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RESEARCH PAPER

Long non-coding RNA ACTA2-AS1 inhibits the cisplatin resistance of non-small cell lung cancer cells through inhibiting autophagy by suppressing TSC2

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ABSTRACT

Long non-coding RNA (lncRNA) ACTA2-AS1 has been reported to play an important role in the progression of multiple human malignancies. The article aims to explore the role of ACTA2-AS1 on the cisplatin resistance of non-small cell lung cancer (NSCLC). RT-qPCR was performed to investigate the expression of ACTA2-AS1 in cisplatin-resistant NSCLC cell lines. Western blot was used to investigate the effects of ACTA2-AS1 on autophagy-related protein expression. RIP assay and RNA pull down were used to analyze the combination of ACTA2-AS1 and enhancer of zeste homolog 2 (EZH2), and CHIP was used to analyze the combination of tuberous sclerosis complex-2 (TSC2) gene promoter and Lys-27 of histone H3 (H3K27me3). In this study, ACTA2-AS1 was downregulated in cisplatin-resistant NSCLC cell lines. ACTA2-AS1 negatively regulated the cell viability and positively regulated the cell apoptosis of cisplatin-resistant NSCLC cell lines. Furthermore, our results demonstrated that ACTA2-AS1 promoted cisplatin-resistant NSCLC cells apoptosis through inhibiting autophagy. The regulation of ACTA2-AS1 to the cisplatin-resistant NSCLC cell autophagy was reversed by TSC2 increasing. Importantly, our results displayed that ACTA2-AS1 bound with EZH2, and TSC2 gene promoter combined with H3k27me3. The inhibition of ACTA2-AS1 to TSC2 expression was recused by EZH2 silencing. In conclusion, ACTA2-AS1 inhibited the cisplatin resistances of NSCLC cell lines through suppressing TSC2 expressing by recruiting EZH2 to TSC2 gene promoter.

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1. Introduction

Lung cancer is one of the most common malignant tumors. Non-small cell lung cancer (NSCLC) is the main type of lung cancer that accounts for more than 85% patients [1]. Many patients with NSCLC are diagnosed in the advanced stage and miss the most treatable stage [2]. The 5-year survival rate of NSCLC patients is only 15.9%. Currently, the standard treatments for NSCLC still are surgical resection, radiotherapy, and targeted therapy for single-drug or combined. However, the drug treatment of most NSCLC patients ended in failure because of the drug resistance. As the "dark matters" of the human genome, in recent years, long non-coding RNAs (lncRNAs) were proved to play a crucial role in the resistance of NSCLC to cisplatin [3].

LncRNA is a type of non-coding RNA with a transcript length of more than 200 nucleotides. In recent years, increasing studies have proved that lncRNAs play a crucial role in the development of malignant tumors and drug resistance. For instance, Lin et al. found that the expression of lncRNA HOXA-AS3 is significantly increased in cisplatin-resistant NSCLC cell lines, and HOXA-AS3 knockdown could enhance the sensitivity of the NSCLC cell lines to cisplatin [4]. Another lncRNA, CCAT1, is high-expressed in the tumor tissues of NSCLC patients and the cell lines of NSCLC induced by gefitinib. CCAT1 decreasing could promote the gefitinib resistance in the cells by regulating miR-218/HOXA1 signaling pathway [5]. Similarly, lncRNA BLACT1, UCA1, LINC01116 also involve in the regulation of the NSCLC cell line resistance [6–8]. ACTA2 antisense RNA 1 (ACTA2-AS1) is a new lncRNA, which has been reported to participate in the development of ovarian cancer, breast cancer and liver cancer [9–11]. However, the expression and function of ACTA2-AS1 in NSCLC and its effect on NSCLC cell resistance are still unclear. According to the report of Ying et al., ACTA2-AS1 was identified as a downregulated lncRNA in lung adenocarcinoma samples and cells [12].

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LncRNAs regulate the biological function of target genes on multiple levels, such as transcription, post-transcription, and epigenetics [13]. Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase that functions as the catalytic subunit of the polycomb repressive complex 2, and participates in the epigenetic regulation of genes [14]. Chen et al. revealed that FOXP4-AS1 is upregulated in gastric cancer tissues, and it could to involve the formation of carcinogenic complex via binding to lysine-specific histone demethylase 1A and EZH2 and positively regulates their expression [15]. Moreover, EZH2 can be recruited to the promoter of the target genes by lncRNAs to regulate the expression of target genes. LncRNA HERES could regulate the activation of Wnt signaling pathway through binding with EZH2 [16]. MALAT1 overexpression could recruit EZH2 to tuberous sclerosis complex-2 (TSC2) gene promoter regions to elevate H3K27me3 and epigenetically inhibited TSC2 transcription [17]. Here, the aim of this study is to investigate the role and mechanism of action of ACTA2-AS1 in the cisplatin resistance of NSCLC cell lines.

2. Materials and methods

2.1. Cell culture

Two NSCLC cell lines, A549 and H1299, were provided by the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cisplatin was used to induce the drug-resistant A549 and H1299, which were named as A549/DDP and H1299/DDP, respectively. The cell culture medium consists of 100 U/ml penicillin (Invitrogen Life Technologies, Carlsbad, CA, USA), 10% fetal bovine serum (FBS; Invitrogen Life Technologies) and RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA). All cells were cultured at 37°C in a humidified incubator with 5% CO_2.

