

Research Article

***Ganoderma lucidum* Polysaccharide (GLP) Inhibited the Progression of Oral Squamous Cell Carcinoma via the miR-188/BCL9/ β -Catenin Pathway**

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Objective. The effects of *Ganoderma lucidum* polysaccharide (GLP) on the proliferation and migration of oral squamous cell carcinoma (OSCC) cells (HSC-3) were investigated in this study. **Methods.** Oral squamous cell carcinoma (OSCC) cells (HSC-3) were cultured in vitro and were treated with different concentrations of GLP. CCK-8 assay and scratch assay were then used to detect the inhibitory effect of GLP on these cells. qRT-PCR and western blot were used to measure the expression changes of the genes involved in the miR-188 and β -catenin signaling pathways. **Results.** GLP had significant cytotoxicity to HSC-3 cells and was capable of inhibiting the proliferation and migration of HSC-3 cells. GLP upregulated the expression of miR-188 in HSC-3 cells and via which inhibited the proliferation and migration of HSC-3 cells. In addition, miR-188 inhibited the activation of the β -catenin signaling pathway through its target BCL9. **Conclusions.** GLP inhibited the proliferation and migration of OSCC cells (HSC-3) by regulating the miR-188/BCL9/ β -catenin signaling pathway. The results in this study provided a theoretical basis for the treatment of OSCC with GLP.

1. Introduction

Oral squamous cell carcinoma (OSCC) is a malignant tumor that occurs in the oral cavity and is the eighth most common type of cancer in the world [1]. In China, the incidence of OSCC is about 3–6 per 100,000 people, and this number is increasing every year. Smoking, alcoholism, and chewing betel nut are believed to play an important role in the etiology of oral cancer. At present, although surgery, radiotherapy, and chemotherapy have made some progress in the treatment of OSCC, the mortality rate of OSCC is still high [2], in which tumor metastasis is the leading cause of death in OSCC patients [3]. Therefore, further research concerning the new or adjuvant treatment of OSCC to improve the prognosis of patients is urgently needed.

Ganoderma lucidum has been widely used as an auxiliary drug for antitumor therapy in clinical practice [4], and polysaccharide is one of the main bioactive

components of *Ganoderma lucidum*. It has many biological functions such as improving immune function, preventing oxidative damage, and protecting the liver, while with little toxicity [5, 6]. Recently, the direct inhibitory effect of *Ganoderma lucidum* polysaccharide (GLP) on tumors has received extensive attention, for example, in colorectal cancer, GLP can promote the apoptosis of colorectal cancer cells by mediating the MAPK/ERK signaling pathway to regulate autophagy [7]; in prostate cancer, GLP can induce PC-3 cells apoptosis through NAG-1 [8] and can also inhibit prostate cancer cell migration through the arginine methyltransferase 6 signaling pathway [9]; in liver cancer, GLP can enhance the radiosensitivity of the liver cancer cell line HepG2 through the Akt signaling pathway [10]. However, the role of GLP in OSCC is unclear. Therefore, in this study, we investigated the effect of GLP on OSCC cells in vitro and explored the associated underlying mechanisms.

2. Material and Methods

2.1. Cell Culture and GLP Processing. Human oral squamous cell carcinoma HSC-3 cells were purchased from ATCC, USA, and the medium was DMEM containing 10% fetal calf serum and cells were cultured at 37°C with 5% CO₂. The cells in each group were treated with different concentrations of GLP including 0 mg/mL (control group), 0.5 mg/mL, 1.0 mg/mL, 2.0 mg/mL, 4.0 mg/mL, and 8.0 mg/mL, respectively. The preparation of GLP followed the method of Chen et al. [11]. The gas chromatographic analysis of GLP is shown in Figure 1. The monosaccharide composition of GLP is glucose and galactose, and the ratio of the amount of substance is 16.8:1.0.

2.2. Cell Transfection. One day before transfection, the cells were seeded into the culture plate in an appropriate number. According to the instructions, miR-188 mimics and miR-188 inhibitor were transfected into the cells using the Lipofectamine 3000 transfection reagent. The final concentration of miR-188 mimic transfection is 50 nM, and the final concentration of miR-188 inhibitor transfection is 100 nM.

