

RETRACTED ARTICLE: ZFAS1 Promotes Cisplatin Resistance via Suppressing miR-421 Expression in Oral Squamous Cell Carcinoma

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Purpose: Oral squamous cell carcinoma (OSCC), with high incidence and mortality, represents one of the main reasons for head and neck malignant tumors. We want to investigate the effect of ZFAS1 on DDP resistance in oral squamous cell carcinoma.

Methods: The proliferation and migration of cells was detected by CCK-8 and Transwell assay. The apoptosis was measured by flow cytometry and Western blot. The interaction of ZFAS1, miR-421, and MEIS2 was verified by luciferase reporter assay. The role of ZFAS1 in DDP resistance in vivo was tested by the nude mouse model. The expression of ZFAS1 in exosomes from cisplatin-resistant patients was also determined.

Results: ZFAS1 overexpression improved OSCC cell growth and inhibited OSCC cell susceptibility to DDP. In addition, the silencing of ZFAS1 promoted DDP-induced apoptosis. ZFAS1 directly binds to miR-421, which was verified by luciferase reporter assay. Inhibition of miR-421 reversed the effect of si-ZFAS1, which promoted the cell viability and decreased the sensitivity of DDP in DDP-resistant cells. The in vivo experiment showed the role of ZFAS1 in increasing the DDP resistance in OSCC tumor. Importantly, this study also showed that ZFAS1 regulated ZFAS1 in serum exosomes derived from cisplatin-resistant patients.

Conclusion: ZFAS1 promotes chemoresistance of oral squamous cell carcinoma to cisplatin and might become a latent therapeutic target for treating OSCC.

Keywords: chemoresistance, cisplatin, oral squamous cell carcinoma, ZFAS1, miR-421, exosome

Introduction

As one of the main reasons for cancers, oral squamous cell carcinoma (OSCC) shows high incidence and mortality, accounting for 95% or more of the head and neck malignant tumors.^{1,2} Despite the advanced treatment over the past decades, it is still unsatisfying in terms of the 5-year survival rate of OSCC, resulting in its poor diagnosis and metastasis.³ Resistance to chemotherapy is a key factor that limits the efficacy of drugs and shows a negative effect on the survival of OSCC patients. As a chemotherapeutic cytotoxic DNA-damaging alkylating drug, cisplatin is not only applied to treating kinds of solid tumors, it is also a key component for treating head and neck malignant tumors, such as OSCC. However, resistance to cisplatin significantly limits its clinical use.⁴ As a result, to develop strategies to advance the cisplatin application in treating OSCC, we need to understand the molecular mechanism of cisplatin resistance better.

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Long non-coding RNAs (lncRNAs) are a new type of transcripts sized from 200 to more than one hundred thousand nucleotides, which shows no potential of protein-coding. Recently, increasing studies have displayed the importance of lncRNAs in various cellular physiological processes. It has caused widespread concern that lncRNAs showed a vital role in modulating gene expression at the epigenetic, transcriptional and post-transcriptional levels in various cancers, which became a target of diagnosis, prognosis and therapy in various cancers.^{5,6} lncRNAs were also involved in the tumorigenesis of OSCC, mainly by acting as miRNA sponges to down-regulate miRNAs on mRNAs.^{7,8} Zinc finger antisense 1 (ZFAS1) is a proto-oncogene that is aberrantly expressed in diverse cancers, including ovarian cancer,⁹ cervical cancer,¹⁰ gastric cancer.¹¹ By interacting with diverse molecules, ZFAS1 regulates different processes including cell cycle, metastasis, invasion, proliferation and apoptosis.¹²

lncRNAs have been widely studied to mediate chemoresistance.^{13–15} In cervical cancer, silencing ZFAS1 inhibits cell migration, invasion and proliferation as well as enhances cisplatin chemosensitivity.¹⁰ Meanwhile, ZFAS1 is corresponding to the Adriamycin-resistant phenotype of T-cell acute lymphoblastic leukemia cell lines.¹⁶ Several other lncRNAs have been demonstrated to involve in chemoresistance of OSCC. HOTAIR was reported to inhibit autophagy, promote apoptosis and increase sensitivity to cisplatin in OSCC. HANR1 also related to cisplatin resistance in OSCC.¹⁸ Cisplatin resistance of OSCC is promoted by ZFAS1 by inhibiting the expression of miR-421.¹⁹ Despite this, the regulatory role of lncRNAs in OSCC chemoresistance remains largely to be uncovered.

In our present study, ZFAS1 was observed upregulated in OSCC. Furthermore, *in vivo* and *in vitro* function of ZFAS1 in OSCC proliferation and drug-sensitivity were examined. We found that ZFAS1 promoted proliferation and chemoresistance of OSCC through sponging miR-421. Meanwhile, miR-421 was demonstrated to target to 3'UTR of MEIS2 and to promote OSCC apoptosis. To sum up, ZFAS1 interacted with miR-421 to suppress its transcription followed by increasing MEIS2 expression to promote proliferation and chemoresistance of oral squamous cell carcinoma to cisplatin. Our results suggested that the ZFAS1/miR-421/MEIS2 pathway showed a regulatory role in OSCC proliferation and chemoresistance to cisplatin, which might provide a better understanding of OSCC chemoresistance.

Materials and Methods

Collection of Clinical Samples

From 2016 to 2018, 45 patients at the Department of Stomatology, Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science suffered from OSCC were employed to collect OSCC and corresponding adjacent non-tumor tissues. All study tissues were collected and frozen in liquid nitrogen immediately and then stored at -80°C . The study was approved by the Ethics Committee of Hubei University of Arts and Science. Written informed consent was acquired from all patients involved in this research.

Cell Culture

Normal human oral keratinocytes (NHOK), DDP-resistant OSCC cells (SCC/DDP) and CAL-27/DDP and OSCC cell lines (CAL-27, Tca8113, SCC9, TSCCA) were all bought from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China).

All these cells were cultured in DMEM (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) at 37°C in an incubator with 5% CO_2 .

