ID1 contributes to cell growth invasion and migration in salivary adenoid cystic carcinoma

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Abstract. Previous studies have reported that inhibitor of DNA binding 1 (ID1) exerts an oncogenic role in a number of tumors. In the present study, the role of ID1 in the growth, invasion and migration of salivary adenoid cystic carcinoma (SACC) cells was investigated. ID1 expression in clinical SACC samples was compared with that in normal salivary tissues using immunohistochemical staining, and the correlation between ID1 expression and clinical pathological characteristics was then determined. Subsequently, ID1 was overexpressed or silenced to investigate the effects of ID1 expression on SACC cell proliferation, invasion and migration. In addition, the gene expression levels of known ID1 target genes, including S100A9, CDKN2A and matrix metalloproteinase 1 (MMP1) was measured using reverse transcription-quantitative polymerase chain reaction to elucidate the potential mechanisms of ID1 in SACC. The results of the present study indicated that the protein expression levels of ID1 were significantly increased in the SACC tissues compared with that in the normal salivary tissues (P<0.001), and a positive correlation between ID1 expression and tumor stage (P=0.001), tumor invasion (P=0.002) and metastasis (P=0.019) in SACC was observed. Knockdown of ID1 in SACC cells significantly inhibited cell growth, invasion and migration (all P<0.01), whereas overexpression of ID1 promoted cell proliferation, invasion and migration (all P<0.01). The gene expression level of MMP1 was significantly reduced following ID1 knockdown in SACC-83 cells when compared with negative controls (P<0.05), whereas S100A9 and CDKN2A expression levels were significantly upregulated (both P<0.05). The results suggest that ID1 may regulate the growth, invasion and migration of SACC cells, and that MMP1, S100A9 and CDKN2A may serve as target genes of ID1 and mediate the effects of ID1 in SACC cells. Therefore, ID1 may present a potential target gene for the treatment of patients with SACC to inhibit cancer cell growth and metastasis.

Introduction

Salivary adenoid cystic carcinoma (SACC) is a common malignant tumor and is characterized by distinctive clinical features and behaviors, including neural and blood invasiveness, aggressive growth, distant metastasis and poor long-term survival (1). SACC occurs in the major and minor salivary glands and spreads to the oral and oropharyngeal mucosa, tracheobronchial tree and the esophagus (2). Previous studies have indicated that 40-60% of patients with SACC develop distant metastases in the soft tissues, lungs and bone (1,3). Typically, distant metastasis is associated with poor patient survival (3). It could be beneficial for patients with SACC to be able to identify potential molecular targets for the early diagnosis, therapy and prognostic analysis (4-9).

Inhibitor of DNA-binding (ID) proteins, including ID1, ID2, ID3 and ID4, belong to the helix-loop-helix (HLH) protein family (10). ID proteins inhibit DNA binding and the transcriptional activity of basic HLH proteins (10). Previous studies suggest that ID1 is overexpressed in various types of cancer, including melanoma, breast and gastric cancer, endometrial carcinoma, osteosarcoma, oral squamous cell carcinoma and lung cancer (11-18). In addition, a correlation between ID1 protein and tumor angiogenesis in SACC has been determined (19). ID1 serves a key role in cell growth, senescence and differentiation. Furthermore, ID1 may mediate

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tumor progression and metastasis by promoting tumor angiogenesis in SACC (20).

In the present study, the role of ID1 in SACC was investigated. The expression of ID1 in clinical SACC samples and normal salivary tissues was determined using immunohistochemistry. In addition, the correlation between ID1 expression and clinical pathological characteristics, including age, gender, tumor stage, tumor invasion and metastasis was examined. ID1 expression was silenced using small interfering RNA (siRNA), or overexpressed using plasmids to investigate its effects on the growth, invasion and migration of SACC cells *in vitro*. To explore the potential mechanisms of ID1 in SACC, the expression of known ID1 target genes, including S100A9, CDKN2A and matrix metalloproteinase-1 (MMP1), was analyzed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis following transfection of SACC cells with ID1 siRNA.

Materials and methods

Cell culture and clinical samples. The SACC-83 cell line was provided by the Peking University School of Stomatology (Beijing, China). The cells were maintained in RPMI 1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and incubated in humidified atmosphere containing 5% CO_2 at 37°C. Surgically resected salivary tissue samples were obtained from the First Affiliated Hospital of the Fujian Medical University (Fuzhou, China) and the Fujian Medical University Union Hospital (Fuzhou, China) between May 2004 and September 2014 and all the samples were reviewed and diagnosed by two independent pathologists. A total of 50 normal tumor-adjacent salivary tissues (24 females and 26 males, age range 23-75, mean age 48.57±14.22) and 68 SACC samples (40 females and 28 males, age range 18-79, mean age 50.13±14.92) were included. The Institutional Review Board of Fujian Medical University (Fuzhou, China) approved the present study, and written informed consent was obtained from each participant.