2.3. Cell Activity Measured with CCK-8 Assay. HSC-3 cells were seeded in 96-well plates at approximately 5,000 cells per well and cultured for 24 h. After that, different treatments were applied to each group. After 24, 48, and 72 h, 10 μ l of the CCK-8 reagent (Dojindo, Japan) was added to each well and mixed. The cells were then incubated for an appropriate period of time, and the absorbance at 450 nm was measured with a microplate reader. Inhibition rate = [(absorbance of control well – absorbance of the experimental well)/(absorbance of control well – absorbance of the blank well)] * 100%.

2.4. In Vitro Scratch Assay. HSC-3 cells were seeded in a 12-well plate and cultured to form a monolayer, and a line was drawn in the layer using a pipette tip and then rinsed with PBS and treated accordingly. Scratches at different time periods were photographed using a microscope and statistical analysis was performed using ImageJ software.

2.5. miRNA Sequencing and Target Prediction. Total RNA was extracted from cells using the Trizol reagent (Invitrogen), followed with high-throughput sequencing of miRNAs on the Illumina platform. The expression profiles of miRNAs were analyzed using Cluster 3.0 software, and targets for miRNAs were predicted on the microRNA.org website.

2.6. qRT-PCR. Total RNA was extracted from the cells using the Trizol reagent (Invitrogen), and the RNA concentration was determined with a spectrophotometer. Reverse transcription was performed using the PrimeScript RT reagent kit (Takara). The resulting cDNA was then subjected to qRT-

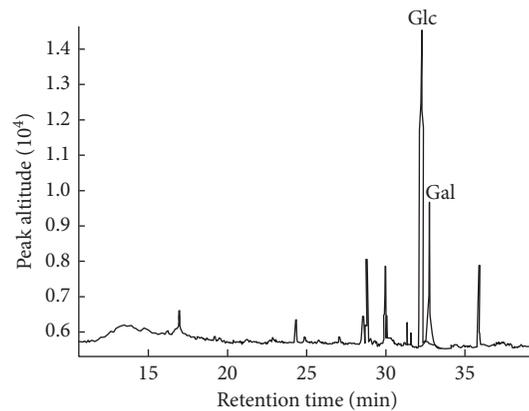


FIGURE 1: Gas chromatographic analysis of GLP.

PCR analysis using the SYBR Premix Ex Taq kit (Takara). U6 snRNA was used as an internal reference for the quantification of miR-188 expression, and for the mRNA expression, β -actin was used as an internal reference. The relative expression level of the target gene was calculated by the $2^{-\Delta\Delta CT}$ method. Primer sequences are as follows: miR-188-F: ATGTACACAAGCACACCTTCTCATT; miR-188-R: TCAGAAAGCTCACCTCCACCAT; U6-F: CTCGCTTCGGCAGCACACA; U6-R: AACGCTTCACGAATTTGCGT;-c-Myc-F: CAAGAGGCGAACACACAACGT; c-Myc-R: GGGCCTTTTCATTGTTTTCCA; CCND1-F: CTGGAGG-TCTGCGAGGAACA; CCND1-R: CTTAGAGGCCACG AACATGCA; Slug-F: CATGCCTGTCATACCACAAC; Slug-R: GGTGTCAGATGGAGGAGGG; Vimentin-F: AAAGTGTGGCTGCCAAGAAC; Vimentin-R: AGCCTCAGAGAGGTCAGCAA; β -acti-F: GGCTCCGGCATGTG-CAAG; β -acti-R: CCTCGGTCAGCAGCACGG.

2.7. Construction of Dual-Luciferase Reporter. The BCL9 3'-UTR sequences containing the wild type (wt) or mutant (mut) miR-188 binding site were synthesized and inserted into the pGL3 luciferase vector (Promega). The luciferase plasmid and miR-188 mimics or NC were cotransfected into cells using the Lipofectamine 2000 reagent (Invitrogen). After 48 h, luciferase activity was measured using the dual-luciferase reporter system (Promega) and Renilla luciferase activity was used as an internal reference.

2.8. Western Blot. Total cellular proteins were extracted with RIPA lysate, and nuclear proteins were extracted using the Nuclear and Cytoplasmic Extraction Reagent kit (Thermo Scientific). Western blot was performed using the standard method. The antibodies used in this experiment were purchased from Cell Signaling Technology.