Plasmid, Cell Transfection and Lentiviral Vector Construction

ZFAS1 overexpression plasmid was constructed via cloning the cDNA of ZFAS1 sequence into pcDNA3.1 vector. An empty pcDNA3.1 vector was used as a negative control. ZFAS1 siRNA and negative control sequences, miR-421 mimic, scrambled mimic control, miR-421 inhibitor and inhibitor control were transfected to cells with RNAiMAX (Invitrogen) in accordance with the manufacturer's instructions. After cells were transfected for 6 h, the medium was changed to complete medium. Next, the cells were incubated for 24 h and infected with constructed sh-ZFAS1 or sh-NC lentivirus. To generate a stable ZFAS1 knockdown cell line, puromycin was used to screen these cells for more than 7 days.

RNA Extraction and qRT-PCR

Total RNA from tissues or cells was extracted with TRIzol reagent (Invitrogen). One-microgram total RNA was reversely transcribed into cDNA with an SYBR Premix Ex Taq II Kit (TaKaRa, Japan) following the manufacturer's instructions. SYBR Green I Master Mix (Roche, Mannheim, Germany) was applied to perform qRT-PCR. $2^{-\Delta\Delta\text{CT}}$

method was applied to calculate the relative fold change of gene expression. All results were normalized to U6 or GAPDH. Primer sequences were listed below: MEIS2: forward 5'-AACCAAGGTCGCACCAGGTG-3', reverse 5'-CGGCCGGCTATCCCTCATAT-3'; ZFAS1: forward 5'-ACCGAGGCTTACCAAGATG-3', reverse 5'-CCCCGTGTACATCTTGCCAT-3'; miR-421: 5'-GCCTAGGATCTGCAT TGACT-3' and U6: 5'-ACGCAAATTCGTGAAGCGTT-3'; GAPDH: forward 5'-CCTGCCGGTGACTAACCCT-3', reverse 5'-AGGCGCCCAATACGACCAAA-3'.

CCK-8 Assay to Detect Cell Viability

CCK-8 (MYBiotech, China) was used to detect cell viability. OSCC cells or DDP-resistant OSCC cells were planted into 96-well plates. After transfection, cells were incubated with DDP and cell proliferation was measured at different times (0, 24, 48, and 72 h). After transfected for 24 h, the OSCC cells were incubated with DDP at the concentration range from 0 $\mu\text{mol/L}$ to 160 $\mu\text{mol/L}$ for 24 h to determine the drug sensitivity. After treatment, 10 mL CCK-8 was added to each well, and then the 96-well plate was incubated at 37°C for 2 h. The optical density (OD) value was detected at a wavelength of 450 nm to determine the cell viability on a microplate reader (Thermo). Each experiment was performed at least in triplicate.

Western Blot

Pre-cold RIPA solution (Beyotime, Shanghai, China) containing protease inhibitor was used to extract whole protein. BCA (bicinchoninic acid) protein Assay Kit (Beyotime, Shanghai, China) was applied to detect protein concentration. After separated by 10% SDS-PAGE, proteins were transferred onto a PVDF (polyvinylidene difluoride) membrane. Membranes containing different proteins were immunoprobed with the corresponding antibodies, MEIS2, Bax, BCL2 and β -actin (Abcam, USA) overnight at 4°C. HRP (horseradish peroxidase)-conjugated secondary antibody was used to incubate the membranes for 1 h at room temperature. The proteins were tested by an enhanced chemiluminescence reagent (Thermo Scientific, Waltham, MA).

Apoptosis Analysis

To evaluate the apoptosis of cells induced by cisplatin, the cells transfected with vectors were incubated with 5 μM DDP or the combined vehicle for 48 h, respectively. Cell apoptosis was examined by Annexin V-FITC PI Apoptosis Detection kit (BD Biosciences, San Diego,

CA, USA). FACSscan flow cytometry (BD Biosciences, San Jose, CA, USA) was applied to identify apoptosis cells. Each experiment was repeated three times and performed in triplicate.

Migration and Invasion Assays

Migration and invasion assays were performed using transwell chambers (8 μm pore size; Corning). Cells in serum-free medium were seeded into the upper chamber. For the invasion assay, the top chamber was precoated with Matrigel. In the lower chamber, it was medium supplemented with 10% FBS. After incubation for 24 h, cells remaining on the top layer were removed with a cotton swab. Cells on the bottom of the membrane were fixed with 4% paraformaldehyde and stained with crystal violet. The stained cells were counted under a microscope. Three independent experiments were performed.

Caspase 3 Activity Assay

Caspase 3 activity was tested by Caspase 3 Activity Assay kit (Beyotime) 48 h after transfection following the manufacturer's protocols.

Luciferase Reporter Assay

A fragment of the GRAP 3'UTR was amplified and cloned into the psiCHECK-2 vectors. For luciferase assay, control psiCHECK-2 vectors, Mut or WT vectors were co-transfected to cells. After the cells were transfected for 48 h, the luciferase activity was detected by the classical Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI) following the manufacturer's instruction. Firefly and Renilla luciferase activities and transfection efficiency were measured for each well.

Treatment and Establishment of Tumor Xenografts

Male BALB/c nude mice (5–6 weeks old and weighing 15–20 g) were obtained from Shanghai Experiment Animal Centre, Shanghai, China and kept in specific conditions. This research gained the approval of the Ethics Committee of Hubei University of Arts and Science. The experiments were performed following the NIH guidelines on animal welfare. CAL-27/DDP cells with sh-ZFAS1 knockdown were dispersed in 100 μL medium. The right flanks of mice were subcutaneously injected with cells. Cisplatin (4 mg/kg) or PBS was intraperitoneally injected

once every 4 days 7 days after subcutaneous injection. Tumor growth was measured at 7, 11, 15, 19, 23, 27, and 31 days after inoculation. Mice were euthanatized for weighing the tumor at the end of the experiment.

Exosome Purification

The serum exosome was extracted by using ExoQuick precipitation kit (SBI, System Biosciences, Mountain View, CA) following the protocol. The characterization of vesicles floated in PBS was conducted using electron microscopy.

