and the down-regulated miRNAs are marked blue. (D) qPCR analysis was performed to determine the indicated miRNAs in CAL27 cells with Ly6D silencing. (E, F) Tu686 and CAL27 cells were transfected with miR-cont, miR-509-5p mimics, NC inhibitor, and miR-509-5p inhibitor for 48 h, the expression of miR-509-5p (E) and CTNNB1 (F) were analyzed by qPCR analysis. (G) Tu686 cells were transfected with miR-cont, miR-509-5p mimic, NC inhibitor, and miR-509-5p inhibitor for 48 h, the expression of β-catenin was detected using western blot. (H) TOP/FOP flash plasmids were co-transfected into Tu686 cells with miR-509-5p mimic or inhibitor, and the luciferase activity was measured 48 h later.

1910-3p and miR-3200-5p were downregulated in Tu686 cells with Ly6D silencing. The depletion of Ly6D in CAL27 cells prominently amplified miR-509-5p and let-7f-1-3p levels, and caused no significant difference in the expression of the other three miRNA (**Figure 6D**). Of note, miR-509-5p exhibited substantially increased upon Ly6D silencing in both LSCC cells. Accumulating evidences showed that miR-509-5p acted as tumor suppressor with low expression in various cancer entities [14, 15]. To address the direct connection between miR-509-5p and CTNNB1, LSCC cells were transduced with miR-509-5p mimic or inhibitor to detect protein and mRNA levels of β-catenin. As shown in **Figure 6E-G**, the protein and mRNA levels of β-catenin were noticeably reduced upon miR-509-5p mimic transfection. In contrast, miR-509-5p depletion by transfection with miR-509-5p inhibitor obviously facilitated β-catenin levels. Additionally, we found that the TOP/FOP fluorescence reporter activity was sharply decreased by transfection of miR-509-5p mimic, and the TOP/FOP fluorescence activity was pronouncedly elevated upon transfection with miR-509-5p inhibitor, indicating that miR-509-5p overexpression restrained the transcriptional activity of β-catenin (**Figure 6H**). Altogether, these data indicate that Ly6D overexpression decreases the levels of miR-509-5p and consequently results in propelling of β-catenin expression.

Downregulation of miR-509-5p is responsible for Ly6D augmenting β-catenin-mediated chemoresistance in LSCC cells

To dissect whether CTNNB1 is directly targeted by miR-509-5p, luciferase report system containing the potential binding region of miR-509-5p in the wild type and mutant CTNNB1 3'UTR was performed (**Figure 7A**). The results showed that transfection of miR-509-5p diminished luciferase activity of CTNNB1 3'UTR wild type and had no obvious effect on the luciferase activity of mutant type (**Figure 7B**). These results indicate that miR-509-5p directly targets to suppress CTNNB1 expression. To clarify the contribution of miR-509-5p on Ly6D-drove the activation of Wnt/β-catenin, miR-509-5p inhibitor was transduced into Tu686 cells with Ly6D silencing. We found that knockdown of Ly6D mediated down-regulation of ABCB1, MYC, CCND1, S100A8, and AXIN2 were reversed by transfection of miR-509-5p inhibitor (**Figure 7C**). To further verify the role of miR-509-5p on Ly6D-mediated chemoresistance, we infected shLy6D cells with NC inhibitor or miR-509-5p inhibitor in the presence of cisplatin. The CCK8 assays indicated that transfection of miR-509-5p inhibitor weakened the enhanced chemosensitivity caused by Ly6D silencing in LSCC cells (**Figure 7D**). Moreover, CCK8 and Colony formation assays were conducted to evaluate the role of miR-509-5p on chemotherapy sensitivity of Ly6D-overexpression cells. As expected, Ly6D-mediated the chemoresistance was compromised by transfection of the miR-509-5p mimics (**Figure 7E** and **7F**). It has been reported that miR-509-5p acts as a negative regulatory factor for MDM2 and FOXO1, thereby inhibiting malignant progression [26, 27]. Numerous studies have shown that MDM2 and FOXM1 play an important role in tumor chemotherapy resistance [28]. Consistent with previous studies, where miR-509-5p directly targets MDM2 and FOXM1, we observed a significant decrease in the expression of MDM2 and FOXM1 in cells overexpressed with miR-509-5p (**Figure 7G**). These data indicated that MDM2 and FOXM1 are also involved in chemoresistance mediated by miR-509-5p. Altogether, these findings suggest that Ly6D overexpression significantly attenuates the expression of miR-509-5p to derepress β-catenin, which in turn exacerbates drug resistance in LSCC cells.