Immunohistochemistry. Salivary tissues were fixed in 10% neutral buffered formalin at 4°C for 24 h, embedded in paraffin and sections (5-μm in thickness) were mounted on slides coated with poly-L-lysine. Following deparaffinization in xylene at 37°C, the sections were rehydrated in a decreasing ethanol series, washed for 10 min in phosphate-buffered saline (PBS; pH 7.2) and incubated in methanol containing 3% H_2O_2 for 10 min at room temperature. Following several washes in PBS, the sections were blocked with a universal 5% blocking reagent (Fuzhou Maixin Biotech. Co., Ltd., Fuzhou, China) for 10 min at room temperature, and subsequently incubated with a primary antibody against ID1 (ab66495; 1:30; Abcam, Cambridge, MA, USA) for 1 h at room temperature. Following several washes with PBS, the sections were incubated with a biotin-conjugated secondary antibody (ready-to-use; Rabbit Anti-Mouse lgG H&L EliVision™ plus kit; KIT-9902-B; Fuzhou Maixin Biotech. Co., Ltd.) for 10 min at room temperature. The sections were then washed several times with PBS and incubated with streptavidin-peroxidase (ready-to-use, ABD-0030; Fuzhou Maixin Biotech. Co., Ltd.) for 10 min at

room temperature. The sections were subsequently rinsed with PBS and the antibody complexes were visualized following incubation with diaminobenzidine chromogen (DAB chromogenic kit; DAB-0031; Fuzhou Maixin Biotech. Co., Ltd.) for 2 min at 37°C. Sections were counterstained with hematoxylin (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 2 min at room temperature, dehydrated and examined by light microscopy. Two pathologists reviewed all slides independently and in a blinded fashion. The staining results were quantified using the following 4-tier scale: Negative, no staining; 1+, weakly-positive staining; 2+, positive staining; and 3+, strongly-positive staining. Cell staining was also assessed using the following four-tier scale: Negative, no staining in the cells; 1+, staining was present in <30% of cells; 2+, indicated staining in 30-50% of cells; and 3+, staining was present in >50% of cells. The immunohistochemical results were graded with 4 different scores (negative, weakly-positive, positive and strongly-positive).

RNA interference (RNAi) and plasmid transfection. Negative control (NC) siRNA and two siRNAs against ID1 (siRNA800 and siRNA858) were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The siRNA sequences are listed in Table I. At 24 h prior to transfection, SACC-83 cells in the exponential phase of growth were trypsinized by Trypsin-EDTA solution (Gibco; Thermo Fisher Scientific, Inc.), counted and plated in 6-well plates at a density of $3x10^5$ cells/well. Cells were transfected with 50 nM siRNAs using Lipofectamine RNAiMAX reagent (Invitrogen; Thermo Fisher Scientific, Inc.), or the ID1 recombinant plasmid pEX-2 (2 μg/well; Shanghai GenePharma, Co., Ltd.) using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. SACC-83 cells were transfected with NC siRNA or empty vector as a negative control for siRNA and plasmid transfection, respectively.

RT-qPCR. At 48 h following transfection with siRNA, total RNA was extracted from SACC-83 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and 2.5 μg total RNA were reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan, Japan) according to the manufacturer's protocols. The cDNA was used as a template to detect gene expression levels of ID1 and known ID1 target genes, S100A9, CDKN2A and MMP1, by RT-qPCR analysis using SYBR Premix Ex Taq™ (Takara Bio, Inc.) The 20 μl PCR mixture contained 10 μl SYBR Premix Ex Taq™ (2X), 0.4 μl PCR Forward Primer (10 μM), 0.4 μl PCR Reverse Primer (10 μM), 0.4 μl ROX Reference Dye I (50X), 2 μl cDNA template and 6.8 μl ddH_2O. GAPDH was used as an internal control. The primers used in the present study are listed in Table II. The reaction was as follows: Denaturation at 95°C for 2 min, followed by 40 cycles at 95°C for 15 sec, 60°C for 30 sec. The fluorescent signal was measured at the end of the annealing phase of every cycle. Target gene expression was quantified using the $2^{-\Delta\Delta Cq}$ method (21).

Western blotting. At 48 h following transfection with siRNA and recombinant ID1 plasmids, total proteins were extracted from SACC-83 cells with IP lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). The protein concentration

Table I. Sequences of siRNAs employed in the present study.

siRNA	Sense (5'-3')	Antisense (5'-3')
ID1-800	GGGAUUCCACUCGUGUGUUTT	AACACACGAGUGGAAUCCCTT
ID1-858	GUUUGGUGCUUCUCAGAUUTT	AAUCUGAGAAGCACCAAACTT
Negative control	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

siRNA, small interfering; ID1, inhibitor of DNA binding 1.

Table II. Reverse transcription-quantitative polymerase chain reaction primers.

Gene	Forward (5'-3')	Reverse (5'-3')
ID1	ATTACGTGCTCTGTGGGTCTCC	TAGTAGGTGTGCAGAGAGGAGC
S100A9	GCTGGAACGCAACATAGAGACC	TCTGCATTTGTGTCCAGGTCCT
CDKN2A	GGCACCAGAGGCAGTAACCAT	GAAAGCGGGGTGGGTTGTG
MMP1	GGGGAGATCATCGGGACAACTC	AGAATGGCCGAGTTCATGAGCT
GAPDH	TGCACCACCAACTGCTTAGC	AGCTCAGGGATGACCTTGCC

ID1, inhibitor of DNA binding 1; MMP1, matrix metalloproteinase 1.

was determined by BCA method and 25 μg of protein was loaded per lane for SDS-PAGE separation. The proteins were separated by 8% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (GE Healthcare; Chicago, IL, USA). Membranes were blocked in 5% bovine serum albumin (VWR International, Radnor, PA, USA) at 4˚C for 60 min. Subsequently, the membranes were immunoblotted overnight at 4˚C with primary antibodies against ID1 (ab66495; 1:1,000; Abcam) or GAPDH (M20006; 1:2,000; Abmart, Inc., Berkeley Heights, NJ, USA). Membranes were then washed three times in Tris-buffered saline with 0.1% Tween 20 and subsequently incubated with a secondary antibody (Goat Anti-Mouse IgG H&L (HRP); ab205719; 1:2,000; Abcam) at room temperature for 60 min. Protein bands were visualized using CDP-Star reagent (Roche Diagnostics, Indianapolis, IN, USA). The signals were detected by exposure to X-Ray film for different times (1-10 min).