2.2. QRT-PCR

Total RNA was extracted from the frozen tissues or cultured cells utilizing TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration of RNA was ensured using the NANODROP 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Next step, the reversely transcribed reaction was carried out to synthetize complementary DNA using the PrimeScript™ RT Reagent Kit (Takara, Tokyo, Japan) and the total RNA as template. Subsequently, qRT-PCR was performed using SYBR Premix Ex Taq™ II (Takara, Japan, DRR820A). The qRT-PCR was carried out on an ABI 7500 system (Applied Biosystems, Foster City, CA, USA). The expression of target genes was normalized by β-actin acting as an internal control. PCR reaction conditions were performed as follows: 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 60 s. The relative expression of ACTA2-AS1 was analyzed using the $2^{-\Delta\Delta CT}$ method for quantification. The primers used in our study were obtained by the Sheng Gong Company (Shanghai, China).

ACTA2-AS1: forward, 5'-GTTCTGGAGGCTG GCTTGATATGG-3', and reverse, 5'- TCCTTCATC GGTAGGCAACAAACG-3';

EZH2: forward, 5'-GCCAGACTGGGAAGAA ATCTG-3', and reverse, 5'- TGTGCT GGAAAA TCCAAGTCA-3';

TSC2: forward, 5'-CTCGCCATCCTGTCCAAT -3', and reverse, 5'- GACAGGCAAT ACCGTC CAAG-3′;

β-actin: forward, 5'-ACCCCCACTGAAAAAG ATGA-3′, and reverse, 5'-ATC TTCAAACCT CATGATG-3′.

2.3. MTS

Cell viability assays were performed using MTS (Promega, Madison, WI). The optical density (OD) was measured at 490 nm by Plate Chameleon Multi-technology plate-reader (Hidex, Turku, Finland).

2.4. Cell apoptosis analysis

The Annexin V-APC/7-AAD apoptosis detection kit was purchased from Vazyme Biotech Co.,Ltd (Nanjing, China), and was used to determine the apoptotic rate of A549/DDP cells and H1299/DDP cells. Briefly, the cells were suspended in 1,000 ml binding buffer. Then, 100 μl of cell suspension was maintained with 5 μl 7-AAD and 5 μl Annexin V-APC for 15 min at 37°C in the dark. After that, each tube was added with 400 μl binding buffer, and then the apoptotic rate of the cells was

determined using flow cytometry (FACSCAN, BD Biosciences) programmed with Cell Quest software (BD Biosciences, San Diego, CA, USA).

2.5. Western blotting analysis

For Western blotting assay, RIPA lysis buffer (Solarbio, Beijing, China) was obtained, and was used to extract total protein. 25 μg of total protein was separated on the 12% SDS-PAGE for 2.5 hours. Then, the proteins were transferred to polyvinylidene fluoride (PVDF) membrane. After that, the membranes were maintained with 5% nonfat milk for 1 hour at 37°C. Following incubation with the antibodies, including rabbit anti-LC3 -I (1:1000), rabbit anti- LC3-II (1:1000), rabbit anti-Beclin-1 (1:1000), rabbit anti-TSC2 (1:1000), rabbit anti-mTOR (1:1000), rabbit anti-p-mTOR (1:1000), and rabbit anti-β-action (1:5000) overnight at 4°C, the membranes were maintained with the goat anti-rabbit secondary antibody (1:1000) for 1 hour at room temperature. The primary antibodies and secondary antibody were obtained from Cell Signaling Technology (CST; Donvers, MA, USA). At last, the membranes were stained with ECL reagent (Solarbio) for 5 min at room temperature to visualize the protein bands. The relative expression of protein was conducted using Image J software.

2.6. Statistical analysis

All values were exhibited as mean ± standard deviation, and were analyzed utilizing SPSS 22.0 statistical software (Chicago, IL, USA) and GraphPad Prism 4.0 (Inc., La Jolla, CA, USA). The independent samples *t*-test was chosen for the statistical analysis between two groups. One-way ANOVA was utilized to the analysis of the significant difference among three and more groups. $P < 0.05$ represented the statistically significant.

3. Results

3.1. ACTA2-AS1 is increased in A549/DDP and H1299/DDP cells

Recombinant cell lines A549/DDP and H1299/DDP were induced using cisplatin. The results showed that the ACTA2-AS1 expression level markedly lower in both A549/DDP and H1299/DDP cells than that in both A549 and H1299 cells (Figure 1).

3.2. ACTA2-AS1 increasing inhibited A549/DDP and H1299/DDP cells viability and promoted cell apoptosis

Here, qRT-PCR was utilized to validate the transfection efficiency of ACTA2-AS1 overexpression plasmid in A549/DDP and H1299/DDP cells. As shown in Figure 2(a), compared with the ACTA2-AS1-NC group, the ACTA2-AS1 expression level were remarkably increased in ACTA2-AS1-OE group in the two cell lines. Importantly, ACTA2-AS1 overexpressing significantly suppressed the cell viability of A549/DDP and H1299/DDP cells (Figure 2(b)). Upregulation of ACTA2-AS1 led to an increased apoptotic rate in A549/DDP and H1299/DDP cells (Figure 2(c)).

3.3. ACTA2-AS1 decreasing enhanced A549/DDP and H1299/DDP cells viability and suppressed cell apoptosis

To explore the influence of ACTA2-AS1 silencing to A549/DDP and H1299/DDP cells viability and apoptosis, the cells were treated with the lentivirus expressing ACTA2-AS1 shRNA and shRNA negative control. The ACTA2-AS1 expression level was

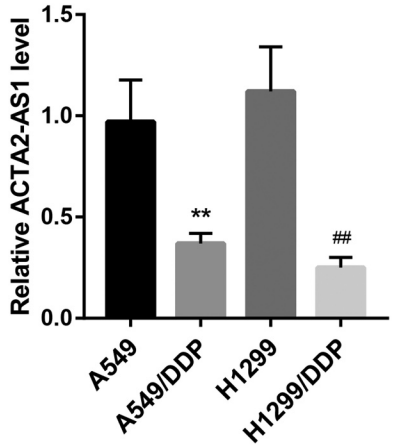

Figure 1. ACTA2-AS1 was decreased in A549/DDP and H1299/DDP cells.