2.9. Statistical Analysis. Data were analyzed using SPSS 18.0 software and expressed as mean \pm standard deviation. $P < 0.05$ was considered statistically significant.

3. Results

3.1. GLP Inhibited HSC-3 Cell Activity. As shown in Figure 2, compared with the control group, the inhibition rates of GLP (0.5, 1.0, 2.0, 4.0, 8.0 mg/mL) on the HSC-3 cells at 72 h were 44.0, 58.3, 59.7, 62.7, 71.7, and 93.3%, respectively. Also the IC₅₀ for GLP at 24 h, 48 h, and 72 h were 5.43, 3.26, and 0.91 mg/mL, respectively. The results showed that GLP significantly reduced cell viability ($P < 0.05$) in a dose- and time-dependent manner, indicating that GLP has potent cytotoxicity against HSC-3 cells.

3.2. GLP Inhibited HSC-3 Cell Migration. As shown in Figure 3, the migration ability of HSC-3 cells was reduced in a dose-dependent manner ($P < 0.05$) after treatment with different concentrations of GLP for 48 h compared with the control group. Therefore, GLP had a significant inhibitory effect on the migration of HSC-3 cells.

3.3. GLP Upregulated the Expression of miR-188. After the treatment of HSC-3 cells with 1.0 mg/ml GLP for 48 h, cellular RNAs were extracted for high-throughput sequencing of miRNA. The result showed that the expression level of miR-188 was significantly increased (Figure 4). Furthermore, HSC-3 cells were also treated with different concentrations of GLP and the sequencing results were verified via qRT-PCR. The results confirmed that GLP upregulated the expression of miR-188 in a dose-dependent manner ($P < 0.05$) (Figure 4).

3.4. GLP Regulated HSC-3 Cell Proliferation and Migration via miR-188. Recovery experiments showed that transfection of the miR-188 inhibitor in GLP (1.0 mg/mL) treated cells significantly reversed GLP-induced inhibition on cell proliferation (Figure 5(a)) and migration (Figure 5(b)) ($P < 0.05$), which indicated that GLP inhibited the proliferation and migration of HSC-3 cells via miR-188.

3.5. miR-188 Regulated BCL9/ β -Catenin Pathway in HSC-3 Cells. With microRNA.org, we found that BCL9 is a potential target for miR-188 (Figure 6(a)) and that was subsequently validated with the luciferase reporter gene (Figure 6(b)). In addition, we also found that miR-188 inhibited the activation of the β -catenin signaling pathway by downregulating the expression of BCL9, which in turn caused a decrease in the level of β -catenin in the nucleus (Figure 6(c)). Not surprisingly, the expression (mRNA and protein) of genes involved in the downstream of the β -catenin pathway including c-Myc, CCND1, Vimentin, and Slug was all downregulated by miR-188 (Figures 6(d) and 6(e)). The results mentioned above suggested that miR-188 regulated the BCL9/ β -catenin pathway in HSC-3 cells.

4. Discussion

Studies have shown that GLP can exert antitumor effects by inhibiting tumorigenesis, tumor growth, and metastasis, as

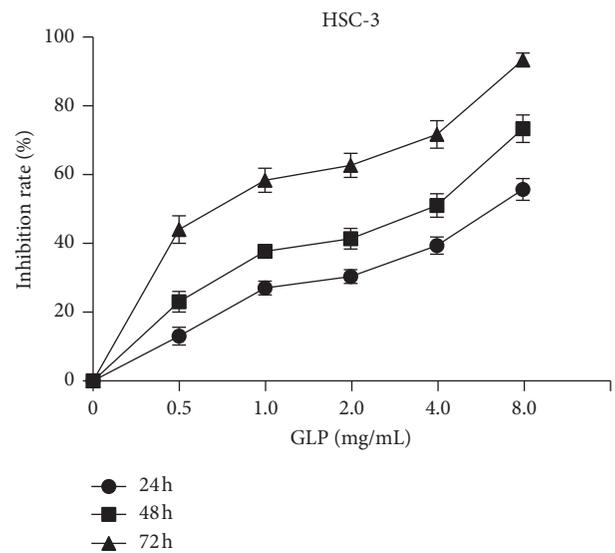


FIGURE 2: Cytotoxic effects of GLP on HSC-3 cells.

well as regulating immunity [12, 13]. However, there are a few research studies focusing on the effects of GLP in OSCC. The present study investigated the antitumor effect and corresponding mechanism of GLP in the OSCC cell line HSC-3.