Cell proliferation assay. The number of viable SACC-83 cells in the logarithmic phase of growth following siRNA or plasmid transfection was measured at 24 h intervals using the Cell Counting Kit-8 assay (CCK-8; CK04; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocols. SACC-83 cells were transfected with siRNAs or plasmids and $2x10^3$ cells/well were seeded in a 96-well plate. CCK8 reagent (10 μl/well) was added to the cells at the same time over 5 consecutive days and incubated at 37˚C for 1 h and the absorbance of each well was measured at 450 nm using a microplate reader (Pharmacia Biotech, Inc.; GE Healthcare). This procedure was performed in triplicate for statistical analysis and to construct the corresponding proliferation curves.

Colony formation assay. At 24 h following siRNA or plasmid transfection, cells were plated in 6-well plates at a density of 500 cells/well, and cultured for 2 weeks. Colonies were fixed with cold 100% methanol for 10 min and stained with 1% crystal violet for 30 min at room temperature and the number of colonies in each well was counted. The experiments were repeated three times.

Wound healing assay. Then, 24 h after seeded in a 6-well plate at a density of $7x10^5$ cells/well, SACC-83 cells were transfected with siRNAs or plasmids. The cells formed a monolayer covering the bottom of the plate, and a 20-μl pipette tip was used to generate a scratch-wound. The medium was replaced with RPMI-1640 supplemented with 0.1% FBS at 0 and 24 h following generation of the scratch-wound. The cells were visualized under a light microscope at both time points, and images were captured. The width of scratch-wound was measured by the ruler to evaluate the cell invasion.

In vitro cell invasion assay. Cell invasion was determined using 24-well Matrigel-coated Transwell chambers (8-μm pore size; 354480; BD Bioscience, Bedford, MA, USA). 24 h after transfection with siRNA or plasmids, SACC-83 cells were serum-starved for 24 h and then harvested and resuspended in RPMI 1640 containing 1% FBS. Cells were subsequently plated in the upper chamber of the Transwell plate at a density of $1x10^5$ cells, and 500 μl RPMI 1640 containing 10% FBS was added to the lower chamber. Following incubation at 37˚C for 48 h, the Matrigel and cells in the upper chamber were removed using a cotton swab and the chamber was stained with 1% crystal violet for 10 min at room temperature. The cells were counted under a light microscope in at least five random fields of view (magnification, x200) and the images were captured.

In vitro cell migration assay. Cell migration assays were performed using 24-well Transwell chambers (8 μm pore

Table III. Expression of ID1 in normal salivary and SACC samples.

Sample	Case	Negative	Weakly positive	Positive	Strongly positive	P-value
Normal salivary	50	46	1	1	2	<0.001
SACC	68	24	16	23	5	

Rank-sum test, Z=-8.097, P<0.001. ID1, inhibitor of DNA binding 1; SACC, salivary adenoid cystic carcinoma.
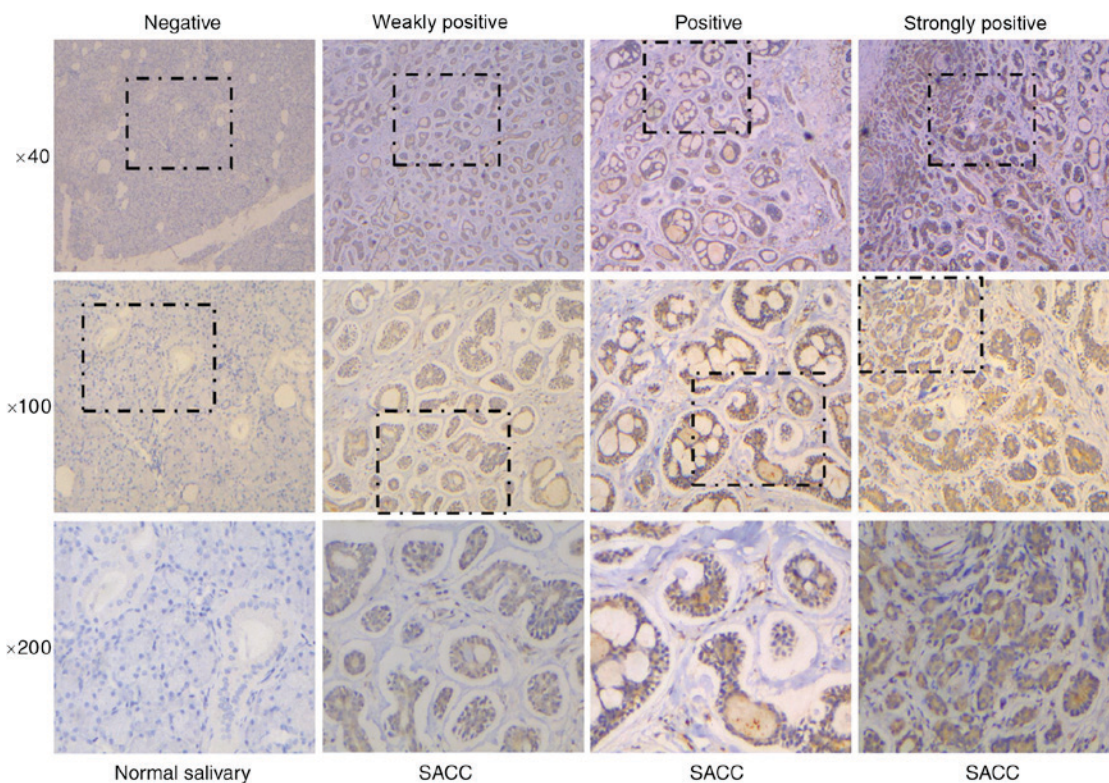

