Effects of ornithine decarboxylase antizyme 1 on the proliferation and differentiation of human oral cancer cells

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Abstract. Ornithine decarboxylase antizyme 1 (OAZ1) is an antizyme targeting ornithine decarboxylase for degradation, subsequently inhibiting polyamine production to prevent cell proliferation. OAZ1 is also involved in other major cellular events, including differentiation and apoptosis. Recent studies have shown that OAZ1 has tumor suppressor activities and its effects on cell proliferation and differentiation have been reported in several cancer cell lines. To explore the role of OAZ1 in human oral cancer, the effects of OAZ1 were studied on the proliferation and differentiation of human malignant oral cancer cell line, SCC15. MTT assay and flow cytometry analysis showed that stable *OAZ1* expression in SCC15 significantly inhibited cell proliferation (P<0.001) and induced G_0/G_1 arrest with the G_1-phase cells increased from 55.83 to 65.26%. Morphological observation revealed the increased formation of epithelial islands. Further results from quantitative reverse transcription-polymerase chain reaction and western blot analysis proved the upregulation of several terminal differentiation marker genes (*K10*, *FLG* and *LOR*) in *OAZ1*-expressed SCC15 cells. To elucidate the possible mechanism of *LOR* upregulation by OAZ1, further experiments were performed and it was found that the *OAZ1* expression inhibited Smad nuclear interacting protein 1 (SNIP1) at the protein level and RNA interference of SNIP1 in SCC15 cells, which increased the expression of *LOR*. These results show that OAZ1 simultaneously inhibits the proliferation and induces the differentiation of oral cancer cells in humans. The effects on cellular differentiation depend partly on the degradation of SNIP1.

Introduction

Ornithine decarboxylase antizyme 1 (OAZ1) is a member of the ornithine decarboxylase (ODC) antizyme family, which

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targets ODC for ubiquitin-independent proteasome degradation, thereby inhibiting the synthesis of polyamines and cell proliferation (1). OAZ1 can also inhibit cell growth through ODC-independent mechanisms, including the inhibition of cellular uptake of polyamines by inactivating the polyamine uptake transporter, promoting cyclin D1 degradation and preventing ubiquitin-independent msp1 degradation (2-5). Studies of cancer have also proved that OAZ1 has tumor suppressor activities and it can affect the apoptosis and proliferation of multiple tumor cell lines (6-8).

Several investigators have studied the involvement of OAZ1 in tongue squamous cell carcinoma (TSCC). In p53-knockout mice, overexpression of the OAZ1 protein inhibited the production of keratinocyte proliferation marker, keratin 6 (*K6*), whereas it upregulated the expression of keratinocyte differentiation marker, loricrin (*LOR*) (9). In hamster malignant oral keratinocytes, ectopic expression of *OAZ1* induced epithelial differentiation with the overexpression of involucrin (*IVL*) (10). In several human oral cancer cell lines, the expression level of the *OAZ1* gene was downregulated (11,12). A previous study has shown that OAZ1 induces the conversion of the human tongue squamous cancer cell line, UM1, to the less metastatic type, UM2, with the hypomethylation of genome DNA and histone H3 lysine 9 dimethylation (12). However, the overall effects of OAZ1 on cellular proliferation and differentiation in human oral cancer cells and the underlying mechanism remain to be studied.

In the present study, the lentiviral vector containing the *OAZ1* gene was constructed and transfected into the human tongue cancer cell line, SCC15, to evaluate the effects of *OAZ1* expression on proliferation and differentiation of the cells. The results showed that the stable expression of *OAZ1* in SCC15 inhibited the cell proliferation rate and induced G_0/G_1 arrest. *OAZ1* expression also induced the formation of epithelial islands with elevation of several differentiation marker genes (*K10*, *FLG* and *LOR*). OAZ1 was also found to inhibit Smad nuclear interacting protein 1 (SNIP1) and silencing of *SNIP1* increased the expression of *LOR* in SCC15 cells. The results of the study proved that OAZ1 can simultaneously inhibit proliferation and induce differentiation of oral cancer cells in humans.