Statistical Analyses

Data were represented as the mean $\pm$ SD and analyzed by GraphPad Prism 5.0 software (La Jolla, CA, USA). Significances between different groups were analyzed by Student's *t*-test and one-way analysis of variance (ANOVA). The SPSS was used to make the ROC analysis. $p < 0.05$ represents statistical difference.

Results

ZFAS1 Upregulation in OSCC Cells, Tissues as Well as DDP-Resistant OSCC Cells

Former studies have identified that ZFAS1 was differentially expressed in diverse cancers. To investigate whether ZFAS1 participated in OSCC, we firstly examined the expression of ZFAS1 in OSCC tumor tissues and normal tissues collected from 45 patients by RT-qPCR assays. We demonstrated that ZFAS1 expression was significantly upregulated in tumor tissues from 27 of 45 OSCC patients (68.8%) when compared to corresponding adjacent normal tissues (Figure 1A). In addition, the overall expression of ZFAS1 was also up-regulated in OSCC tissues (Figure 1B). What is more, the expression pattern of ZFAS1 in OSCC cell lines (CAL-27, Tca8113, SCC-9, TSCCA), as well as normal human oral keratinocyte (NHOK), was examined. The results showed that compared with the

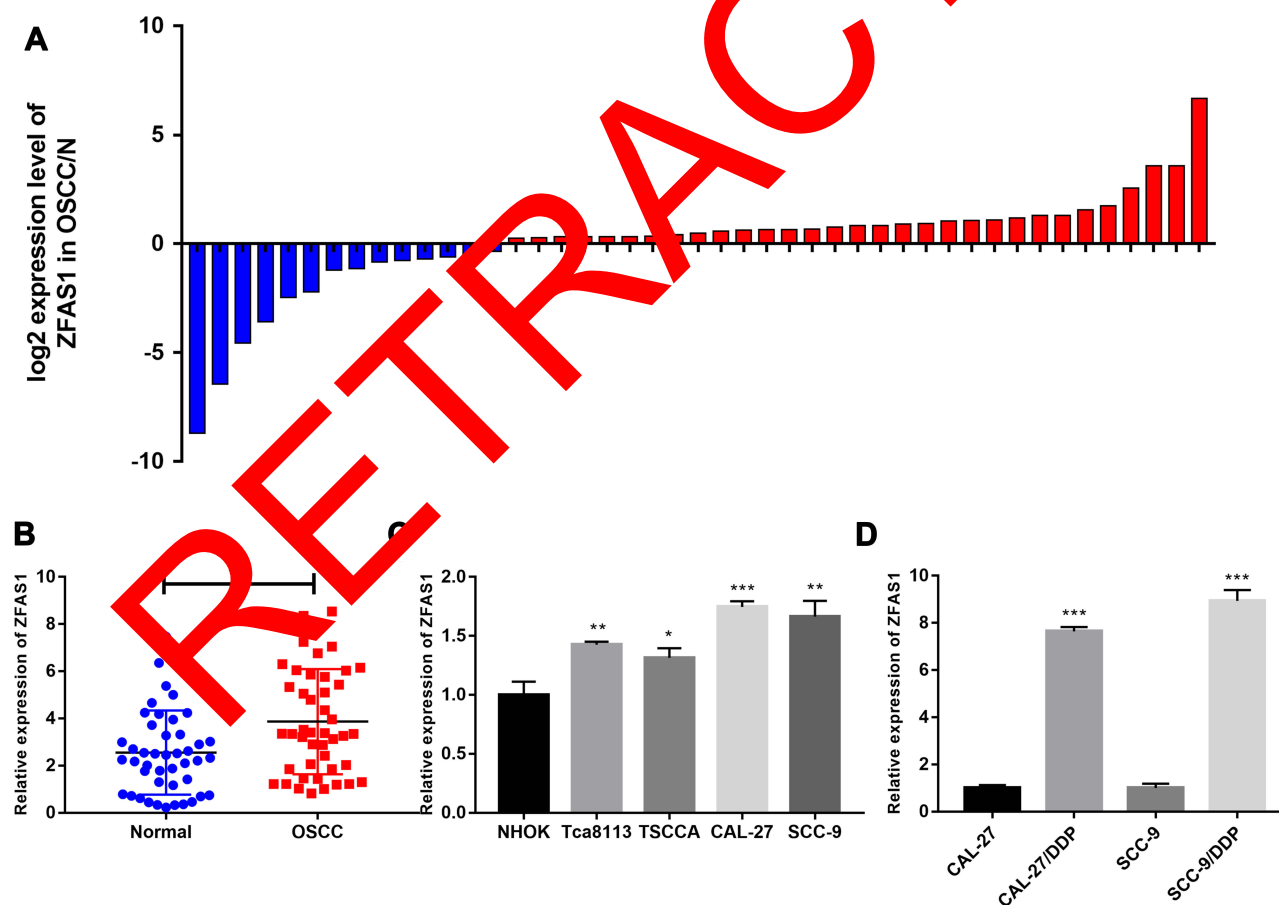


Figure 1 Upregulation of ZFAS1 levels in OSCC cell lines and tumor samples. **(A and B)** ZFAS1 levels in tumor samples as well as their corresponding noncancerous samples from 45 OSCC patients were analyzed. **(C)** ZFAS1 levels in OSCC cell lines (TSCCA, SCC-9, CAL-27, Tca8113) as well as normal human oral keratinocyte cell line (NHOK). **(D)** Analysis of ZFAS1 levels in OSCC cell lines (SCC-9, CAL-27) as well as corresponding DDP-resistant cell lines (SCC-9/DDP and CAL-27/DDP). *** $P < 0.005$, ** $P < 0.01$, * $P < 0.05$.

normal cells, ZFAS1 expression in OSCC cell lines were obviously elevated (Figure 1C). In order to confirm the participation of ZFAS1 in drug resistance, we also checked the expression of ZFAS1 in the DDP-resistant CAL-27 cell (CAL-27/DDP) and DDP-resistant SCC-9 cell (SCC-9/DDP). In accordance with our expectation, DDP-resistant OSCC cells demonstrated remarkably upregulation of ZFAS1 (Figure 1D), which suggested that ZFAS1 might be involved in OSCC progression and resistance to cisplatin of OSCC cells.