Figure 1. ID1 protein expression is upregulated in SACC. Representative images of the expression of ID1 in SACC and normal salivary tissue sections, as determined by immunohistochemical analysis. Tissues were analyzed at magnifications of x40, x100 and x200 using the following four-tier scale: Negative, weakly positive, positive and strongly positive. SACC, salivary adenoid cystic carcinoma; ID1, inhibitor of DNA binding 1.

size; 353097; BD Biosciences, Bedford, MA, USA). The same procedure for the cell invasion assay was followed with the exception that the plate was not Matrigel-coated.

Statistical analysis. The results were analyzed using the SPSS 22.0 statistics software package (IBM Corp., Armonk, NY, USA). Data are presented as the mean ± standard deviation. Rank-sum tests were used to compare the two groups of immunohistochemistry data, the Mann-Whitney U test was used to correlate ID1 expression with clinicopathological features and multi-sample mean one-way analysis of variance tests with Dunnett's test were used for group comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

ID1 is upregulated in SACC. The salivary tissue samples consisted of 68 SACC and 50 normal tumor-adjacent tissue samples. As shown in Fig. 1 and Table III, tissue samples were divided into four groups depending on ID1 staining intensity. The results demonstrated that ID1 protein expression was upregulated in SACC tissue samples when compared with normal tissues (P<0.001; Table III). Subsequently, the association of ID1 protein expression levels with clinical features of SACC was determined. The results shown in Table IV demonstrate that the expression of ID1 was significantly increased in the late-stage tumors when compared with early-stage tumors (P=0.001), and ID1 expression levels were significantly correlated with tumor invasion (P=0.002) and tumor metastasis (P=0.019) in patients with SACC. These results indicate that ID1 may serve an important role in SACC.

ID1 promotes SACC cell proliferation in vitro. To investigate the role of ID1 in the proliferation of SACC cells, siRNAs targeting ID1 (siRNA800 and siRNA858) were transfected into SACC-83 cells to knockdown ID1 expression. As indicated by RT-qPCR (Fig. 2A; P<0.01) and western blotting (Fig. 2B) analyses, both siRNAs targeting ID1 efficiently

Table IV. Association between the inhibitor of DNA binding 1 expression and the clinical and pathological characteristics of patients with salivary adenoid cystic carcinoma.

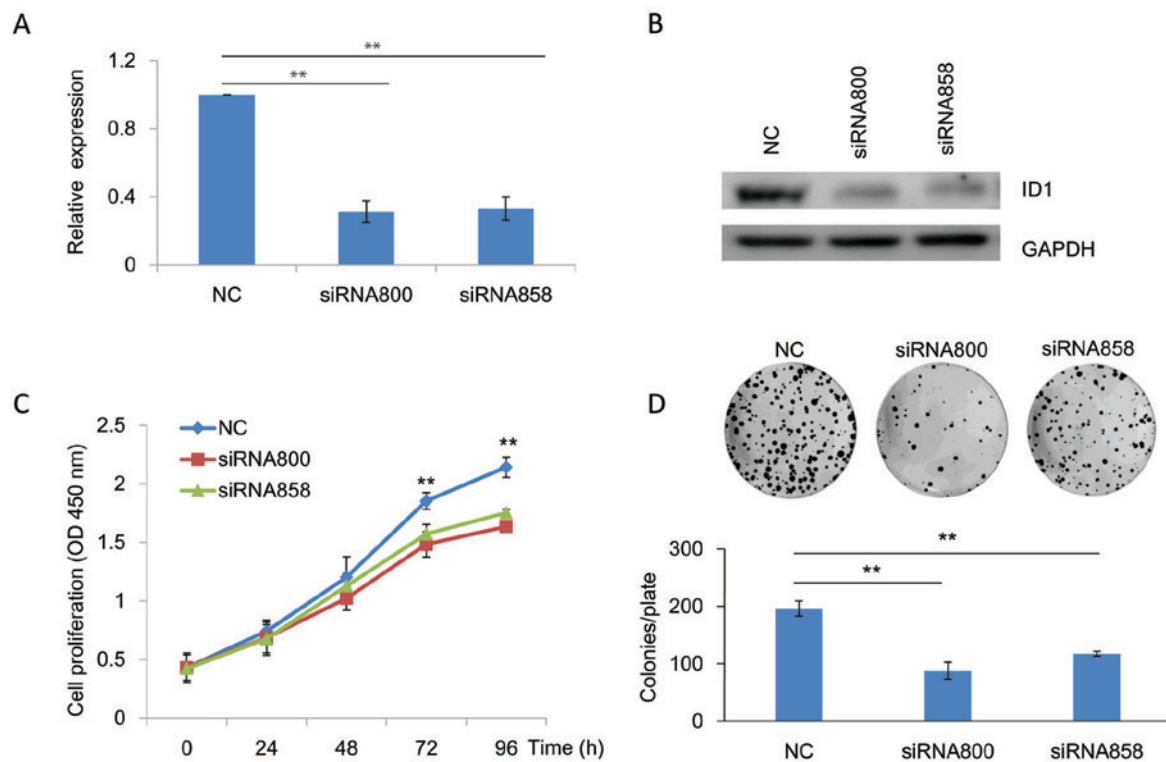| Characteristics	Staining intensity				Total	P-value
	Negative	Weakly positive	Positive	Strongly positive		
Gender						
Female	13	10	14	3	40	0.661
Male	11	6	9	2	28	
Age (years)						
≤55	15	9	13	4	41	0.942
>55	9	7	10	1	27	
Stage						
Early	18	5	7	1	31	0.001
Late	6	11	16	4	37	
Invasion						
No	15	7	6	0	28	0.002
Yes	9	9	17	5	40	
Metastasis (lymph node and distant)						
No	20	14	14	2	50	0.019
Yes	4	2	9	3	18	