Materials and methods

Cell culture and transfection. The human oral cancer cell line, SCC15, was obtained from the American Type Culture

Table I. Primers used for RT-qPCR.

Primer	Sequence (5'→3')	Product length (bp)
OAZ1 (NM_004152)	F: TCTCCCTCCACTGCTGTAGTAACC	198
	R: GTTGAGAATCCTCGTCTTGTCGTT	
E-cadherin (NM_004360)	F: CTACAATGCCGCCATCGCTTA	98
	R: CACTGATGACTCCTGTGTTCCTGTT	
K1 (NM_006121)	F: CGGAACTGAAGAACATGCAG	128
	R: CATATAAGCACCATCCACATCC	
K6A (NM_005554)	F: CAAGGCCCAATATGAGGAGA	135
	R: GCAATCTCCTGCTTGGTGTT	
K10 (NM_000421)	F: AAACCATCGATGACCTTAAAAATC	134
	R: GCGCAGAGCTACCTCATTCT	
K19 (NM_002276)	F: GCCACTACTACACGACCATCC	126
	R: CAAACTTGGTTCGGAAGTCAT	
FLG (NM_002016)	F: TTTCGGCAAATCCTGAAGAATCC	195
	R: ACTGTGCTTTCTGTGCTTGTG	
IVL (NM_005547)	F: CTGCCTCAGCCTTACTGTGA	133
	R: TGGGTATTGACTGGAGGAGG	
LOR (NM_000427)	F: GCACCGATGGGCTTAGAG	130
	R: AGAAACCAAAGAGGCTAAACAG	
SNIP1 (NM_024700)	F: AAGAAGCAAGTCTCCTCGCAG	128
	R: GTTCTGATGGTTCCCTGTGCT	
GAPDH (NM_002046)	F: CGCTGAGTACGTCGTGGAGTC	172
	R: GCTGATGATCTTGAGGCTGTTGTC	

Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's modified Eagles medium/F12 medium supplemented with 10% (v/v) fetal bovine serum at 37°C in 5% CO_2. The lentiviral vector (pLVX-Neo-IRES-ZsGreen) containing the green fluorescent protein (GFP) gene, *ZsGreen*, was ligated to *OAZ1* cDNA (loss of the frameshift site) to construct the stable expression vector of *OAZ1* (13). The cells were transferred to 6-well plates and the *OAZ1*-containing vector or control vector was transfected. When GFP was expressed, G418 (1,000 μg/ml) and flow cytometry were used to screen the stably transfected clones. SCC15 cells transfected with the *OAZ1*-expressed vector or the negative-control vector are referred as SCC15/*OAZ1* and SCC15/GFP.

Cell proliferation assay. Cells (1x10^4/well) were transferred in 3 replicates to 96-well plates and cultured on standard conditions. At the time-points of 24, 48 and 72 h, the viability of the cells was analyzed after the addition of 20 μl MTT (5 mg/ml) and dimethyl sulfoxide. Absorbance was determined using the Multiskan MK3 Microplate Reader (Thermo Scientific, Waltham, MA, USA) at a wavelength of 490 nm.

Cell cycle assay. Cells (2x10^5/well) were transferred in 3 replicates to 6-well plates. After 24 h of starvation, the cells were recovered for 36 h before analysis. The cells were treated according to the manufacturer's instructions for the cell cycle detection kit (Nanjing KeyGen Biotech, Nanjing, China) and submitted to flow cytometric analysis.

Morphological observation. Cells (1x10^3/well) were plated in 100x20 mm plates and observed under an inverted microscope after 1 week.