ZFAS1 Promoted Proliferation and Migration

For further measurement of the function of ZFAS1 in OSCC progression and drug resistance, we applied ZFAS1 over-expressing plasmid in SCC-9 and CAL-27 cells and the siRNA of ZFAS1 in CAL-27/DDP and SCC-9/DDP cells. After transfecting with pcDNA-ZFAS1, ZFAS1 expression was significantly elevated (Figure 2A). In accordance, siRNA of ZFAS1 also down-regulated the expression of ZFAS1 in these two cell lines (Figure 2B). It was showed that overexpression of ZFAS1 also caused higher cell viability in OSCC cells (Figure 2C). We performed CCK-8 assays after transfecting cells with ZFAS1 over-expressing plasmid, and the results exhibited that OSCC cell growth was markedly improved by ZFAS1 overexpression (Figure 2E). Supporting with the above results, siRNA of ZFAS1 also suppressed the DDP-resistance OSCC cell proliferation, and dramatically enhanced OSCC cell susceptibility to DDP (Figure 2D and F). The migration and invasion assay showed that lower expression of ZFAS1 inhibited the migration of CAL-27 and SCC-9 cells (Figure 2G).

Silencing of ZFAS1 Promoted DDP-induced Apoptosis

Regarding the above results, silencing of ZFAS1 dramatically enhanced OSCC cell susceptibility to DDP, we further verified the anti-apoptotic role of ZFAS1 in DDP-resistant OSCC cells. Through the flow cytometry results, we discovered that apoptosis induced by DDP in DDP-resistant OSCC cells (Figure 2H) was markedly augmented by si-ZFAS1. We also measured the expressions of several apoptosis-related genes, including BCL2, Bax and caspase-3. After silencing ZFAS1, the activity of caspase-3 was significantly elevated, which mirrored the results of the increased apoptotic ratio (Figure 2I).

Furthermore, the anti-apoptosis gene BCL2 and pro-apoptosis gene Bax were decreased and increased, respectively, in DDP-resistant OSCC cells treated with DDP (Figure 2J). These results indicated that the silencing of ZFAS1 promoted DDP-induced apoptosis in DDP-resistant OSCC cells treated with DDP.

ZFAS1 Inhibited miR-421 Expression

Studies have found competing endogenous RNA (ceRNA) exerts regulatory dialogues functions between different RNAs, such as lncRNAs and miRNAs. Interactions between miRNAs and lncRNAs in various cancer progressions have gained increasing attention. Bioinformatics prediction analysis was used to investigate the potential target miRNAs of ZFAS1 using starBase website. It was shown that ZFAS1 directly bound to miR-421 (Figure 3A). Therefore, the direct interaction between ZFAS1 and miR-421 was verified by luciferase reporter assay. Co-transfection of miR-421 or miR-NC with WT-ZFAS1 or MUT-ZFAS1 luciferase reporter plasmids was performed. We showed that the luciferase activity of WT-ZFAS1 was apparently blocked by miR-421 mimic. However, miR-421 mimic showed no effect on luciferase activities of MUT-ZFAS1 (Figure 3B), which validated the direct interaction between these two lncRNAs. To further investigate the regulation of ZFAS1 on miR-421, we transfected OSCC cells with ZFAS1 over-expressing plasmid and DDP-resistance OSCC cells with siRNA of ZFAS1. Data showed that ZFAS1 significantly suppressed the expression of miR-421, while blocked the ZFAS1 down-regulated miR-421 (Figure 3C and D), which suggested that the function of ZFAS1 in DDP resistance might be mediated by miR-421.

The Effect of ZFAS1 on Proliferation, Apoptosis, and DDP-Sensitivity in DDP-Resistant OSCC Cells Was Mediated by miR-421

Regarding the regulation of ZFAS1 on miR-421, we further tried to uncover whether miR-421 was the mechanism of ZFAS1 on cell proliferation, apoptosis, and DDP-sensitivity in DDP-resistant OSCC cells. To validate this supposition, si-ZFAS1 and anti-miR-421 were transfected into DDP-resistant OSCC cells. The results revealed that the proliferation of DDP-resistant OSCC cells was significantly reduced by si-ZFAS1, and anti-miR-421 could abolish this effect (Figure 3E and F). Meanwhile, anti-miR-421 significantly