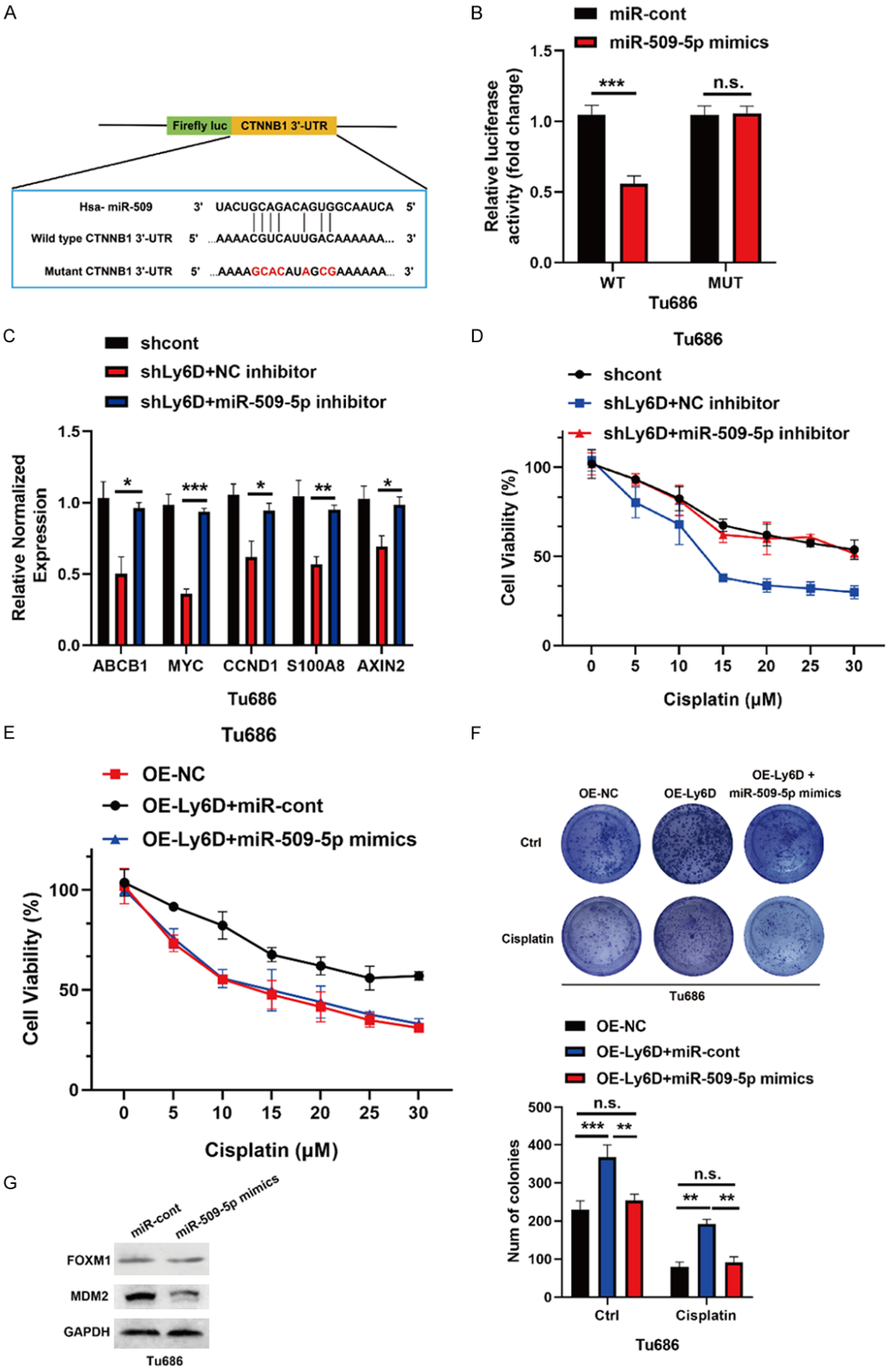
A
Firefly luc
CTNNB1 3'-UTR
Hsa- miR-509 3' UACUGCAGACAGUGGCAAUCA 5'
Wild type CTNNB1 3'-UTR 5' ...AAAACGUCAUUGACAAAAAA... 3'
Mutant CTNNB1 3'-UTR 5' ...AAAAGCACAUAGCGAAAAAA... 3'
B
miR-cont
miR-509-5p mimics
Relative luciferase activity (fold change)
***
n.s.
WT
MUT
Tu686
C
shcont
shLy6D+NC inhibitor
shLy6D+miR-509-5p inhibitor
Relative Normalized Expression
ABCB1
MYC
CCND1
S100A8
AXIN2
Tu686
D
Tu686
shcont
shLy6D+NC inhibitor
shLy6D+miR-509-5p inhibitor
Cell Viability (%)
Cisplatin (μM)
E
Tu686
OE-NC
OE-Ly6D+miR-cont
OE-Ly6D+miR-509-5p mimics
Cell Viability (%)
Cisplatin (μM)
F
OE-NC
OE-Ly6D
OE-Ly6D + miR-509-5p mimics
Ctrl
Cisplatin
Tu686
OE-NC
OE-Ly6D+miR-cont
OE-Ly6D+miR-509-5p mimics
Num of colonies
n.s.
Ctrl
Cisplatin
Tu686
G
miR-cont
miR-509-5p mimics
FOXM1
MDM2
GAPDH
Tu686