RNA interference. SNIP1-specific small hairpin RNA (shRNA) (GeneChem Co., Ltd., Shanghai, China) including sh-400 (5'-ATGCTGCTTTGAACAAGAC-3'), sh-401 (5'-TCGATGTATGTACATGACT-3'), sh-402 (5'-TTAGAC GTTCTCCTTTGGT-3') and the negative control, sh-NC (5'-TTCTCCGAACGTGTCACGT-3'), were synthesized. SCC15 cells were transferred to 6-well plates. When the cells reached 80% confluent, shRNA was transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in 250 μl Opti-MEM (Gibco, Carlsbad, CA, USA). Sh-NC was used as the negative control.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated using the RNAiso plus kit (Takara Bio, Inc., Shiga, Japan). Full-length cDNA was generated using the PrimeScript RT reagent kit (Takara, Dalian, China). cDNA was subjected to RT-qPCR analysis using SYBR-Green I (Takara) and results are reported as the ratio alteration among groups. The primers used are presented in Table I.

Immunoblotting. The cells were washed twice in phospate-buffered saline and lysed in radio-immunoprecipitation assay lysis buffer (with phenylmethanesulfonyl fluoride). Equal
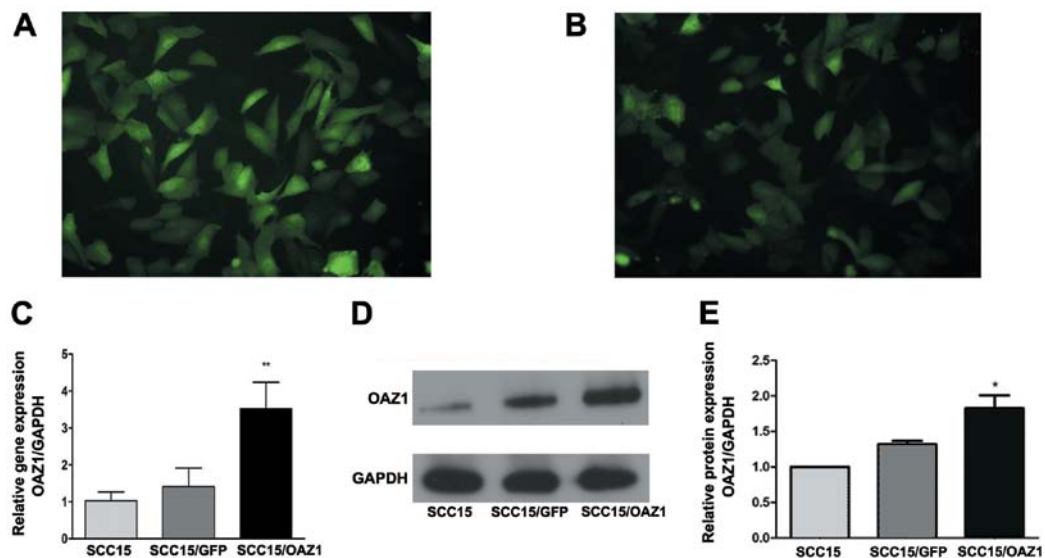
Figure 1. Expression of ornithine decarboxylase antizyme 1 (OAZ1) in SCC15 cells. (A and B) Green fluorescent protein (GFP) expression detected by florescence microscope (magnification, x200) in SCC15 cells transfected with the (A) *OAZ1*-expressed vector or (B) control vector. (C-E) Confirmation of OAZ1 expression by (C) reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and (D and E) western blotting. *P<0.05 and **P<0.01 compared to the other groups.
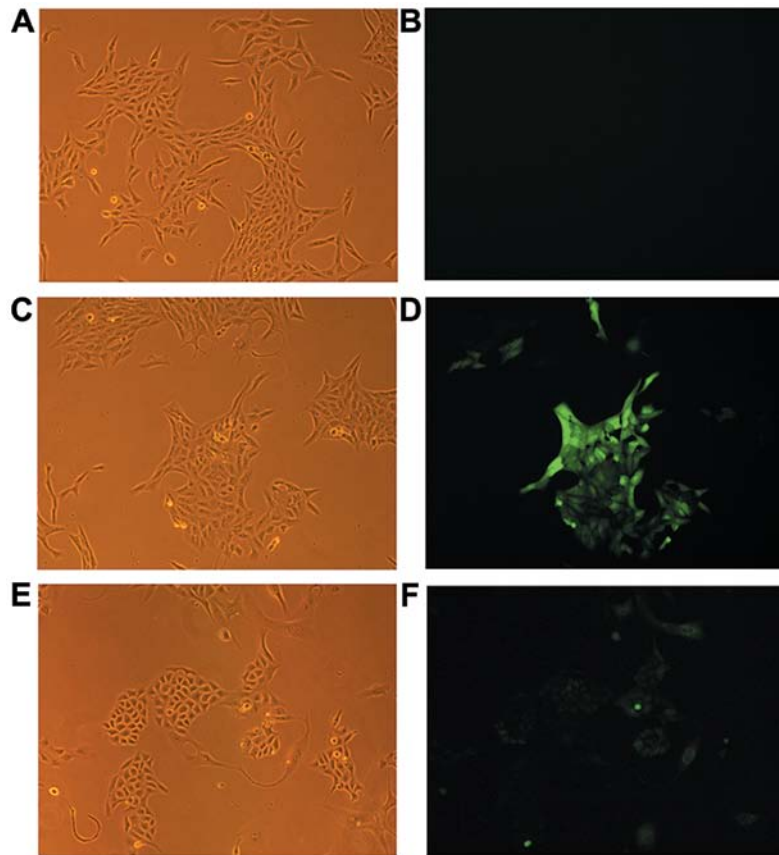