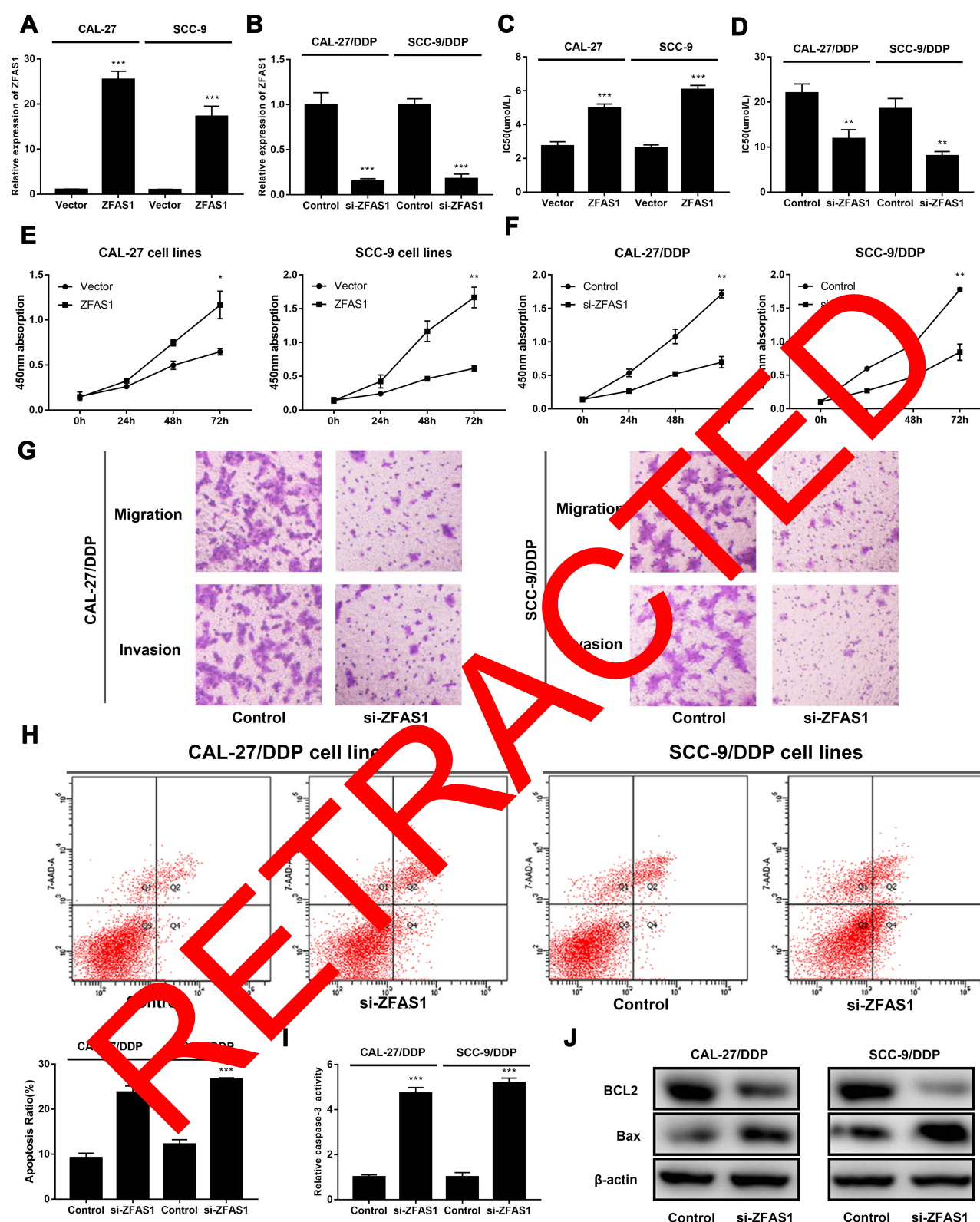


Figure 2 ZFAS1 promoted DDP resistance and proliferation of OSCC cell lines. (**A** and **B**) The overexpression efficiency of ZFAS1 si-RNA and plasmid were confirmed by RT-qPCR assays. (**C**) Influence of ZFAS1 upregulation on OSCC cells was detected by CCK-8 with IC₅₀ value calculation. (**D**) DDP resistance affected by ZFAS1 downregulation was detected by using CCK-8 to calculate the IC₅₀ value. (**E**) OSCC cell proliferation affected by ZFAS1 overexpression was measured by CCK-8 assays. (**F**) The proliferation of DDP-resistant OSCC cells affected by ZFAS1 knockdown was determined by CCK-8. Five $\mu\text{mol/L}$ DDP treatment for CAL-27/DDP and SCC-9/DDP cells induced by si-NC or si-ZFAS1 for 48 h. (**G**) The migration and invasion of cells treated with ZFAS1 siRNA. Apoptotic rate was calculated to analyze the effect of si-ZFAS1 on cell apoptosis (**H**) caspase-3 activity (**I**) Expression of Bax/BCL2 (**J**). *** $P < 0.005$, ** $P < 0.01$, * $P < 0.05$.