The expression level of ACTA2-AS1 in A549/DDP, A549, H1299/DDP and H1299 cells was determined by RT-qPCR. **p < 0.01, ##p < 0.01.

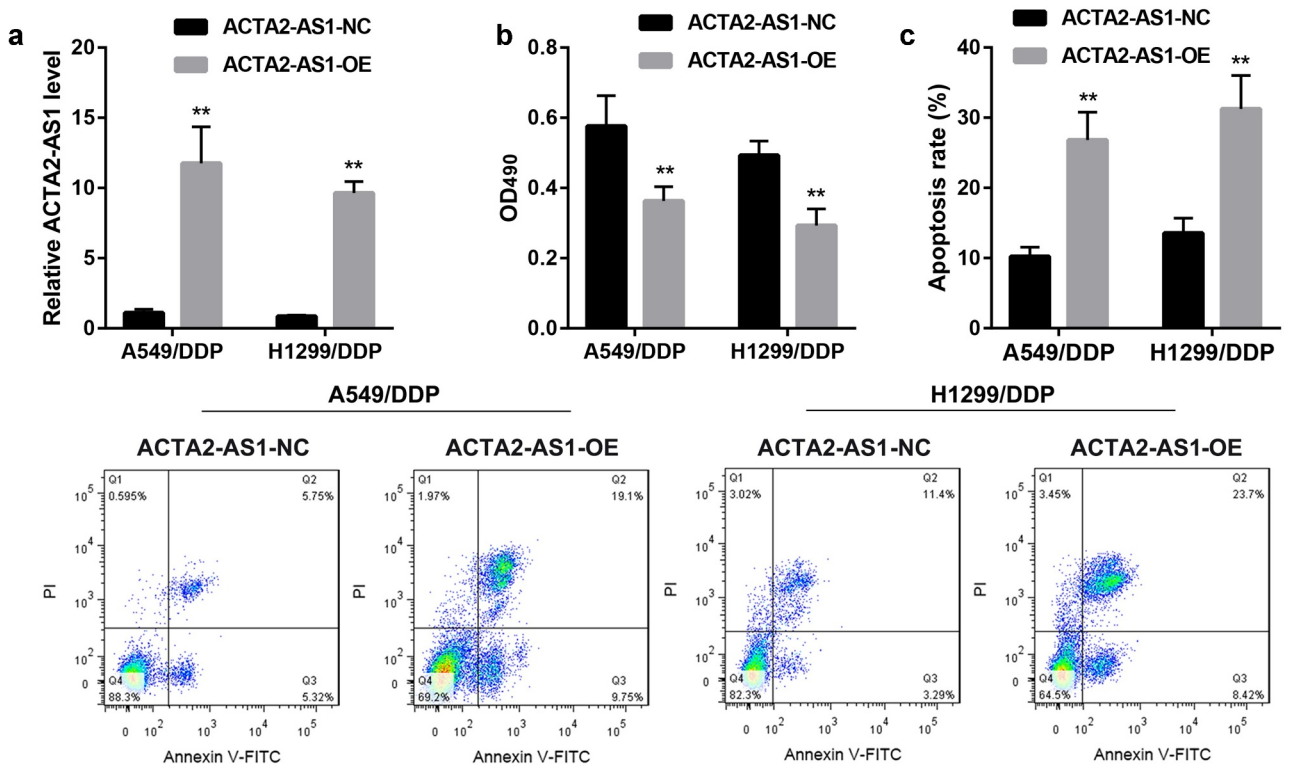

Figure 2. ACTA2-AS1 increasing inhibited the viability and promoted the apoptosis of the cisplatin-resistant A549 and H1299 cells. A549/DDP and H1299/DDP cells were transfected with the ACTA2-AS1 overexpression plasmid and control plasmid (empty vector). Subsequently, (a) the ACTA2-AS1 expression in A549/DDP and H1299/DDP cells was detected using qRT-PCR. (b) the cell viability was determined utilizing MTS assay. (c) flow cytometry was performed to detect cell apoptosis. **p < 0.01.

reduced by ACTA2-AS1 shRNA treatment (Figure 3(a)). ACTA2-AS1 silencing enhanced the cell viability (Figure 3(b)) and inhibited the cell apoptosis (Figure 3(c,d)) of A549/DDP and H1299/DDP cells.

3.4. ACTA2-AS1 facilitated A549/DDP and H1299/DDP cells apoptosis via inhibiting autophagy

To investigate whether ACTA2-AS1 regulates cell apoptosis through autophagy, we detected the LC3-II/LC3-I and Beclin-1 expression levels in both the A549/DDP and H1299/DDP cells transfected with or without ACTA2-AS1 overexpression plasmid. Our data showed that ACTA2-AS1 increasing decreased the levels of LC3-II/LC3-I ratio and Beclin-1 expression in A549/DDP and H1299/DDP cells (Figure 4(a)). In addition, the A549/DDP and H1299/DDP cells transfected with the ACTA2-AS1 overexpression plasmid were treated with rapamycin (an activator of autophagy). Our data demonstrated that rapamycin treatment increased the cell viability of A549/DDP and H1299/DDP cells, and the inhibition of ACTA2-AS1 was also partly reversed by rapamycin treatment (Figure 4(b)). Moreover, rapamycin treatment suppressed the cell apoptosis of A549/DDP and H1299/DDP cells, and the promotion of ACTA2-AS1 to cell apoptosis was also partly reversed by rapamycin treatment (Figure 4(c)). Above results indicated that ACTA2-AS1 promoted the A549/DDP and H1299/DDP cells apoptosis via regulating autophagy.

