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Original Article

Overexpressed DNA-binding protein inhibitor 2 as an unfavorable prognosis factor promotes cell proliferation in nasopharyngeal carcinoma

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The aim of the present study was to analyze the expression of DNA-binding protein inhibitor 2 (ID2) in nasopharyngeal carcinoma (NPC) and its correlation with clinicopathological features. It was found that the expression of *ID2* was significantly increased in NPC cells when compared with that in NP69 cell line. Similar level of ID2 cytoplasmic expression was observed in NPC when compared with that in non-cancerous nasopharynx tissues. However, the level of ID2 in nucleus was increased in NPC when compared with that in normal nasopharynx tissues. Furthermore, the higher expression level of nuclear ID2 was significantly associated with tumor size (T classification), lymph node metastasis (N classification), and clinical stage. Patients with increased ID2 expression level had poorer overall survival rates than those with low ID2 levels. The inhibition of ID2 expression in NPC cell line SUNE1 by lentiviral-mediated short hairpin RNA could suppress cell proliferation and colony formation, but did not disrupt cell migration. Knocking down the expression of ID2 by RNA interference could down-regulate the expression of Snail, suggesting that ID2-promoted cell growth, partially attributing to the regulation of Snail activity in NPC. Our study demonstrated that overexpression of ID2 protein is an unfavorable prognostic factor which promotes cell proliferation in NPC.

Keywords DNA-binding protein inhibitor 2; nasopharyngeal carcinoma; prognosis; proliferation

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Introduction

Nasopharyngeal carcinoma (NPC) is a common malignancy in Southern China and Southeast Asia. Decades of epidemiological studies have displayed that NPC has unique prevalence features, including obvious racial, regional, and familial aggregation. Five provinces in Southern China, including Guangdong, Hainan, Guangxi, Hunan, and Fujian, have higher incidence rates of NPC. However, the highest rates are noted in the Cantonese who inhabit the cities and counties in Pearl River Delta and West Pearl River Valley, with a rate of 27.2/100,000 person-years in men and 11.3/100,000 person-years in women in the year 2003 [1]. Synergetic effects of *Epstein–Barr virus* (EBV) infection, non-viral environmental factors including consumption of salted fish and other nitrosamine containing preserved foods, formaldehyde, and wood dust exposure and genetic susceptibility are believed to be risk factors for the development of NPC, which mainly contributes to cause abnormal gene expression, such as the activation of oncogenes and the inactivation of tumor suppressor genes [2–7].

Members of the inhibitor of DNA binding (ID) family are transcriptional regulators that contain a helix–loop–helix (HLH) domain but not a basic domain. The expression of ID proteins is activated in many human cancers, for example, in prostate, breast, bladder, colon and pancreatic cancer, as well as high-grade astrocytoma and T-cell lymphomas [8–13]. ID proteins can induce proliferation, anaplasia, and invasion, and serve as prognostic markers in several types of human cancers [14,15]. ID2, one of the ID family members, plays different roles in different types of cancer, such as in pancreatic cancer, neuroblastoma, prostate cancer, sarcoma, lung cancer, ovarian cancer, and colorectal carcinoma. ID2 also is an oncogenic factor to promote tumors progression [8,13,16–21]. On the contrary, ID2 acts as a tumor suppressor participating in the pathogenesis of some tumors including liver cancer, breast cancer, and ocular cancer [22–24]. However, the

expression pattern and functional mechanism of ID2 in NPC is still unclear.

In order to clarify the role of ID2 in the pathogenesis of NPC, in the present study, we investigated the expression pattern of ID2 in NPC, atypical hyperplasia nasopharynx, squamous nasopharynx, and normal nasopharynx tissues, the correlation of its protein expression with clinicopathological features in NPC patients, and the effects of ID2 on cell growth and migration. We found that ID2 was over-expressed in NPC. Accumulated nuclear ID2 was favorable for NPC progression and poor prognosis. Furthermore, ID2 knockdown in NPC cells could significantly decrease cell proliferation, but had no effect on cell migration. Our studies demonstrate that over-expressed ID2 promotes cell proliferation and its nuclear protein accumulation is an unfavorable factor for NPC progression and prognosis.