Figure 7. Inhibition of miR-509-5p by Ly6D is essential for β-catenin-drove chemoresistance of LSCC cells. (A) Graphic representation of construction of luciferase reporter gene with conserved miR-509-5p binding motif in 3'UTR of wild-type or mutant CTNNB1. (B) Luciferase reporter activity in 293T cells cotransfected with wild-type or mutant CTNNB1 3'UTR with mimics for miR-509-5p. (C) Tu686 cells with Ly6D silencing were transfected with NC inhibitor and miR-509-5p inhibitor for 48 h, and the indicated mRNA expressions were analyzed by qPCR assay. (D) Tu686 cells with Ly6D silencing were transfected with NC inhibitor and miR-509-5p inhibitor for 48 h in the presence of various concentrations of cisplatin, and then cell viability was analyzed by CCK8 assay. (E, F) CCK8 assay (E) and clonogenic assay (F) were conducted to analyze Tu686 cells with Ly6D overexpression were transfected with miR-cont and miR-509-5p mimics for 48 h in the presence of various concentrations of cisplatin. (G) Tu686 cells were transfected with miR-cont and miR-509-5p mimic for 48 h, the expression of MDM2 and FOXM1 was detected using western blot.

Discussion

LSCC is a kind of head and neck tumor with high incidence rate and mortality, which seriously endangers human health. Currently, the bottleneck of LSCC treatment is chemotherapy resistance, but its underlying mechanism remained elusive. Deeply exploring the drug resistance mechanism of LSCC and searching for novel and precise biomarkers are of great significance for overcoming drug resistance and improving patient survival [29]. Studies have shown that Ly6D expression is increased in multiple types of cancer and serves as a marker of poor prognosis [4, 5]. Comparatively more studies have focused on the function of Ly6D as a biomarker, but the mechanism of Ly6D in chemotherapy resistance is still unclear. In this study, we found that high expression of Ly6D is closely related to chemoresistance in LSCC cells. Mechanistically, high expression of Ly6D reduces the expression of miR-509-5p, thereby releasing the inhibitory effect on the target gene CTNNB1, leading to an increase in the expression of β-catenin and activation of Wnt/β-catenin signaling, ultimately promotes the resistance of LSCC to chemotherapy drugs (**Figure 8**).

It is well established that Wnt/β-catenin signaling is abnormally activated in various tumors and closely related to chemotherapy resistance [30]. In tumor cells, macropinocytosis serves as a nutrient delivery pathway, enabling tumor cells to survive in nutrient-deficient environments. Previous studies have demonstrated that Wnt/β-catenin signaling activation drives micropinocytosis to facilitate tumor survival [31]. Although the biosynthetic process is mostly blocked by chemotherapeutic drugs, cancer cells can still take up essential biological materials through macropinocytosis, thus promoting chemoresistance [32]. Previous studies have shown that Ly6D located on the cell membrane interacts with integrin β1, activates downstream FAK-Src family kinase (SFK) signaling, induces macropinocytosis, and consequently promotes the survival of senescent cells [4, 33]. This study found that overexpression of Ly6D can up-regulate the expression level of β-catenin and promote chemoresistance in LSCC cells. Whether this process is caused by the induction of macropinocytosis requires further investigation.

Mounting evidences have demonstrated that miRNAs play a key role in the occurrence and development of tumors [10]. The results showed that Ly6D regulates the expression of β-catenin mainly via affecting the mRNA stability (**Figures 4E**, **4F**, **6A** and **6B**). Numerous reports have shown that miRNA is involved in mRNA stability [18]. We screened miRNAs that could regulate CTNNB1 expression in LSCC cells with Ly6D knockdown by PCR array, and identified the Ly6D-mediated miRNA, miR-509-5p (**Figure 6C** and **6D**). Several studies have shown that miR-509-5p acts as a tumor suppressor gene in various tumors such as lung cancer, ovarian cancer, and colon cancer [34]. A previous study revealed that miR-509-5p is negatively correlated with poor prognosis, and overexpression of miR-509-5p could increase the sensitivity of pancreatic cancer cells to gemcitabine [35]. We found that LSCC cells with high expression of Ly6D attenuated miR-509-5p levels, thereby promoting β-catenin expression, which in turn led to drug resistance (**Figures 6C**, **7D-F**). Ly6D is well known as a cell-surface protein that lacks any cytoplasmic domain. What is most puzzling is the molecular mechanism by which Ly6D regulates miRNA expression in the cytoplasm. Recent studies have shown that the conditional deletion of PTEN in the murine prostate epithelium leads to the expansion of transformed $Ly6D^+$ progenitor cells without compromising the property of tumor stem cells [36]. Ly6D secretes amphi-