3.5. TSC2 overexpression reversed the inhibition of ACTA2-AS1 to A549/DDP and H1299/DDP cells autophagy

We next detected the TSC2, mTOR and p-mTOR expression levels in both the A549/DDP and H1299/DDP cells transfected with or without ACTA2-AS1 overexpression plasmid. The results revealed that ACTA2-AS1 overexpression obviously upregulated

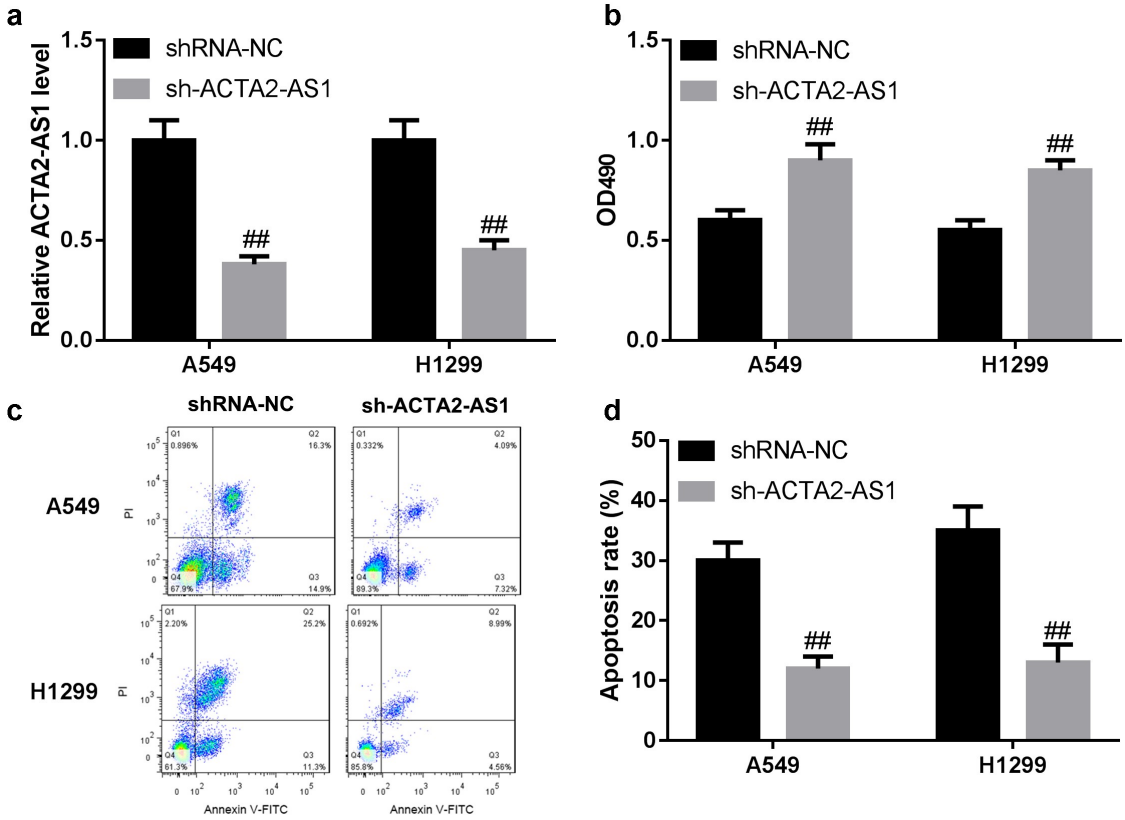

Figure 3. ACTA2-AS1 silencing promoted the viability and inhibited the apoptosis of the cisplatin-resistant A549 and H1299 cells. The A549/DDP and H1299/DDP cells were treated with the lentivirus expressing ACTA2-AS1 shRNA or shRNA negative control. Then, (a) the expression level of ACTA2-AS1 in A549/DDP and H1299/DDP cells was determined using qRT-PCR. (b) the cell viability was determined utilizing MTS assay. (c) flow cytometry was performed to detect cell apoptosis. ##$p < 0.01$.

the p-mTOR/mTOR ratio and downregulated TSC2 expression in both A549/DDP and H1299/DDP cells (Figure 5(a)). The LC3-positive cell number in ACTA2-AS1-OE group was higher than that in ACTA2-AS1-NC group (Figure 5(b)). Meanwhile, our results indicated that TSC2 overexpressing suppressed the phosphorylation of mTOR but increased the ratio of LC3-II/LC3-I and the expression of Beclin-1. The promotion of ACTA2-AS1 to p-mTOR/mTOR ratio and the inhibition of ACTA2-AS1 to LC3-II/LC3-I ratio and Beclin-1 expression were partly reversed by TSC2 overexpression (Figure 5(c)).

3.6. ACTA2-AS1 combined with EZH2 protein

To explore the regulatory mechanism of ACTA2-AS1 to TSC2 expression, we detected the subcellular localization of ACTA2-AS1. As shown in Figure 6(a), the major subcellular localization of ACTA2-AS1 is cytoplasm. The level of ACTA2-AS1 in cytoplasm is significantly higher than that in nuclear (Figure 6(b,c)). The combination of ACTA2-AS1 and EZH2 was proved using RNA pull down (Figure 6(f,g)) and RIP assay (Figure 6(d,e)). These data revealed that ACTA2-AS1 maybe regulate the expression of TSC2 through EZH2.