Figure 2. (A and B) Effect of ornithine decarboxylase antizyme 1 (OAZ1) overexpression on cell morphology in SCC15 cells (magnification, x100). SCC15 cells without treatment under a (A) regular or (B) fluorescence microscope. (C and D) SCC15 cells transfected with the control vector under a (C) regular or (D) florescence microscope. (E and F) SCC15 cells transfected with *OAZ1*-contained vector under a (E) regular or (F) florescence microscope.

amounts of total protein were separated by 12% SDS-PAGE, transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA) and probed with OAZ1 (Abcam, Hong Kong, China); K1, K10, IVL, LOR, filaggrin (FLG) and SNIP1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA);

and GAPDH (Cell Signaling Technology, Shanghai, China) antibodies and subsequently with the horseradish peroxidase-conjugated secondary antibody. The blots were evaluated with the ECL detection system (Advansta, Menlo Park, CA, USA). The protein bands of interest were quantified using
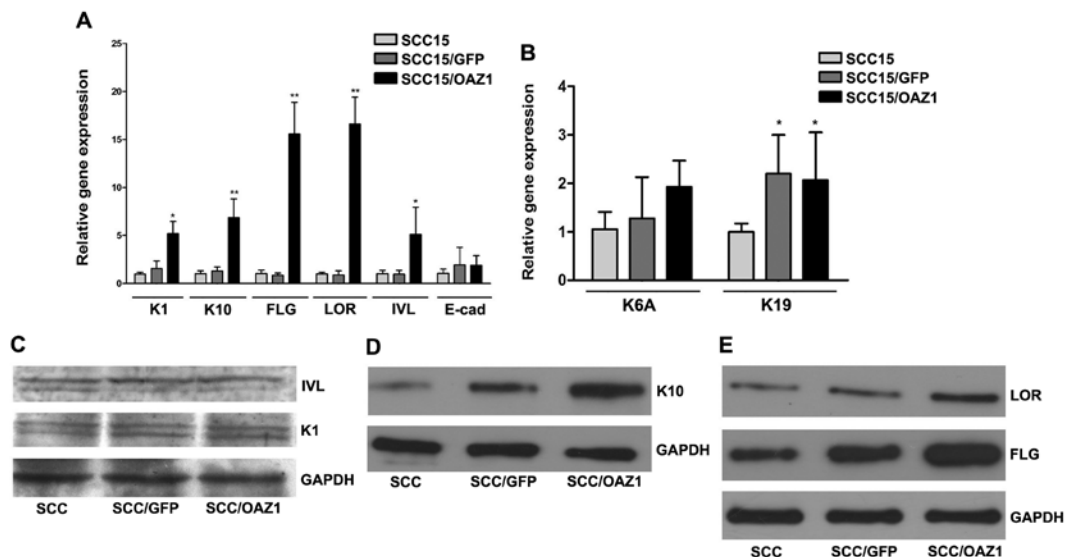
Figure 3. Effects of ornithine decarboxylase antizyme 1 (OAZ1) on differentiation and proliferation markers in SCC15 cells. (A and B) Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of the (A) differentiation [keratin 1 (*K1*), *K10*, filaggrin (*FLG*), involucrin (*IVL*), loricrin (*LOR*) and E-cadherin] and (B) proliferation markers (*K6A* and *K19*) among the different groups. (C-E) Western blotting detection of (C) K1, IVL, (D) K10, LOR and (E) FLG among the different groups. *P<0.05 and **P<0.01 compared to the other groups.