Figure 2. Knockdown of ID1 inhibits the proliferation of SACC-83 cells. At 48 h following transfection with ID1 siRNA, the expression of ID1 in SACC-83 cells was measured by (A) reverse transcription-quantitative polymerase chain reaction (**P<0.01 vs. NC) and (B) western blotting. At 24 h following transfection of SACC-83 cells with ID1 siRNA or plasmids, the proliferation of the SACC-83 cells was detected using the (C) Cell Counting kit-8 assay (**P<0.01 vs. siRNA800 and P<0.01 vs. siRNA858), and (D) a colony formation assay (**P<0.01 vs. NC as indicated). The results are presented as the mean ± standard deviation (n=3). ID1, inhibitor of DNA binding 1; siRNA, small interfering RNA; SACC, salivary adenoid cystic carcinoma; NC, negative control; OD, optical density.

reduced ID1 expression levels in SACC-83 cells when compared with the NC. The results of the CCK-8 assay demonstrated that the growth of SACC-83 cells at 72 and 96 h following knockdown of ID1 expression was significantly
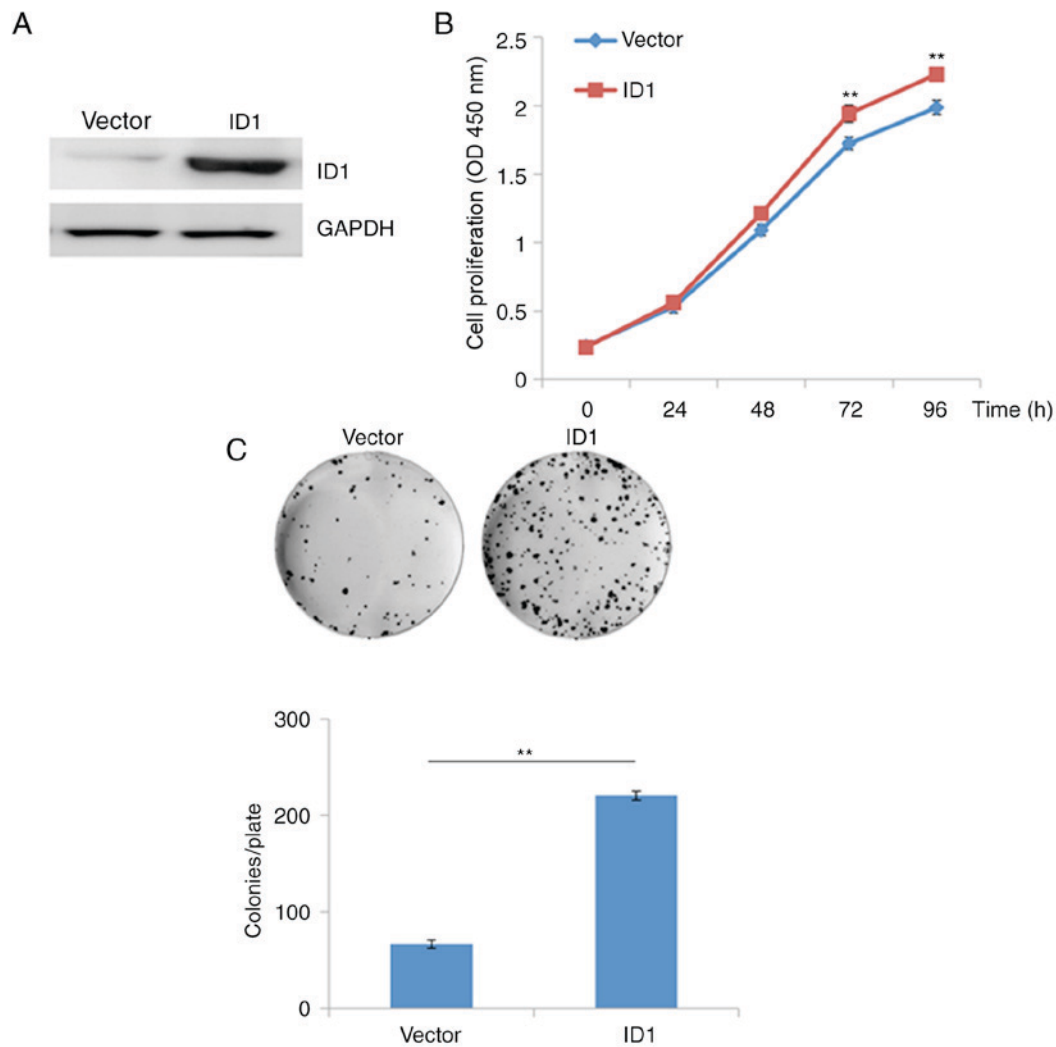
Figure 3. Overexpression of ID1 promotes the proliferation of SACC-83 cells. (A) Western blotting analysis of ID1 protein expression levels in SACC-83 cells transfected with a recombinant plasmid (pEX-2) containing the ID1 sequence or empty vector controls. Following plasmid transfection, the proliferation of SACC-83 cells was detected using the (B) Cell Counting kit-8 assay at 0-96 h (**P<0.01 vs. vector-only transfected cells), and a (C) colony formation assay (**P<0.01 as indicated). The results are presented as the mean ± standard deviation (n=3). ID1, inhibitor of DNA binding 1; SACC, salivary adenoid cystic carcinoma; OD, optical density.