3.7. ACTA2-AS1 recruited EZH2 to elevate Lys-27 of histone H3 and epigenetically inhibited TSC2 expression

Previous studies demonstrated that EZH2 could regulate gene expression in ways besides Lys-27 of histone H3 (H3K27me3) [18]. In this study, the results of CHIP assay showed that TSC2 promoter was enriched with H3K27me3 in both A549/DDP and H1299/DDP cells (Figure 7(a,b)). EZH2 increasing inhibited TSC2

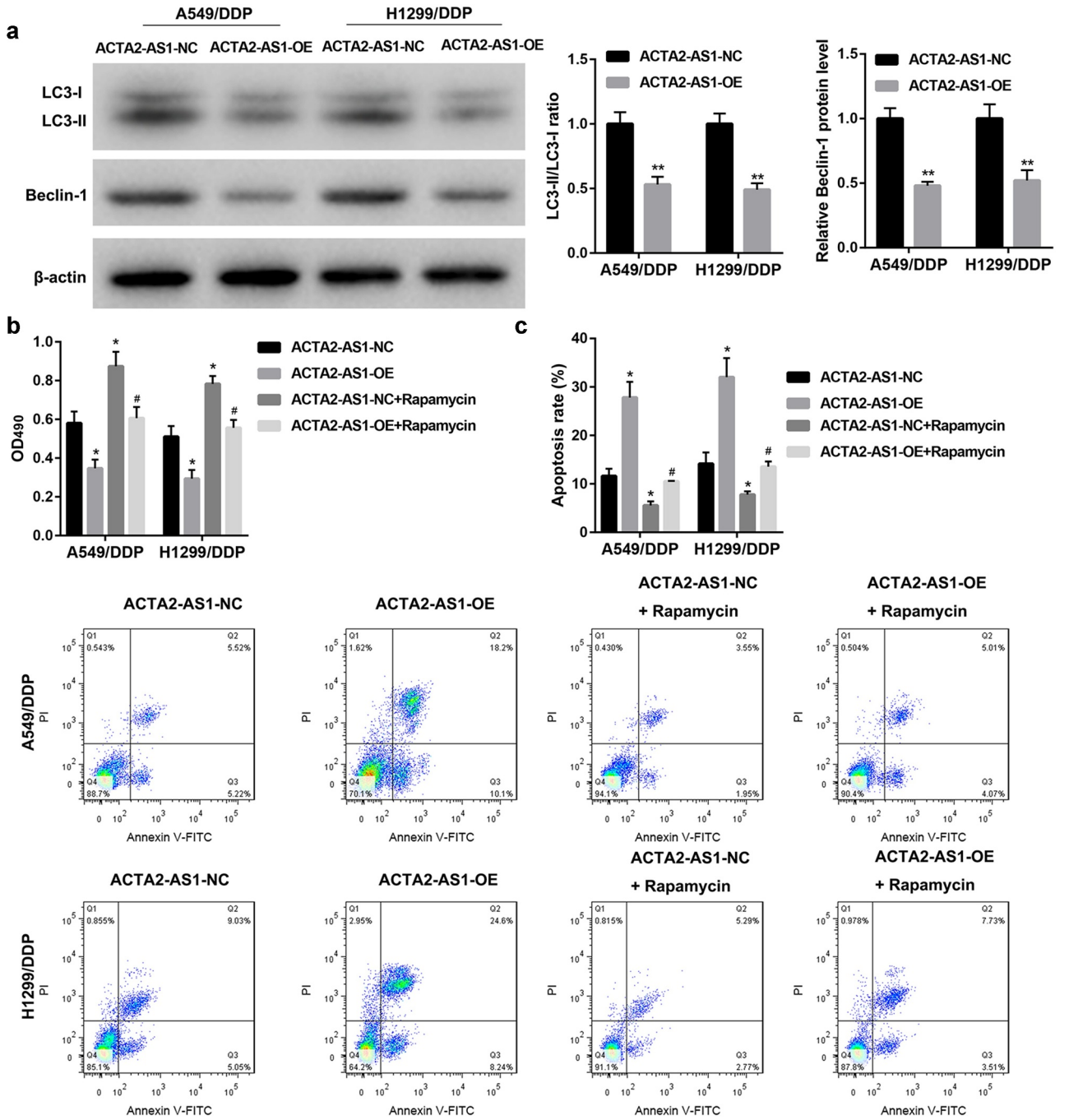

Figure 4. ACTA2-AS1 regulated A549/DDP and H1299/DDP cells apoptosis through affecting autophagy.

A549/DDP and H1299/DDP cells were transfected with the ACTA2-AS1 overexpression plasmid and control plasmid. (a) protein levels of LC3-I, LC3-II and Beclin-1 in the A549/DDP or H1299/DDP cells were analyzed using Western blot. The β-actin served as control. Next, the A549/DDP and H1299/DDP cells transfected with the ACTA2-AS1 overexpression plasmid were treated with an autophagy activator, and were cultured in the medium containing 16 μg/ml cisplatin. (b) the cell viability was detected using MST assay. (c) the cell apoptosis was determined using flow cytometry. *$p < 0.05$, **$p < 0.01$, and $^{\#}p < 0.05$.

expression and promoted H3K27me3 expression (Figure 7(c–e)). Furthermore, EZH2 silencing promoted TSC2 expression and inhibited TSC2 expression. The inhibition of ACTA2-AS1 to TSC2 expression and promotion to H3K27me3 expression were partly reversed by EZH2 silencing (Figure 7(f–h)). Overall, our results displayed that ACTA2-AS1 inhibited TSC2 expression through EZH2.