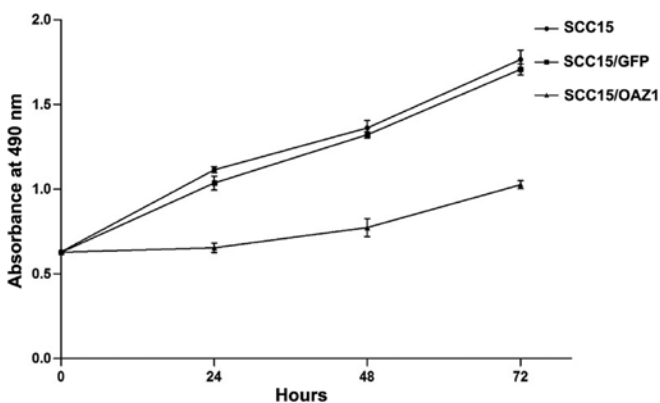

Figure 4. Ornithine decarboxylase antizyme 1 (OAZ1) inhibits cell proliferation in SCC15 cells. The curves show the viability of SCC15 cells detected by MTT analysis in the different groups.

FluorChem 8900 image analysis software (Alpha Innotech, San Leandro, CA, USA), and the integrated signal densities were normalized to GAPDH first, and subsequently expressed in terms of the fractional abundance relative to the control cells. These experiments were replicated 3 times.

Statistical analysis. SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Data are presented as means ± standard deviation. One-way analysis of variance was used and P<0.05 was considered to indicate a statistically significant difference.

Results

Overexpression of OAZ1 induces morphological changes in SCC15 cells. The lentiviral vector expressing *OAZ1* and GRF (*ZsGreen*), and the control vector expressing GFP were transfected into SCC15 cells separately and stable expression of GFP was observed in the two groups (Fig. 1A and B).

Quantitative analysis showed that the mRNA level of *OAZ1* was significantly elevated in the *OAZ1*-transfected group (Fig. 1C). The OAZ1 protein expression was verified by western blotting (Fig. 1D). The cells transfected with the control vector (Fig. 2C and D) demonstrated the typical appearance of SCC15 (Fig. 2A and B). However, the appearance of the *OAZ1* transfectants exhibited evidence of epithelial island formation and increased cellular junctions indicating the terminal differentiation of the cells (Fig. 2E and F).