Firstly, we demonstrated that GLP is cytotoxic to HSC-3 and can significantly inhibit HSC-3 cell proliferation and migration. Our results demonstrated that GLP has a significant antitumor effect in OSCC cells.

Secondly, we explored the mechanism of the antitumor effects of GLP. We found that GLP promoted the expression of miR-188 in HSC-3 cells. Studies have shown that GLP can regulate the expression of some miRNA and exert its antitumor effect through these miRNAs [14, 15]. With the help of high-throughput sequencing, we found that GLP induced changes in miRNA expression in HSC-3 cells, and we subsequently focused on miR-188 in the present study. miR-188 has shown anticancer effects in many tumors, including OSCC [16]. The results of our experiments showed that GLP can inhibit HSC-3 cell proliferation and migration by upregulating the expression of miR-188.

There are few studies focusing on miR-188 in OSCC; therefore, we further explored the role of miR-188 in OSCC. We found and confirmed that BCL9 is a direct target for miR-188 and that miR-188 can inhibit the expression of BCL9 by binding to its' 3'-UTR. The BCL9 gene was originally discovered in lymphoblastic leukemia cells, which typically are overexpressed in a variety of malignancies and can activate the Wnt/ β -catenin signaling pathway to promote cell proliferation, migration, invasion, and metastasis [17]. Our results showed that by inhibiting BCL9, miR-188 can inhibit the activation of the β -catenin signaling pathway and thus inhibits the expression of genes involved in the downstream of the β -catenin signaling pathway including c-Myc and CCND1, Vimentin and Slug, as shown by Zhuang et al. [18].

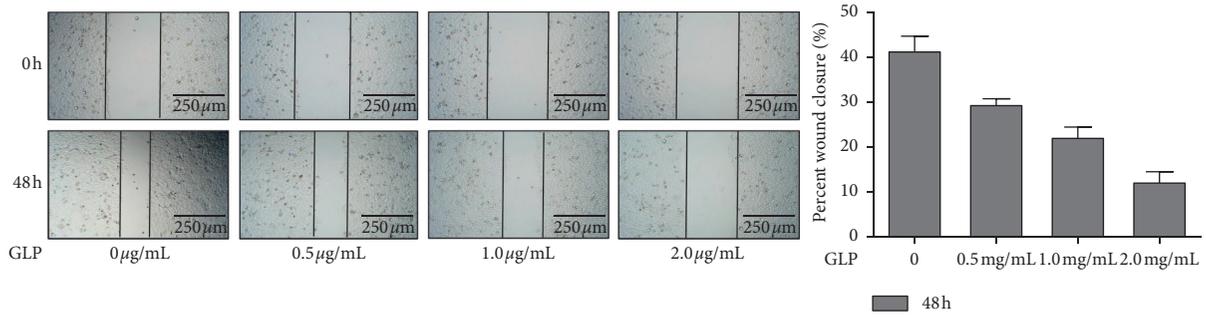


FIGURE 3: GLP inhibited HSC-3 cell migration.