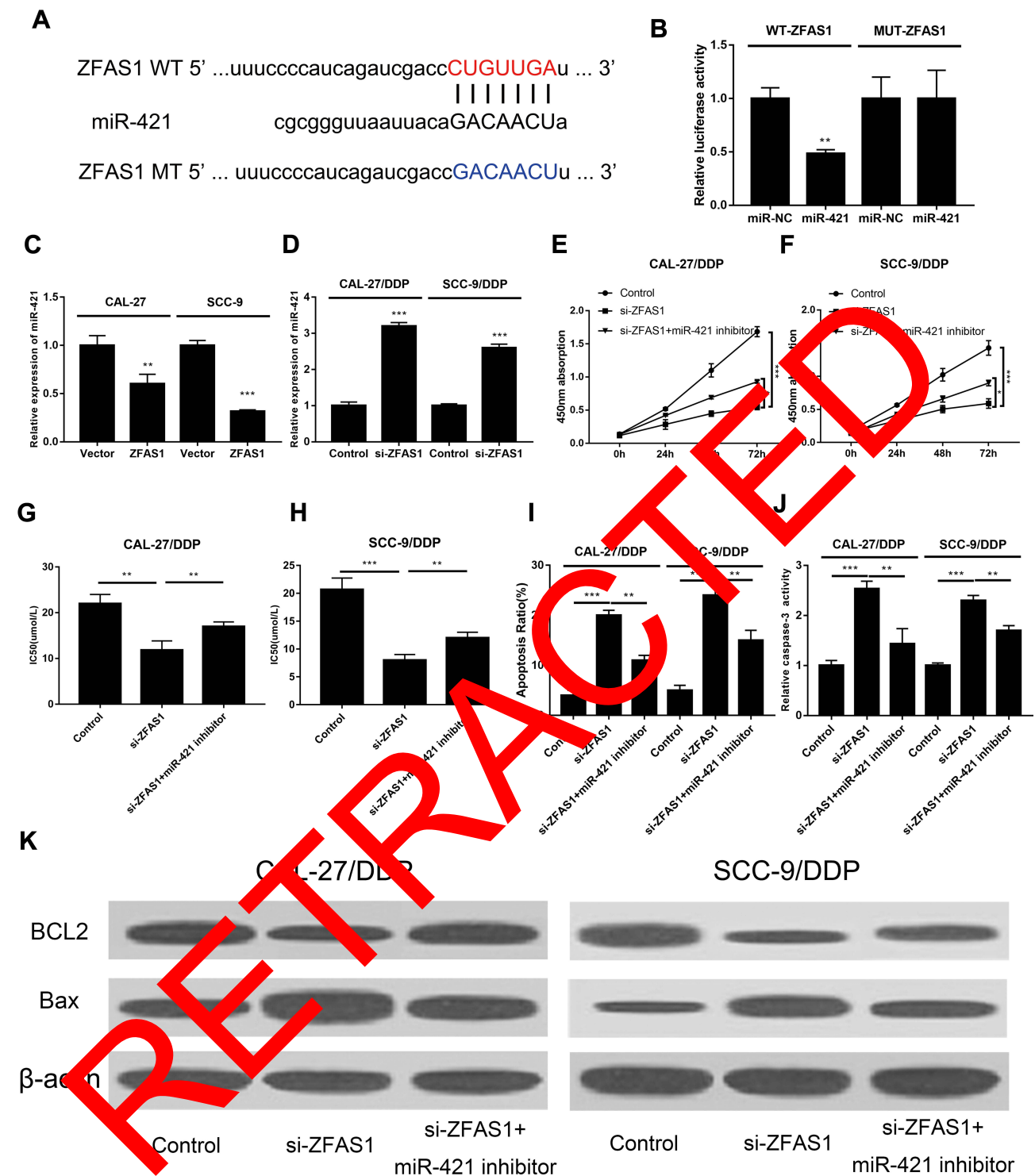


Figure 3 ZFAS1 interacted with miR-421 to modulate cell proliferation and apoptosis. **(A)** The putative binding site as well as the corresponding mutant region of miR-421 to ZFAS1. **(B)** Luciferase activity of MUT-ZFAS1 and WT-ZFAS1 reporter systems in 293T cells affected by miR-421 was detected using luciferase reporter assay. **(C and D)** The expression of miR-421 affected by ZFAS1 knocking-down or overexpression was determined in OSCC cells **(C)** and DDP-resistance OSCC cells **(D)** by qRT-PCR. Transfection of CAL-27/DDP and SCC-9/DDP cells with anti-miR-421 and si-UCA1 or si-UCA1 alone. **(E and F)** Cell growth was determined by CCK-8. **(G and H)** DDP sensitivity was detected by calculating IC50 value using CCK-8. **(I)** Cell apoptosis detected by flow cytometry analysis. **(J)** Analysis of caspase-3 activity. **(K)** Expressions of Bax and BCL2 detected by Western blot. *** $P < 0.005$, ** $P < 0.01$, * $P < 0.05$.

promoted the cell viability of DDP-resistant cells with the treatment of DDP. In another word, miR-421 promoted the sensitivity of DDP-resistant cells to DDP (Figure 3G and H).

In addition to proliferation, we also measured the effect of anti-miR-421 on cell apoptosis. The elevated ratio of apoptosis in DDP-resistant cells induced by si-ZFAS1 was

significantly reversed by anti-miR-421 (Figure 3I). Additionally, we also found that the caspase-3 activity (Figure 3J) and Bax expression (Figure 3K) was decreased while BCL2 expression (Figure 3K) was increased by anti-miR-421

ZFAS1 Promoted the Expression of MEIS2 by Sponging miR-421

Base-pair matching with the 3' untranslated region (3'UTR) of target mRNA transcripts was used to regulate

gene expression by miRNAs. To investigate the possible mechanism of miR-421 on DDP-sensitivity in DDP-resistant OSCC cells, we investigated whether there was a potential target of miR-421 that works in DDP-resistant OSCC cells. MEIS2 was identified as a candidate target of miR-421 by using TargetScan online software (Figure 4A). To further verify the direct interaction of miR-421 and MEIS2, we performed the luciferase reporter assay. It was shown that miR-421 mimic greatly suppressed the luciferase activities of WT-MEIS2, while miR-421 mimic