OAZ1 affects the expression of marker genes in cell differentiation. *OAZ1*-transfected SCC15 cells showed morphological alterations indicating terminal differentiation. To further confirm the effects of OAZ1 on the differentiation of SCC15, the expression of several reported differentiation marker genes were examined. Elevation of early terminal differentiation genes, *K1*, *K10* and *IVL*, was observed in *OAZ1*-expression cells. As for late terminal differentiation genes, the elevation of *FLG* and *LOR* was also observed, whereas the mRNA level of E-cadherin remained stable (Fig. 3A). When detected by western blot analysis, K10, LOR and FLG showed increased expression on protein level (Fig. 3C-E).

OAZ1 inhibits the proliferation of SCC15 cells. MTT assay was performed to detect the effects of *OAZ1* expression on the proliferation of SCC15. Compared to the control, SCC15 cells stably expressing *OAZ1* showed a significant reduction in the cell growth rate (P<0.001 compared to the other groups) (Fig. 4), indicating that *OAZ1* expression inhibited the proliferation of SCC15.

The cell cycle profiles of the *OAZ1*-transfected SCC15 cells and the control cells were further examined (Fig. 5). The flow cytometry analysis showed that the cells in the G$_1$ phase were increased in *OAZ1*-expressed SCC15 cells (65.26%) compared to the control (55.83%). Whereas cells in the S phase were decreased to 30.07% compared to the control
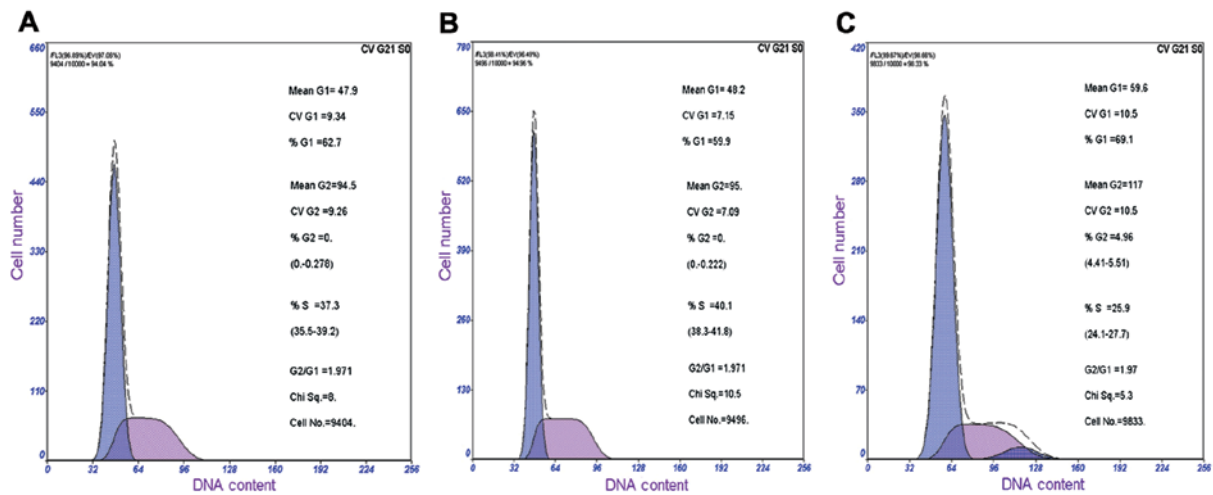
Figure 5. Ornithine decarboxylase antizyme 1 (OAZ1) affects the cell cycle in SCC15 cells. Flow cytometry analysis of (A) untreated SCC15 cells, (B) cells transfected with the control vector and (C) cells transfected with the *OAZ1*-contained vector.
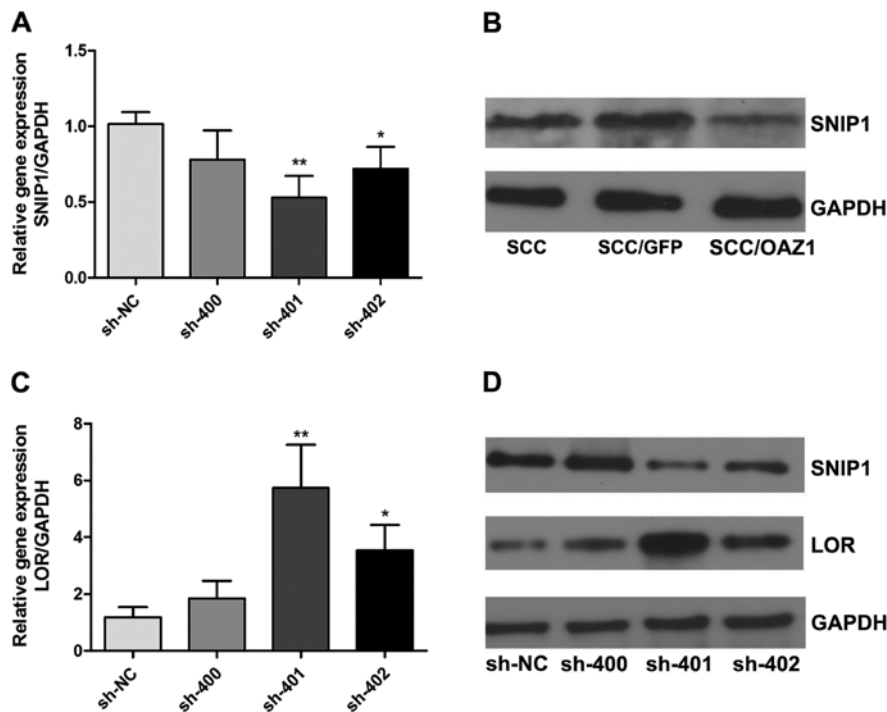