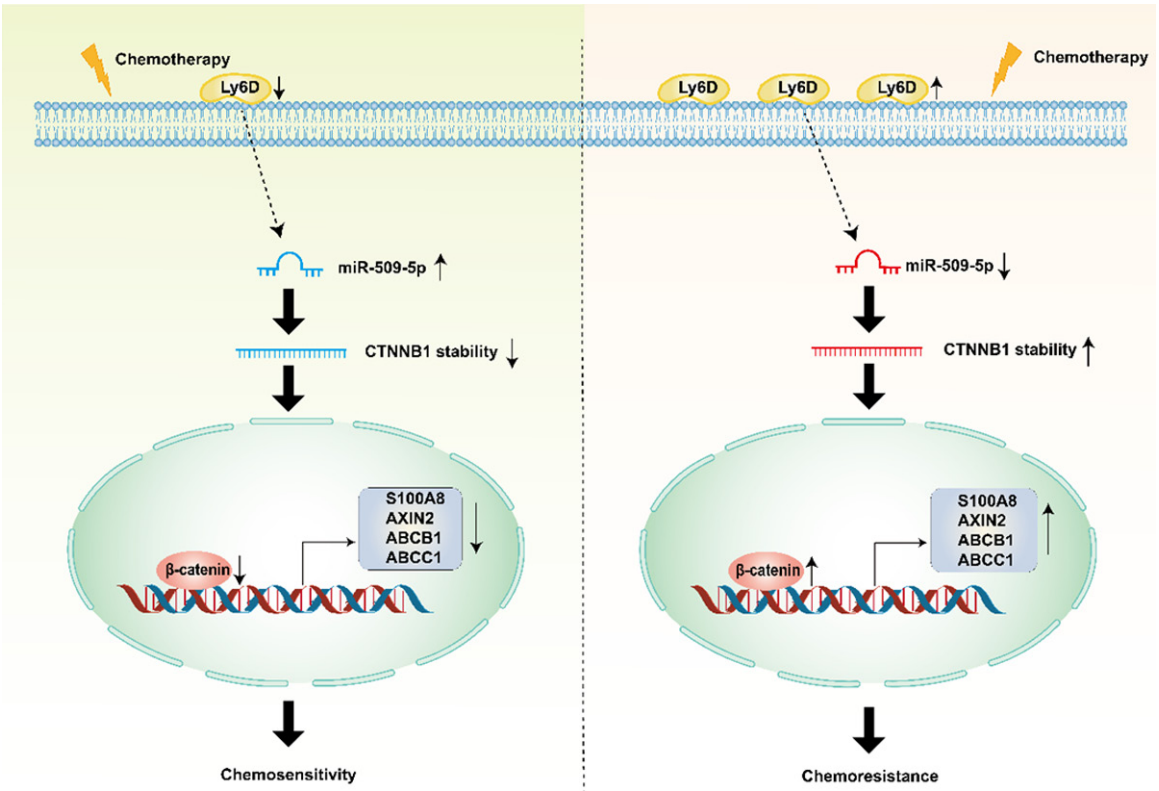

Figure 8. Schematic diagram of the molecular mechanism of Ly6D promoting chemoresistance in LSCC.

regulin (AREG) from malignantly transformed progenitor cells of the prostate, which binds to its cell surface epidermal growth factor receptor (EGFR) to activate the MAPK signaling pathway, and promotes castration-resistant prostate cancer growth via an autocrine positive feedback loop [36]. It is reported that under hypoxic conditions, EGFR can phosphorylate the Tyr393 site of argonaute 2 (AGO2), thereby regulating the maturation process of miRNAs [37]. Studies have shown that knockdown of EGFR in glioblastoma leads to upregulation of miR-200c, thereby attenuating ZEB1 expression and ultimately inhibiting tumor cell migration [38]. The results of proteomics and co-immunoprecipitation showed that LY6D located on the cell membrane interacted with the transmembrane integrin β1, thereby transmitting extracellular signals into the intracellular to activate FAK-SFK signaling, and promotes the formation of vacuoles in senescent cells [4, 30]. In addition, treatment of prostate cancer cells with inhibitors of the downstream target protein Src of integrin β1 can induce the expression of the tumor suppressor gene miR-30 family members, thereby inhibiting epithelial-mesenchymal transition [39]. Therefore, we speculated that ly6D may indirectly regulate miR-509-5p expression in the cytoplasm by regulating the secreted protein-EGFR pathway or interacting with transmembrane integrin β1. Additional research effort will be needed to elucidate that Ly6D-mediated miR-509-5p expression rely on integrin β1 or other signal transduction mediators.