reduced when compared with the NC (P<0.01; Fig. 2C). These results were consistent with the colony formation assay, which demonstrated that knockdown of ID1 was associated with a significant reduction in the number of colonies (P<0.01; Fig. 2D). To further verify these results, SACC-83 cells were transfected with a recombinant plasmid (pEX-2) containing the coding sequence of ID1. As demonstrated in Fig. 3A, transfection recombinant expression plasmids containing the ID1 sequence was associated with a marked increase in the protein expression levels of ID1 compared with empty vector controls (Fig. 3A). Overexpression of ID1 in SACC-83 cells significantly promoted cell growth at 48, 72 and 96 h following transfection when compared with vector-only transfected cells (P<0.01; Fig. 3B). Similarly, the results of the colony formation assay demonstrated that overexpression of ID-1 significantly increased the growth of SACC-83 cells when compared with vector-only transfected cells (P<0.01; Fig. 3C). These results suggest that overexpression of ID1 may promote the proliferation of SACC cells *in vitro*, and ID1 may therefore exert an oncogenic role in SACC.

ID1 promotes cell migration and invasion in vitro. The functional role of ID1 in the migration and invasion of SACC cells was investigated. As demonstrated in Fig. 4, knockdown of significantly inhibited the migration (P<0.01, n=3) and invasion (P<0.01, n=3) of SACC-83 cells when compared with controls. By contrast, overexpression of ID1 in SACC-83 cells significantly promoted cell motility when compared with controls, as indicated by the wound healing (Fig. 5A) and Transwell assays (P<0.01, n=3; Fig. 5B and C). As demonstrated in Fig. 5B and D, ID1 overexpression was associated with a significant increase in cell invasiveness (P<0.01, n=3). These results provide further evidence to suggest that ID1 may function as an oncogene in SACC, and contribute to the migration and invasion of SACC cells.