Figure 6. Ornithine decarboxylase antizyme 1 (OAZ1) induced *LOR* expression through degradation of Smad nuclear interacting protein 1 (SNIP1). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of (A) *SNIP1* and (C) Loricrin (*LOR*) expression in the different siRNA silencing groups (sh-400, sh-401 and sh-402) of *SNIP1* and the control (sh-NC). (B) Western blot analysis of SNIP1 in the OAZ1-transfected group and control. (D) Western blot analysis of SNIP1 and LOR in the SNIP1-silencing group and the control. *P<0.05 and **P<0.01 compared to the control group.

(42.47%) (Table II). These results indicate that OAZ1 may inhibit the cell proliferation through induction of G_0/G_1 arrest.

The expression of two genes reported to be elevated during epithelial cell proliferation, *K6A* and *K19*, were also investigated. RT-qPCR results showed that *K19* expression was significantly elevated (P<0.05), and the level of *K6A* remained stable (Fig. 3B).

OAZ1 inhibits LOR by promoting SNIP1 degradation. OAZ1 can induce the degradation of SNIP1, leading to the release of CREB-binding protein (CBP), a key factor in the regulation of *LOR* transcription (14,15). To further explore the mechanism

of *LOR* upregulation by OAZ1, the expression of SNIP1 in *OAZ1*-transfected cells was examined. The SNIP1 protein level was downregulated (Fig. 6B) in *OAZ1* transfectants. The RNA interference experiment showed that inhibition of *SNIP1* in the SCC15 cells induces the expression of *LOR* (Fig. 6A, C and D). These results indicate that OAZ1 may increase *LOR* expression through the degradation of SNIP1.

Discussion

OAZ1 is an important regulator of cellular differentiation and proliferation. Studies have focused on its unique frameshift

Table II. Effect of OAZ1 on cell cycle in SCC15 cells.