Conclusions

In summary, our data revealed that overexpression of Ly6D blocked miR-509-5p expression to derepress its target CTNNB1, thereby activated Wnt/β-catenin signaling and ultimately facilitated β-catenin-mediated transactivation of S100A8, Axin2, ABCB1, and ABCC1 to drive chemoresistance of LSCC cells. Furthermore, MDM2 and FOXM1 are also involved in chemoresistance mediated by miR-509-5p. Therefore, these findings suggest that Ly6D will serve as a potential biomarker to determine the chemosensitivity of LSCC, and targeting Ly6D may be a new strategy for LSCC treatment.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No.

82003146 and No. 32200321) and the Shanxi Provincial Key Research and Development Program (Grants No. 201903D321090).

Disclosure of conflict of interest

None.

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Table S1. Primers used in the experiments

Primer name	Primer sequence (5'-3')
Ly6D-qF	GTGAAGAAGGACTGTGCGGA
Ly6D-qR	CAGGCTGGGGGCTAAGATGA
ABCB1-qF	CAGGCTTGCTGTAATTACCCA
ABCB1-qR	TCAAAGAAACAACGGTTCGG
ABCC1-qF	CTCTATCTCTCCCGACATGACC
ABCC1-qR	AGCAGACGATCCACAGCAAAA
MYC-qF	TAGTGGAAAACCAGCAGCCT
MYC-qR	AGAAATACGGCTGCACCGAG
CCND1-qF	CTGATTGGACAGGCATGGGT
CCND1-qR	GTGCCTGGAAGTCAACGGTA
BCL2-qF	GGAGGATTGTGGCCTTCTTT
BCL2-qR	GCCCAATACGACCAAATCCGTTGA
CCNE1-qF	TTTCAGGGTATCAGTGGTG
CCNE1-qR	ACATGGCTTTCTTTGCTC
CCND1-qF	CTGATTGGACAGGCATGGGT
CCND1-qR	GTGCCTGGAAGTCAACGGTA
AXIN2-qF	TAACCCCTCAGAGCGATGGA
AXIN2-qR	AGAGACAGGCATGGGTTTGG
JAG1-qF	TGCCAAGTGCCAGGAAGT
JAG1-qR	GCCCCATCTGGTATCACACT
LEF1-qF	TGGTAAACGAGTCCGAAATCA
LEF1-qR	TGTGTTTGTCCGACCACCT
S100A7-qF	CTTCCCCAACTTCCTTAGTG
S100A7-qR	GTAGTCTGTGGCTATGTCTC
S100A8-qF	TCCTTGCGATGGTGATAAAA
S100A8-qR	GGCCAGAAGCTCTGCTACTC
β-catenin-qF	CTGAGGAGCAGCTTCAGTCC
β-catenin-qR	GGCCATGTCCAACTCCATCA
GAPDH-qF	AAGGTCGGAGTCAACGGATTT
GAPDH-qR	CCTGGAAGATGGTGATGGGATT
Axin2-ChIPqF	CTGGAGCCGGCTGCGCTTTGAT
Axin2-ChIPqR	CGGCCCCGAAATCCATCGCTCT
CCND1-ChIPqF	AGGCGCGGCGGCTCAGGGAT
CCND1-ChIPqR	ACTCTGCTGCTCGCTGCTACT
ABCB1-ChIPqF	CCCTATTAAGTAAGCCGCTGTG
ABCB1-ChIPqR	GTACGCGCAAGCAGACAGT
shLY6D-1F	CCGGGAGGACCTGTGCAATGAGAAGCTCGAGCTTCTCATTGCACAGGTCCTCTTTTTG
shLY6D-1R	AATTCAAAAAGAGGACCTGTGCAATGAGAAGCTCGAGCTTCTCATTGCACAGGTCCTC
shLY6D-2F	CCGGGCAATGAGAAGCTGCACAACGCTCGAGCGTTGTGCAGCTTCTCATTGCTTTTTG
shLY6D-2R	GCAATGAGAAGCTGCACAACGCTCGAGCGTTGTGCAGCTTCTCATTGC