Knockdown of ID1 reduces the expression of MMP1 and promotes the expression of S100A9 and CDKN2A. To explore the potential mechanisms of ID1 in SACC further, the gene expression levels of known ID1 target genes (S100A9, CDKN2A and MMP1) were determined following the transfection of
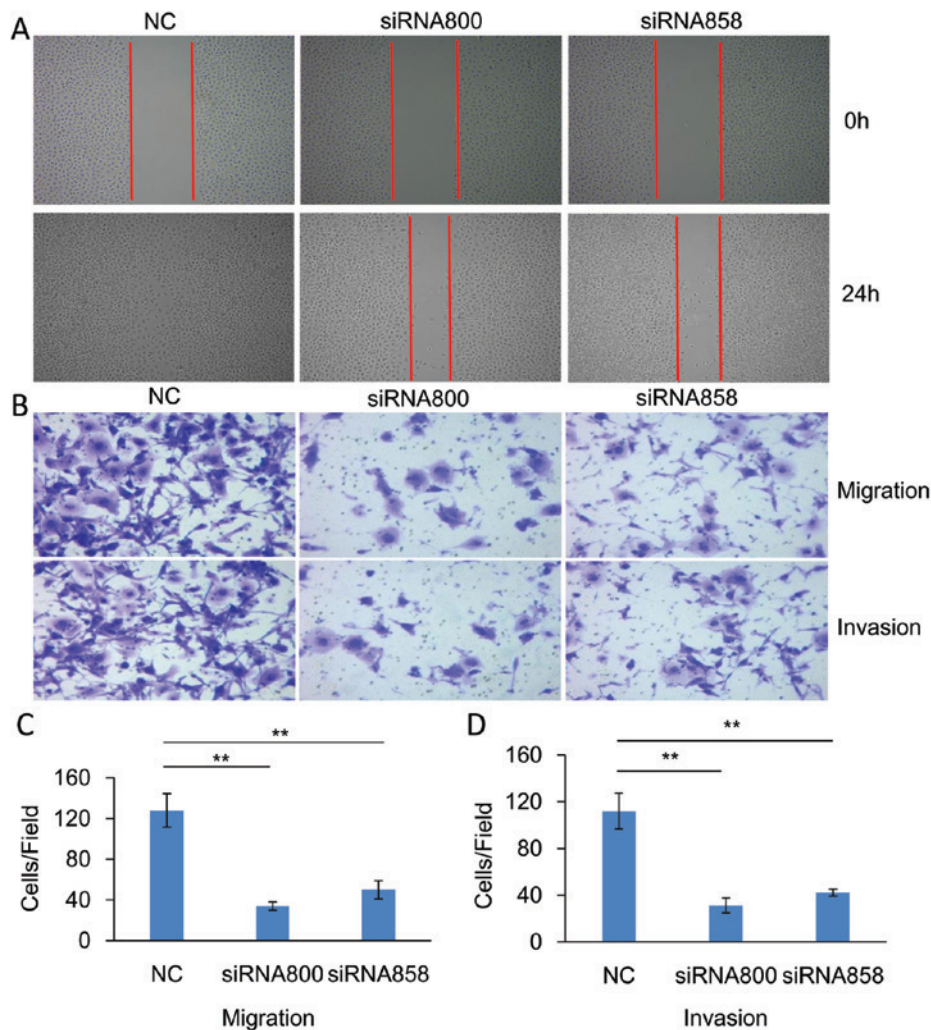
Figure 4. Knockdown of ID1 inhibits the migration and invasion of SACC-83 cells. (A) Representative images of SACC-83 cell migration after transfection with siRNA800, siRNA858 or NC at 0 and 24 h following generation of the scratch-wound (magnification, 40x). (B) Representative images of the Transwell migration (upper panels) and invasion (lower panels) assays following transfection of SACC-83 cells with ID1 siRNAs or NCs (magnification, x200). (C) The number of cells that migrated through the uncoated filters (no Matrigel), represented the migratory ability of the SACC-83 cells. (D) The number of cells that passed through filters pre-coated with Matrigel represented the invasive ability of SACC-83 cells. The cell counts are presented as the mean values/field of view from at least five randomly selected low-powered fields from three independent experiments. The results are presented as the mean ± standard deviation. [**]P<0.01 as indicated. ID1, inhibitor of DNA binding 1; SACC, salivary adenoid cystic carcinoma; siRNA, small interfering RNA; NC, negative control.

SACC-83 cells with ID1 siRNA800 or NC controls using RT-qPCR. Compared with NC, the expression level of MMP1 was significantly downregulated following knockdown of ID1 (P<0.05), whereas the S100A9 and CDKN2A gene expression levels were significantly upregulated in SACC-83 cells (both P<0.05; Fig. 6). These results indicate that MMP1, S100A9 and CDKN2A may be involved in ID1-mediated alterations in SACC cell proliferation, migration and invasion.

Discussion

ID1 serves an important role in the regulation of a number of biological behaviors in various tumors. Silencing of ID1 was demonstrated to induce apoptosis and inhibit the growth of osteosarcoma cells, and the level of phosphorylated AKT was downregulated by ID1 RNAi (17). Previous studies have indicated that ID1 serves a role in promoting cell survival and proliferation and it is involved in tumor differentiation (21-25). In addition, ID1 contributes to cell invasion in thyroid tumor cells by inducing mesenchymal features (26). Pillai *et al* (27) revealed that ID1 facilitates the growth and metastasis of non-small cell lung cancer.

In a previous study, the expression of ID1 in SACC was increased by 65.2% compared with normal salivary tissues (20). In the present study, the expression of ID1 in 68 cases of SACC and 50 tumor-adjacent normal tissue samples was compared using immunohistochemical staining. The results suggested that the expression of ID1 was significantly higher in SACC tissues compared with that in the normal salivary tissues. In addition, the results demonstrated a positive correlation between ID1 expression and tumor stage, invasion and metastasis in patients with SACC. This suggests that ID1 may serve an oncogenic role in SACC.