Groups	G_1, %	S, (%)	G_2, %
SCC15	55.83±5.95	42.47±4.49	1.73±1.57
SCC15/GFP	54.83±4.43	44.17±3.69	1.36±1.52
SCC15/OAZ1	65.26±3.32[a]	30.07±4.42[b]	4.64± 2.58

[a]$P<0.05$ and [b]$P<0.01$ compared to the other groups. OAZ1, ornithine decarboxylase antizyme 1.

regulation mechanism, function in regulating polyamine metabolism and ability of inducing ubiquitin-independent degradation of numerous large molecules. Previously, it has been reported that OAZ1 has tumor suppressor activities and it can affect the apoptosis and proliferation of multiple tumor cell lines (6-8,16). There is also evidence that overexpression of *OAZ1* can induce the differentiation of certain cancer cells (10,11,17). The mechanism underlying the tumor suppressor activity of OAZ1 remains unclear and further exploration may help with the understanding.

TSCC is one of the most common malignant oral tumors. Previous studies of the oral cancer cell line UM1 have proved that TSCC is an appropriate model to investigate the detailed molecular mechanisms of differentiation and proliferation regulation in oral cancer development (11,12). In the present study, the human TSCC cell line SCC15 was studied and a lentivirial vector was constructed that stably expresses the frame shift form of *OAZ1* to continuously trigger the degradation of ODC. The expression of *OAZ1* in SCC15 was verified by western blot analysis. Morphological study showed that the cells overexpressing *OAZ1* exhibit the formation of more epithelial islands, which is a marker of terminal differentiation of epithelial cells. This is also consistent with the study on the hamster oral cancer cell line, HCPC-1 (10).

To elucidate the mechanism of OAZ1-induced cell differentiation in SCC15 cells, the expression of several terminal differentiation-associated genes was examined, including *K1*, *K10*, *FLG*, *IVL* and *LOR*. All these genes are marker genes in epithelial differentiation that have decreased expression in epithelial cancer cells, which is resumed during differentiation (18-25). In hamster oral cancer cells, *OAZ1* has been proved to promote the expression of *LOR* and *IVL* (9,10). In the present study, *OAZ1*-expressed SCC15 cells showed significantly increased expression of the majority of the marker genes assessed, confirming the positive role of OAZ1 in epithelial differentiation. The gene involved in cellular junction, E-cadherin, was also examined as closer conjunction of cells expressing *OAZ1* was observed and E-cadherin is reduced in squamous carcinoma (26). However, the mRNA level of E-cadherin showed no significant difference between the *OAZ1*-transfected SCC15 and control cells, which is consistent with a previous study (11). This suggests that factors other than E-cadherin may be involved in the induction of closer cellular junctions for this condition.

The mechanism underlying the induction of *LOR* expression in *OAZ1*-expressed SCC15 cells was further explored. LOR is the component of the cell membrane in terminal differentiated keratinocytes and p300/CBP can function as a bridge during its transcription-activator complex formation (15). SNIP1 has been reported to competitively inhibit the binding of CBP in the promotor region, leading to the inhibition of transcription (14,27). The present study showed that *OAZ1* overexpression promoted the degradation of SNIP1 and the silencing of SNIP1 by RNA interference resumed the level of LOR in SCC15 cells. These results indicate that OAZ1 may induce *LOR* expression partly through SNIP1 degradation.

For the study of cell proliferation in *OAZ1*-expressing SCC15 cells, the results showed that overexpression of the antizyme significantly inhibited cell proliferation with G_0/G_1 arrest. Two genes, *K6A* and *K19*, were further examined that are elevated during epithelial proliferation (9,26). However, the results of RT-qPCR showed that *K19* expression was elevated instead of reduced and the level of *K6A* remained stable. OAZ1 is assumed to inhibit the proliferation of SCC15 cells without the involvement of regulating *K6A* and *K19* expression.

In conclusion, the study showed that OAZ1 induced the formation of epithelial islands with significant upregulation of epithelial terminal differentiation marker genes in SCC15. *OAZ1* expression also inhibited cell proliferation and induced G_0/G_1 arrest. OAZ1 simultaneously inhibited proliferation and induced differentiation of oral cancer cells in humans. This may be a novel perspective to consider in the induction of tumor cell differentiation, particularly in human oral cancer.

Acknowledgements

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