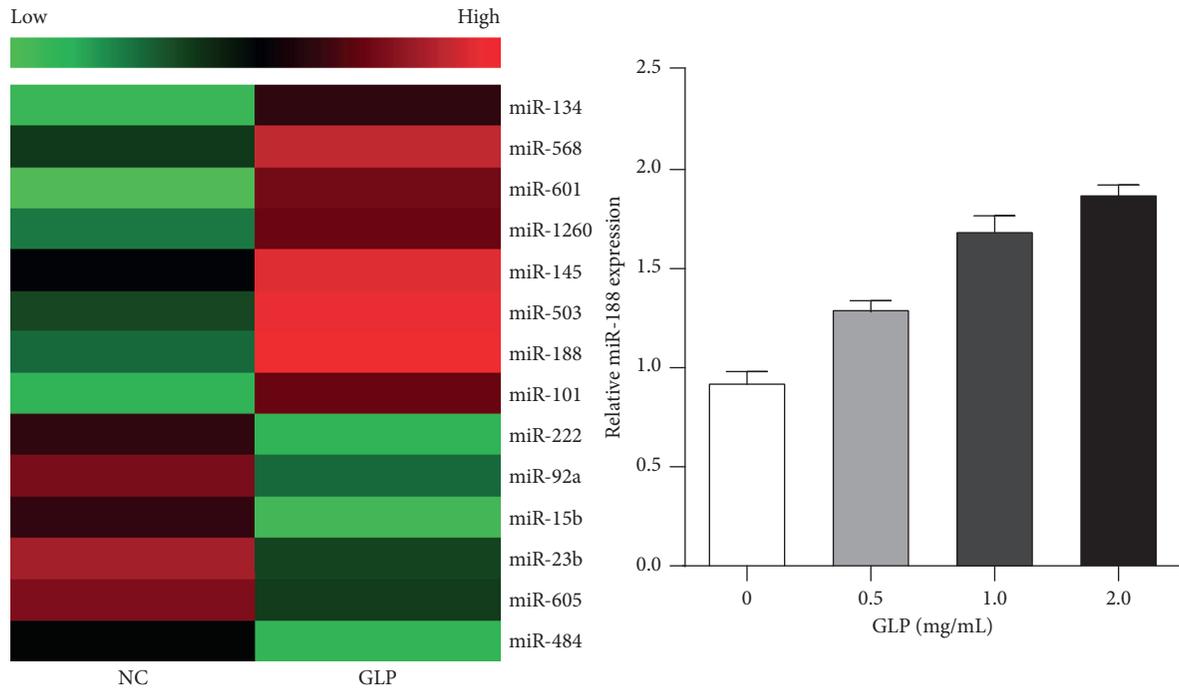


FIGURE 4: GLP upregulated the expression of miR-188.

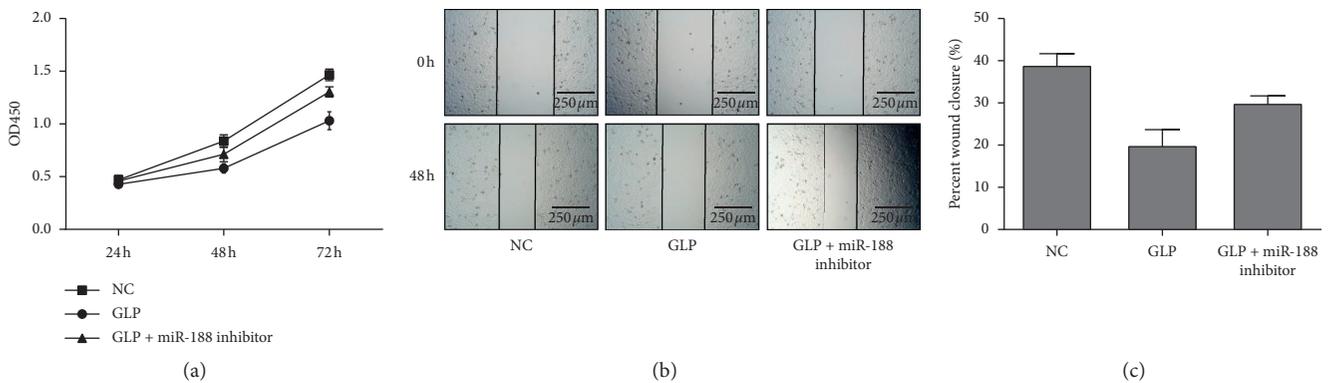


FIGURE 5: GLP inhibited the proliferation and migration of HSC-3 cells via miR-188. (a) Cell proliferation detected by the CCK-8 test; (b and c) cell migration measured by the scratch test.