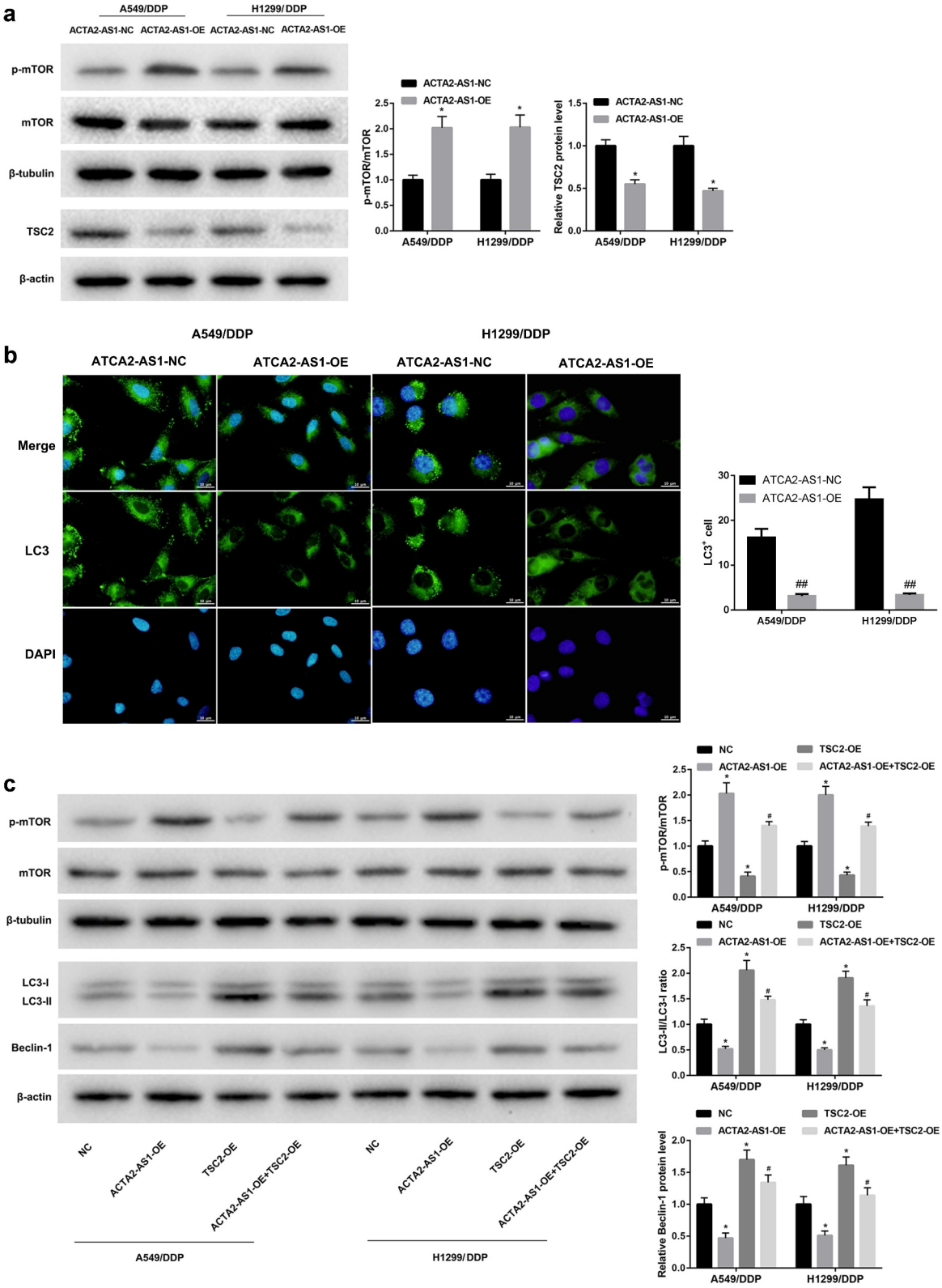

Figure 5. ACTA2-AS1 inhibited A549/DDP and H1299/DDP cells autophagy through targeting TSC2.

A549/DDP and H1299/DDP cells were transfected with the ACTA2-AS1 overexpression plasmid and control plasmid. (a) the protein levels of TSC2, mTOR and p-mTOR in A549/DDP and H1299/DDP cells were analyzed by Western blot. Here, β-actin served as control. (b) immunofluorescence was performed to examine the LC3-positive A549/DDP and H1299/DDP cell numbers. (c) the A549/DDP and H1299/DDP cells were co-transfected with the ACTA2-AS1 overexpression plasmid and TSC2 overexpression plasmid, and then the proteins levels of mTOR, p-mTOR, LC3-I, LC3-II, Beclin-1 was determined using Western blot. *$p < 0.05$, and $^{\#}p < 0.05$.