In the present study, knockdown and overexpression of ID1 in SACC cells by siRNA or plasmid transfection, respectively, was performed to investigate the effect of ID1 on cell growth using CCK-8 and colony formation assays.
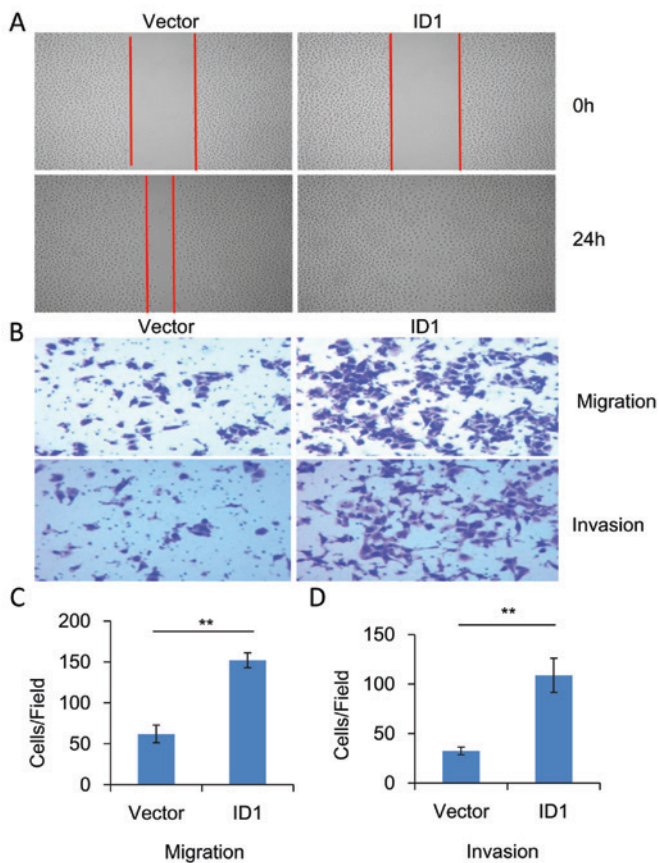
Figure 5. Overexpression of ID1 increased the migration and invasion of SACC-83 cells. (A) Representative images of SACC-83 cell migration after transfection with recombinant plasmids (pEX-2) containing the ID1 sequence or empty vector controls at 0 and 24 h following generation of the scratch-wound (magnification, 40x). (B) Representative images of the Transwell migration (upper panels) and invasion (lower panels) assays following transfection of SACC-83 cells with ID1 plasmids or empty vector controls (magnification, 200x). (C) The number of cells that migrated through the uncoated filters (no Matrigel), represented the migratory ability of the SACC-83 cells. (D) The number of cells that passed through filters pre-coated with Matrigel represented the invasive ability of SACC-83 cells. The cell counts were presented as the mean values/field of view from at least five randomly selected low-powered fields from three independent experiments. The results are presented as the mean ± standard deviation. **$P<0.01$ as indicated. ID1, inhibitor of DNA binding 1; SACC, salivary adenoid cystic carcinoma; NC, negative control.
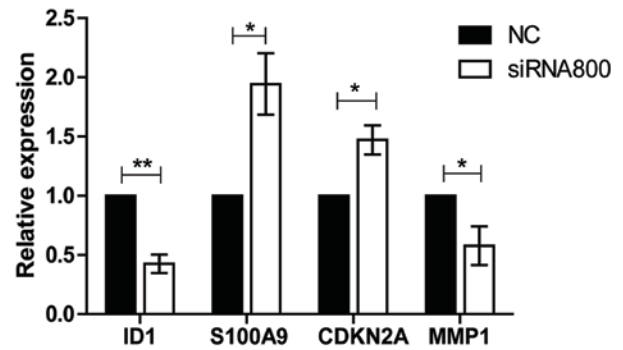

Figure 6. Knockdown of ID1 in SACC-83 cells reduces the expression of MMP1 mRNA and promotes the expression of S100A9 and CDKN2A mRNA. At 48 h following transfection of SACC-83 cells with siRNA800 the mRNA expression levels of ID1, MMP1, S100A9 and CDKN2A in SACC-83 cells were measured using reverse transcription-quantitative polymerase chain reaction analysis. The results are presented as the mean ± standard deviation (n=3). *$P<0.05$ and **$P<0.01$ as indicated. ID1, inhibitor of DNA binding 1; SACC, salivary adenoid cystic carcinoma; MMP1, matrix metalloproteinase 1; siRNA, small interfering RNA; NC, negative control.

The results demonstrated that ID1 significantly promoted the proliferation of SACC cells in vitro, thereby supporting the role of ID1 as an oncogene in SACC. Metastasis and invasion are two important additional factors that affect the prognosis and recurrence of patients with SACC. In the current study, knockdown of ID1 significantly inhibited the migration and invasion of SACC-83 cells, whereas overexpression of ID1 significantly promoted the migration and invasion of the SACC-83 cells. This suggests that ID1 may regulate the migration and invasion of SACC cells; however, the molecular mechanism of ID1 in SACC remains to be fully elucidated. In breast cancer, ID1 promotes tumor metastasis by regulating S100A9 (28). The results of the current study are consistent with those of previous studies (28-30) indicating that MMP1, S100A9 and CDKN2A are ID1 target genes. Knockdown of ID1 significantly inhibited the expression level of MMP1 mRNA, and significantly promoted

the expression of S100A9 and CDKN2A mRNA. These results demonstrated that the expression of these genes was altered in response to ID1 silencing, indicating that MMP1, S100A9 and CDKN2A may serve a role in mediating the effects of ID1 in SACC cells. However, additional studies are required to investigate the potential mechanisms by which ID1 functions to regulate the proliferation and metastasis of SACC cells. In prostate cancer, overexpression of ID1 promotes angiogenesis through the activation of vascular endothelial growth factor (31). Li et al (32) suggested that the extracellular signal-related-dependent downregulation of S-phase kinase-associated protein 2 reduced myc activity with hepatocyte growth factor, which lead to the inhibition of hepatocyte proliferation via a decrease in ID1 expression. In addition, Cheng et al (33) revealed that ID1 promotes the proliferation of lung cancer cells and lung tumor growth via the Akt signaling pathway. Similarly, Yang et al (34) demonstrated that downregulation of ID1 in gastric cancer inhibits cell growth via the Akt signaling pathway. In the present study, ID1 expression was associated with the proliferation, invasion and migration of SACC cells. The observed inhibition of SACC cell growth, invasion and migration following knockdown of ID1 expression in the present study, may have been due to restoration of the balance between oncogenic and tumor-suppressive effects resulting from changes in the expression of downstream genes or associated proteins. Further studies are required to determine the molecular mechanisms of ID1 in SACC. The results of the current study suggest that ID1 may present a novel therapeutic target for the treatment of patients with SACC.

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