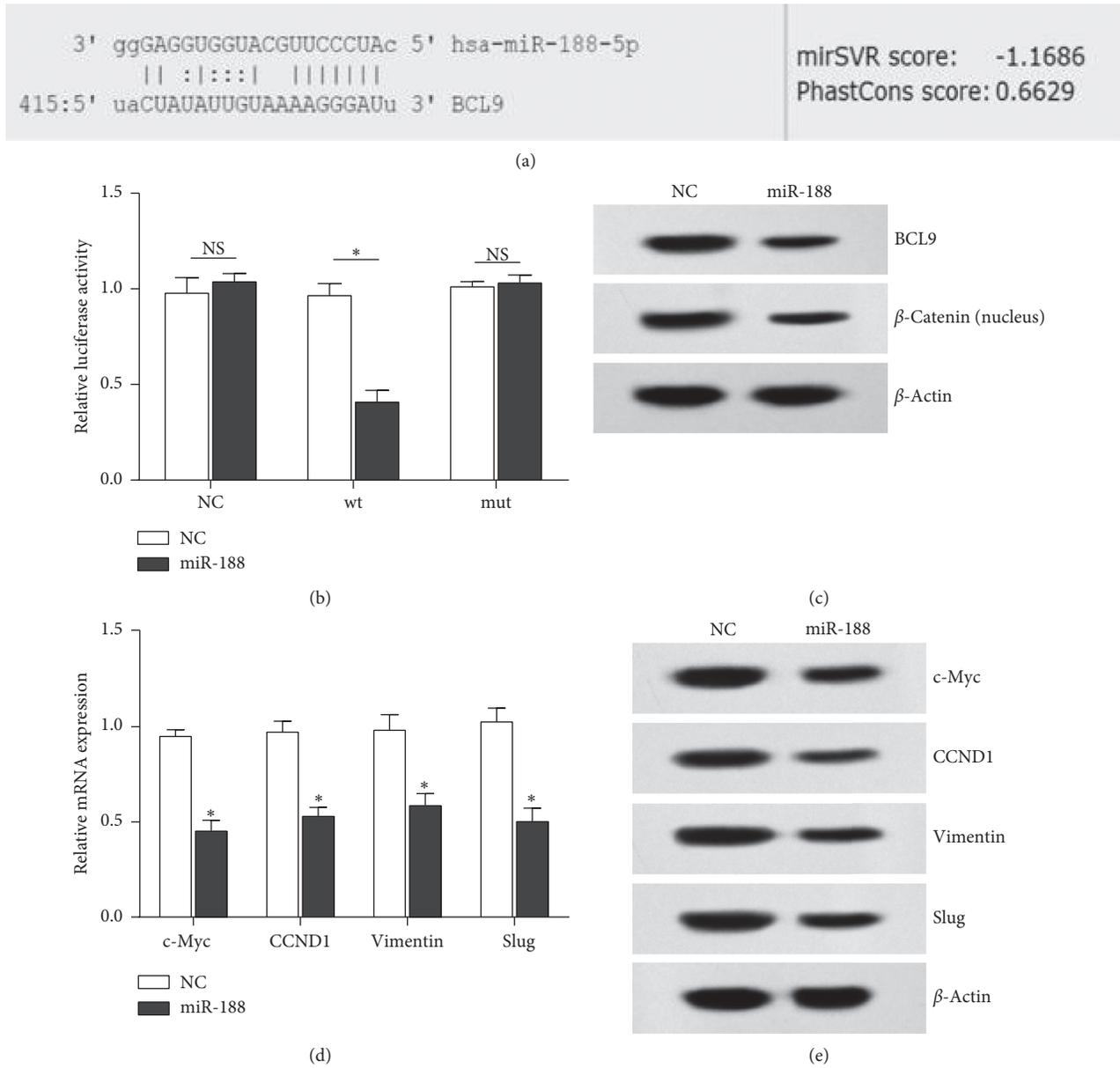


FIGURE 6: miR-188 regulated the β -catenin pathway by targeting BCL9. (a) BCL9 and miR-188 binding sites were predicted on the microRNA.org website; (b) verification of the binding sites via the luciferase reporter gene experiment; (c) miR-188 regulates BCL9 and nuclear β -catenin expression; (d and e) miR-188 regulates mRNA and protein expression of β -catenin downstream genes. * $P < 0.05$

5. Conclusion

Taken together, our results demonstrated that GLP can inhibit the proliferation and migration of OSCC cells (HSC-3) by regulating the miR-188/BCL9/ β -catenin signaling pathway. Therefore, the results in this study provide a theoretical basis for the treatment of OSCC with GLP.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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