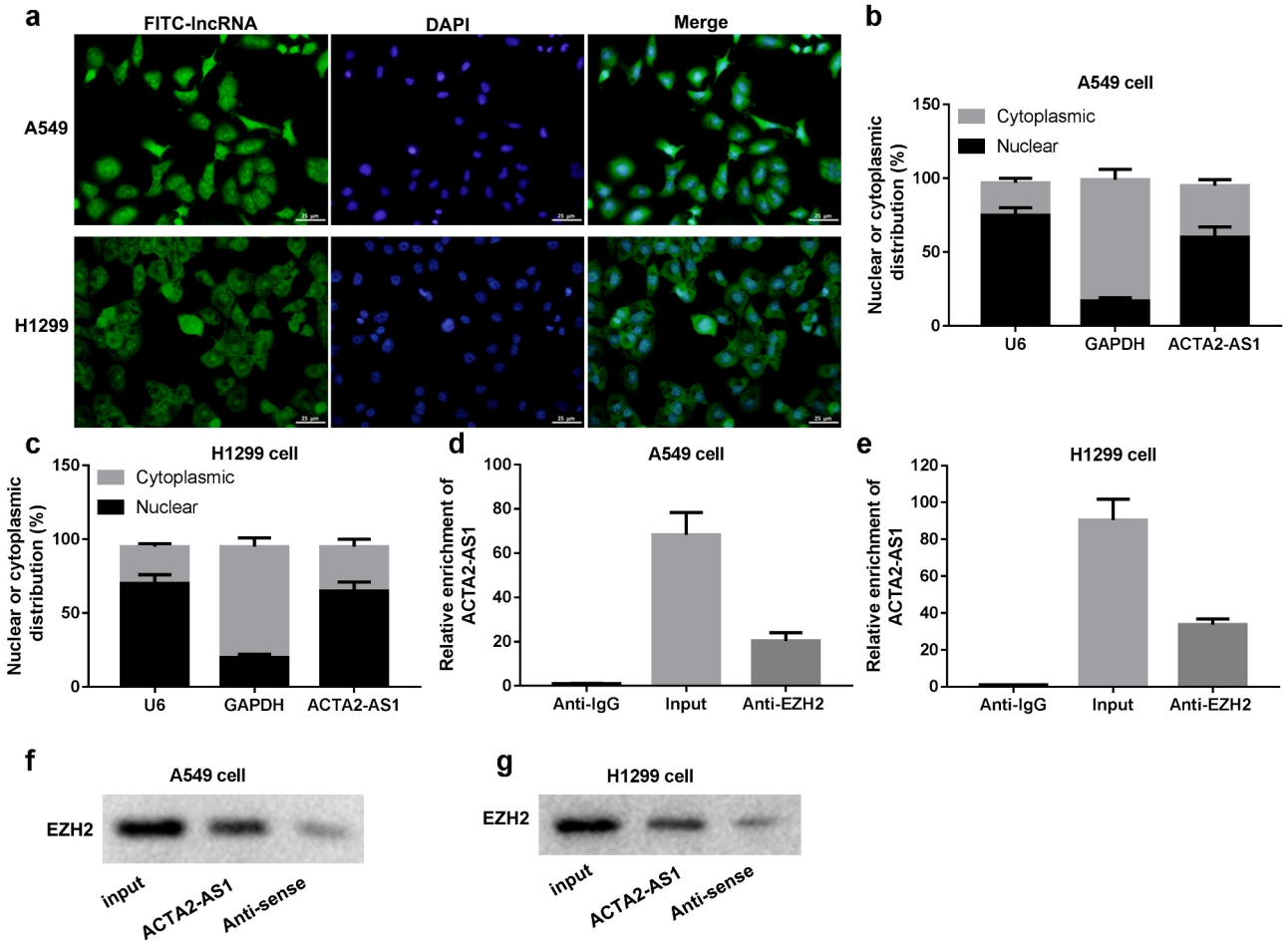

Figure 6. ACTA2-AS1 bound with EZH2.

(a) RNA-FISH was performed to determine the subcellular localization of ACTA2-AS1 in A549/DDP and H1299/DDP cells. (b and c) qRT-PCR was performed to determine the expression level of ACTA2-AS1 in cytoplasmic and nuclear. (d and e) the combination of ACTA2-AS1 to EZH2 in A549/DDP and H1299/DDP cells was detected using RIP assay. (f and g), the combination of EZH2 to ACTA2-AS1 was detected using RNA pull down.

4. Discussion

NSCLC, a histological subtype of pulmonary carcinoma, has a high mortality rate and low survival rate globally [19]. Although a kinds of chemotherapy drugs are available for the treatment of lung cancer, the resistance of the lung cancer cells to these drugs is a key reason for the poor prognosis [20]. Hence, more and more researchers pay attention to the mechanism of cisplatin resistance in NSCLC cell lines. Xiong et al. demonstrated that lncRNA GSTM3TV2 could upregulate L-type amino acid transporter 2 and oxidized low-density lipoprotein receptor 1 by competitively sponging let-7 to promote gemcitabine resistance in pancreatic cancer [21]. Recently, more and more lncRNAs have been reported to regulate the cancer cell resistance. Here, our data displayed first that ACTA2-AS1 is decreased in the cisplatin-resistant NSCLC cell lines. We further analyzed the influence of ACTA2-AS1 to the cell apoptosis and viability of cisplatin-resistant NSCLC cells. The data showed that ACTA2-AS1 negatively regulates the cell viability and positively regulates the cell apoptosis of cisplatin-resistant NSCLC cell lines.

Autophagy is a conserved cellular process involving the formation of autophagosomes that enclose cytoplasmic cargo, and is regulated by multiple proteins including LC3, Beclin-1, and autophagy-related proteins [22,23]. Recently, increasing evidences have demonstrated that lncRNAs could affect the drug resistance in cancer cells partly through regulating