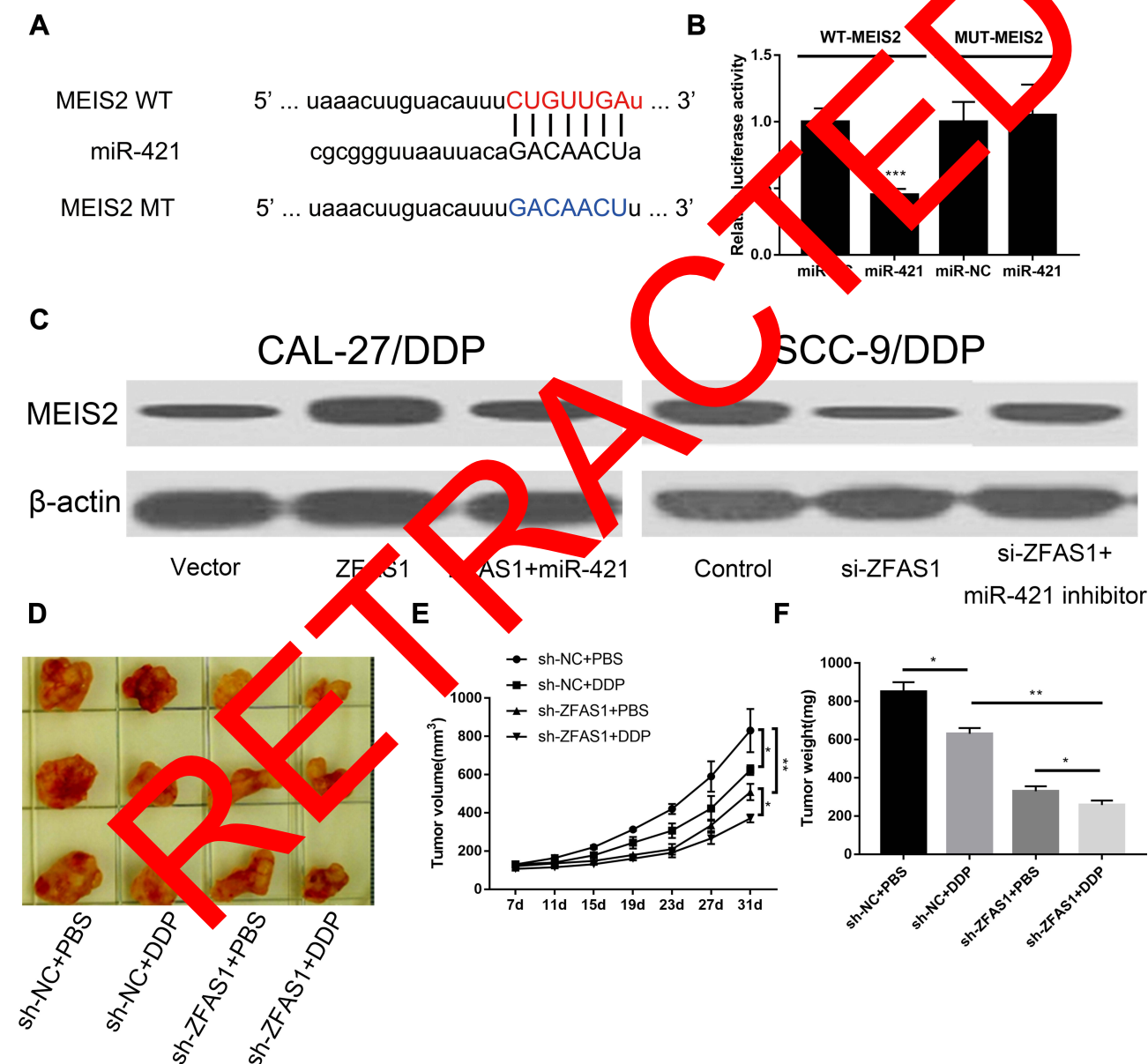


Figure 4 The upregulation of MEIS2 expression by ZFAS1 was partially inhibited by miR-421 overexpression. **(A)** Putative binding sites of miR-421 on MEIS2 3'UTR and mutated binding sequences of miR-421 on MEIS2-3'UTR were presented in graphic. **(B)** Dual-luciferase reporter analysis to confirm the real binding site of miR-421 and MEIS2-3'UTR by co-transfecting MUT-MEIS2-3'UTR or WT-MEIS2-3'UTR reporter with miR-421 mimic or miR-NC in 293T cells. **(C)** Expressions of MEIS2 protein were detected in DDP-resistance cells following various treatments using immunoblotting. Influence of DDP treatment or ZFAS1 knockdown on the growth **(D and E)** and weight **(F)** of xenografts derived from CAL-27/DDP cells. ***P < 0.005, **P < 0.01, *P < 0.05.

did not affect the MUT-MEIS2 (Figure 4B). Former reports have indicated that lncRNAs could function as ceRNA of miRNAs to inhibit the negative regulation of miRNAs on target genes. We also investigated the interaction of ZFAS1, miR-421 and MEIS2. We firstly examined the expression of MEIS2 after transfecting cells with the ZFAS1 overexpressing plasmid. We detected an elevation of MEIS2 after transfected with MEIS2 in DDP-resistant OSCC cells. This elevation was obviously blocked by miR-421 overexpression (Figure 4C). To further ensure this effect, we also applied siRNA of ZFAS1 and anti-miR-421. In line with our expectation, the block of ZFAS1 effectively down-regulated enhanced MEIS2 expression induced by si-ZFAS1 (Figure 4C). In summary, ZFAS1 promoted the expression of MEIS2 by sponging miR-421.

Inhibition of ZFAS1 Enhanced the Sensitivity to DDP in vivo

We have witnessed the role of ZFAS1 in cell proliferation, apoptosis, and DDP-sensitivity in DDP-resistant OSCC cells. In vivo experiments were also conducted to observe the ZFAS1 function in tumor growth. DDP-resistant cell line with stable ZFAS1 deficiency was injected into mice

to construct an animal model with ZFAS1 down-regulation. Tumor growth was measured at 7, 11, 15, 19, 23, 27 and 31 days after inoculation. The results showed that DDP significantly reduced the tumor volume and tumor weight, both of which were further inhibited by si-ZFAS1 (Figure 4D–F). To sum up, we demonstrated that inhibition of ZFAS1 enhanced the sensitivity to DDP in vivo.

ZFAS1 Was Secreted into Exosomes

Exosomes could transfer lncRNAs between cancer cells, transmitting signals and phenotypes. In this study, exosomes were extracted from serum samples of cisplatin-sensitive or cisplatin-resistant patients to identify ZFAS1 expression in exosomes. We characterized these vesicles by electron microscopy (Figure 5A). High expression levels of exosomal marker Tsg101 and HSP70 verified the purity of exosomes in the serum (Figure 5B). The findings of this study indicated that ZFAS1 was highly expressed in cisplatin-resistant serum samples (Figure 5C). Moreover, the AUC of the ROC is 0.82 (Figure 5D), which indicates the diagnosis role of exosomal ZFAS1 in cisplatin-resistant serum.

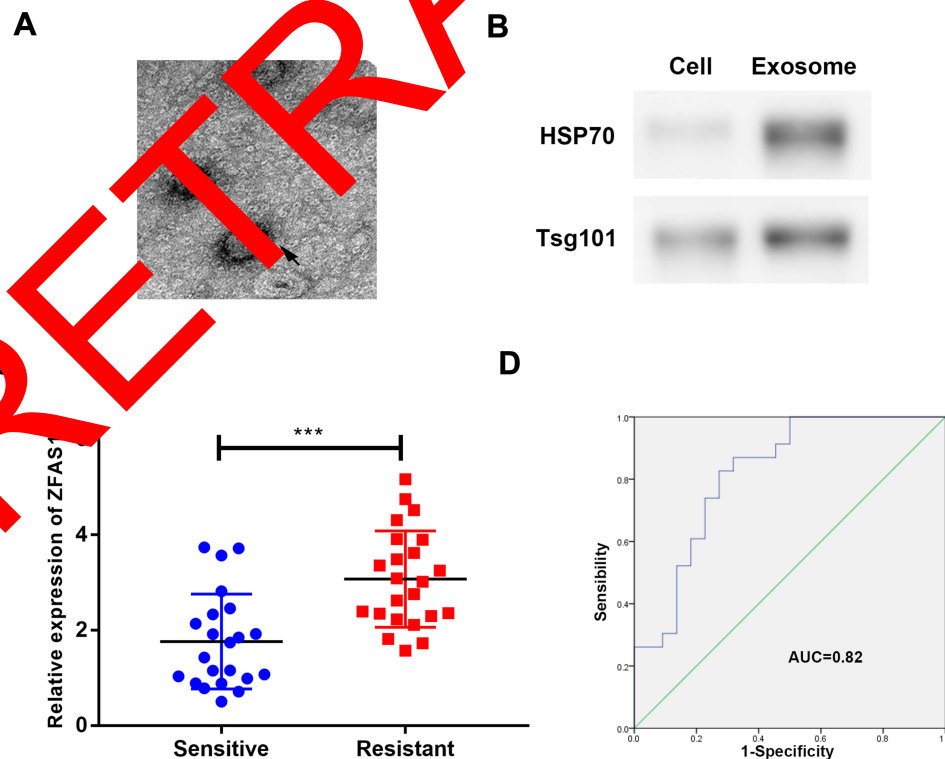


Figure 5 ZFAS1 was secreted into exosomes. **(A)** Typical electron microscope image of exosomes from patient; **(B)** Western blotting for biomarkers of exosomes from purified serum exosomes; **(C)** qRT-PCR results of the abundance of ZFAS1 in serum exosomes; **(D)** ROC analysis of serum exosomal ZFAS1. ***P < 0.005.

Discussion

Nowadays, due to low cure rate and high mortality, with a great individual and socioeconomic burden, OSCC has become a global public health problem. More and more evidence indicates that lncRNAs play a vital role in OSCC initiation and progression, including proliferation, apoptosis. HOXA11-AS was reported to promote the OSCC progression via targeting the miR-518a-3p/PDK1 axis.²⁰ The development of OSCC was regulated by TUG1 through sponging miR-524-5p to mediate DLX1 expression.²¹ However, the effect of lncRNAs on chemoresistance to DDP in OSCC remains largely unknown.

In the present study, we detected a significant increased expression of ZFAS1 in OSCC cell lines, tissues, and DDP-resistant OSCC cell lines, which underlined that ZFAS1 might be involved in OSCC proliferation and drug resistance to cisplatin. ZFAS1 was found to be highly expressed in several cancers, including cervical cancer, gastric cancer, and ovarian cancer. In cervical cancer, ZFAS1 promoted chemosensitivity to cisplatin.¹⁰ ZFAS1 was also associated with the Adriamycin-resistance in T-cell acute lymphoblastic leukemia cell lines.¹⁶ In ovarian cancer, ZFAS1 may participate in platinum resistance.²² Furthermore, ZFAS1/miR-150-5p/Sp1 axis was also reported to be involved in enhancing migration activity, proliferation rate and chemoresistance development in epithelial ovarian cancer. However, whether there is a functional role of ZFAS1 in OSCC remains unknown. Here in our study, we revealed that OSCC cell growth was markedly improved by ZFAS1 overexpression and caused higher cell viability in DDP-resistant cells. In addition, ZFAS1 also plays an anti-apoptotic role in DDP-resistant OSCC cells, possibly by regulating the activity of caspase-3, BAX and BCL2 expression. The above results indicated that ZFAS1 promoted chemoresistance of OSCC to cisplatin, which is in line with results in cervical cancer.

Extensive studies have exhibited that miRNAs were involved in tumorigenesis, development, metastasis and chemoresistance of different cancers.²³ Here, miR-421 was considered as a potential target miRNA of ZFAS1 by using bioinformatics prediction assay. Furthermore, luciferase reporter assay and qRT-PCR ensured the direct interaction between ZFAS1 and miR-421. MiR-421 has been demonstrated to play a significant role in several cancers, including non-small cell lung cancer,²⁴ esophageal adenocarcinoma,²⁵ hepatocellular carcinoma.²⁶ In gastric cancer, miR-421 was reported to promote metastasis, inhibit apoptosis, and induce cisplatin resistance.²⁷ In accordance, we discovered

that anti-miR-421 inhibited the sensitivity of DDP-resistant cells to DDP, which is characterized by enhanced proliferation and decreased apoptosis in DDP-resistant OSCC cells.

MiRNAs regulate gene expressions by targeting 3'-UTR binding sites to inhibit mRNA translation and degradation, thus inhibit gene expression and further regulate the pathophysiological processes of various diseases.^{28,29} By using TargetScan online software, MEIS2 was considered as a candidate target of miR-421. This result was later validated by luciferase reporter assay and qRT-PCR. MEIS2 promoted cell invasion and migration in colorectal cancer,³⁰ prostate cancer,³¹ bladder cancer.³² MEIS2 had the highest correlation with L-OH⁺ resistance in colorectal cancer.³³ MEIS2 was also critical to MN1-induced leukemia, by regulating self-renewal, proliferation, disease progression and impairment of differentiation.³⁴ The elevation of MEIS2 was obviously blocked by miR-421 overexpression, which suggested that ZFAS1 promoted MEIS2 expression through sponging miR-421. These results suggested that ZFAS1 functions as an oncogene by regulating miR-421/MEIS2 in OSCC cell lines. In vivo experiments also showed inhibition of ZFAS1 enhanced the sensitivity to DDP, evidenced by decreased tumor volume and weight. All these results provided the possibility that strategy targeting ZFAS1 might be effective to enhance susceptibility to DDP susceptibility of OSCC patients.

All in all, we studied the new mechanism of the ZFAS1/miR-421/MEIS2 axis in OSCC cells and the nude mouse model of xenograft. Our results showed that marked up-regulation of ZFAS1 in OSCC tissues and DDP-resistant cells promoted proliferation, migration and resistance to DDP in OSCC cells by sponging miR-421 and regulating MEIS2 expression. Our findings might offer a new strategy targeting the ZFAS1/miR-421/MEIS2 axis to improve the treatment of OSCC patients with DDP resistance in clinical practice.

Disclosure

The authors report no conflicts of interest in this work.

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