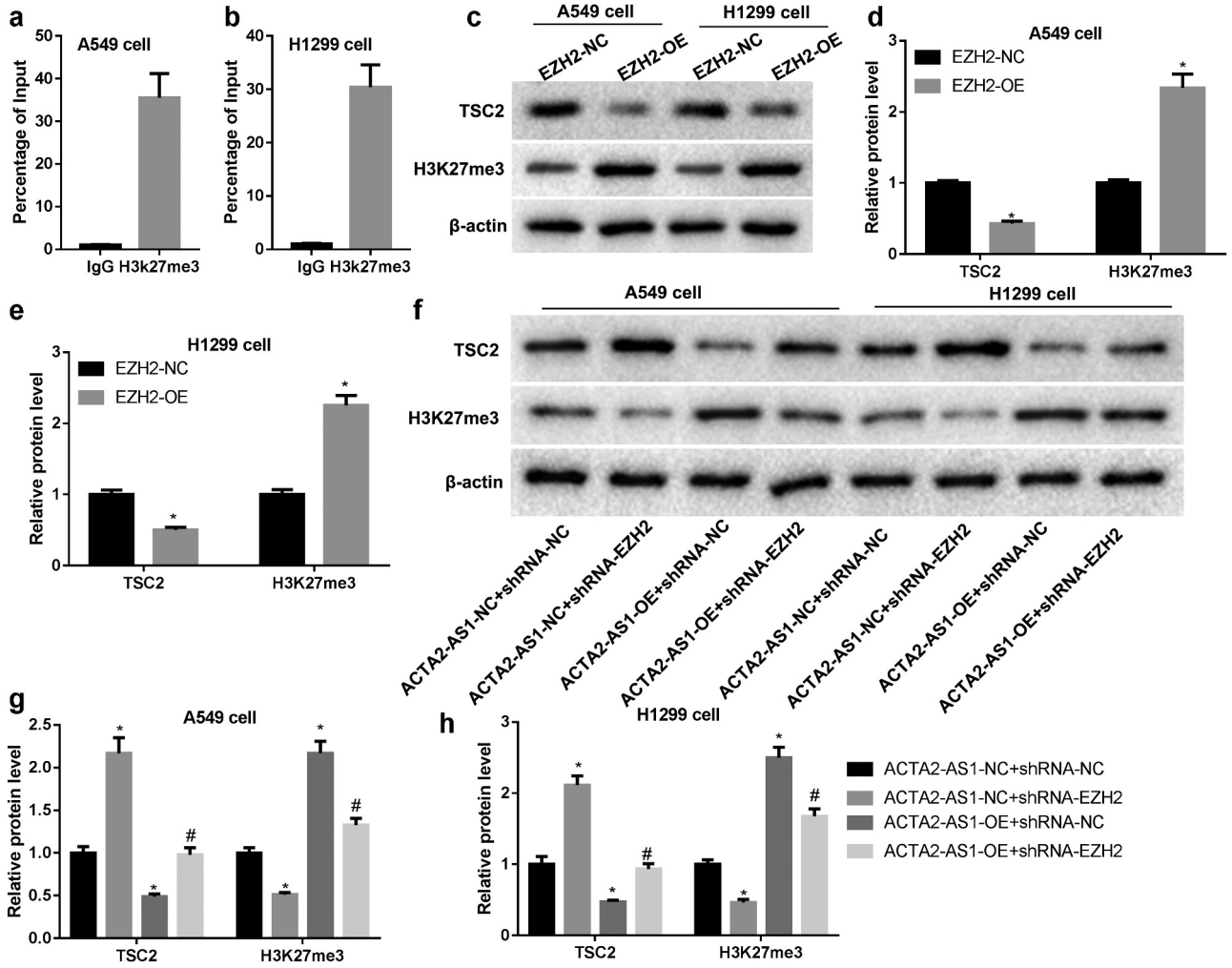

Figure 7. ACTA2-AS1 regulated TSC2 expression through EZH2.

(a) the combination of TSC2 promoter and H3k27me3 in A549/DDP was determined using CHIP. (b) the combination of TSC2 promoter and H3k27me3 in A549/DDP was determined using CHIP. (c–e), A549/DDP and H1299/DDP cells were transfected with the EZH2 overexpression plasmid and control plasmid, and then the expression levels of TSC2 and H3k27me3 were determined using Western blot. (f–h), the protein levels of TSC2 and H3k27me3 were determined using Western blot. *P < 0.05, and #P < 0.05.

autophagy [24–26]. Wang et al. revealed that lncRNA H19 could promote the tamoxifen resistance in breast cancer by activating autophagy by SAHH/DNMT3B signaling pathway [27]. Inhibition of cancer cell autophagy might be a potential mechanism to improve the drug resistance of cancer cells [28]. In this study, ACTA2-AS1 increasing inhibited autophagy-related protein expression. Importantly, autophagy activation could reverse the effecting of ACTA2-AS1 to cisplatin-resistant NSCLC cell lines.

Several studies have shown that downregulated TSC2 promotes cancer cell apoptosis, and is associated with the drug resistance in different kinds of cancers [29,30]. Some lncRNA could regulate the transcription of TSC2. For instance, lncRNA MALAT1 enhanced the apoptosis of cardiomyocytes by inhibiting autophagy by regulating TSC2-mTOR signaling pathway [17]. mTOR is an inhibitor of autophagy [31]. Inoki et al. displayed that TSC2 is phosphorylated and inhibited by AKT and suppressed mTOR signal pathway, indicating that TSC2 plays an important role in tumor suppressor functions [32]. Mutations in TSC1, TSC2, and mTOR are associated with the response to rapalogs in the patients with metastatic renal cell carcinoma [33]. In our study, the results revealed that ACTA2-AS1 increasing promotes the phosphorylation of mTOR, but inhibits the expression of TSC2. The inhibition of ACTA2-AS1 to the autophagy of the cisplatin-resistant NSCLC

cancer
cells was reversed by TSC2 increasing.

EZH2 is one component of the polycomb repressive complex 2, which catalyzes the trimethylation of H3K27me3 to suppress the transcription of target genes [34]. EZH2 could mediate the epigenetic regulation of lncRNAs to its target genes [35]. Here, our data showed that ACTA2-AS1 was major expressed in the cytoplasmic of the A549/DDP and H1299/DDP cells, and was bound with EZH2 protein. Furthermore, TSC2 was bound with the gene promoter of H3K27me3. The inhibition of ACTA2-AS1 to TSC2 was recused by EZH2 silencing.

In summary, our data demonstrated that ACTA2-AS1 attenuates the cisplatin resistance of NSCLC cell lines through suppressing autophagy by inhibiting TSC2 expression via binding to EZH2. These findings imply that ACTA2-AS1 may be a prospective target for NSCLC therapy. The exact biological functions of ACTA2-AS1 need to be explored in the future.

Disclosure statement

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