Materials and Methods

Cell culture and sample collection

Eight NPC cell lines 5−8F, 6−10B, CNE2, CNE1, C666−1, HONE1, HNE1 and SUNE1 were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% newborn calf serum (NBCS) (PAA Laboratories, Inc., Pasching, Austria). NP69, an immortalized human nasopharyngeal epithelial cell line, was grown in defined Keratinocyte serum-free medium supplemented with epidermal growth factor (EGF) (Invitrogen, Carlsbad, USA). All of these cell lines were incubated in a humidified chamber with 5% CO_2 at 37°C. A total of 154 paraffin-embedded undifferentiated NPC specimens and 72 paraffin-embedded non-cancerous nasopharynx specimens (including 42 normal epithelium, 14 squamous epithelium, and 16 atypical hyperplasia epithelium) were obtained at the time of diagnosis before any therapy from People's Hospital in Zhongshan City (Guangdong, China). In these 154 NPC cases, there were 111 male and 43 female with age ranging from 20 to 75 years (median, 50.38 years). The clinical follow-up time of patients ranged from 8 to 73 months. The clinical processes were approved by the Ethics Committees of People's Hospital of Zhongshan City. The patients were confirmed with the informed consents. The pathological stage of all specimens was confirmed according to the 1997 NPC staging system of the WHO.

Reanalysis of microarray data

Raw microarray data from eight pairs of NPC and normal nasopharynx tissue samples were reanalyzed [5,25]. The differentially expressed genes were screened with a selection criteria based on our previous investigation [5].

Real-time polymerase chain reaction

The mRNA levels of *ID2* in NP69, 5−8F, 6−10B, CNE2, CNE1, C666−1, HONE1, HNE1, and SUNE1 cell lines and the mRNA levels of epithelial-mesenchymal transition (EMT)-associated genes (*SIP1*, *Twist*, *Snail*, *CDH1*, and *VIM*) in shRNA-ID2 and Ctr-ID2 NPC cells were measured using real-time polymerase chain reaction (PCR). *GAPDH* was used as the internal control. The primer pair sequences for these genes (**Supplementary Table S1**) were designed with at least an intron to distinguish possibly amplified cDNA products from the genomic DNA. The PCR reaction was carried out using SYBR Green Mix reagent (Takara, Shiga, Japan) and repeated three times.

Immunohistochemistry

Paraffin sections (4 μm) from samples were deparaffinized in 100% xylene and re-hydrated in descending ethanol series and water according to standard protocols. Heat-induced antigen retrieval was performed in 10 mM citrate buffer for 2 min at 100°C. Endogenous peroxidase activity and non-specific antigen were blocked with peroxidase blocking reagent containing 3% hydrogen peroxide and serum, followed by incubation with rabbit anti-human ID2 antibody (1: 100) (Spring Bioscience, Fremont, USA) for overnight at 4°C. After washing, the sections were incubated with biotin-labeled rabbit anti-goat antibody for 10 min at room temperature, and subsequently were incubated with streptavidin-conjugated horseradish peroxidase (HRP) (Maixin, Fuzhou, China). The peroxidase reaction was developed using 3,3-diaminobenzidine (DAB) chromogen solution in DAB buffer substrate. Sections were visualized with DAB and counterstained with hematoxylin, mounted in neutral gum, and analyzed using a bright field microscope.

Evaluation of staining

The immunohistochemically stained tissue sections were reviewed and scored separately by two pathologists blinded to the clinical parameters. Expression of ID2 in the nucleus and in the cytoplasm was independently evaluated. For cytoplasmic staining, the score was evaluated according to the sum of cytoplasm staining intensity and the percentage of positive staining areas of cells. The staining intensity was scored as previously described (0−3) [3] and the percentage of positive staining areas of cells was defined as a scale of 0−3 (0: $<$10%, 1: 10−25%, 2: 26−75%, and 3: $\geq$76%). For nuclear staining, the staining score was defined based on the sum of nuclear staining intensity and the number of positive nuclear staining. Nuclear staining intensity score was consistent with cytoplasm. The positive nuclear staining scores were defined as follows: 0: $<$20%, 1: 20−49%, 2: 50−79%, and 3: $\geq$80%. The sum of the cytoplasm and nuclear staining scores was used as the final

staining score for ID2 (0−12). For statistical analysis, a final staining score of 0−4 and 5−6 in cytoplasm or 0−3 and 4−6 in nucleus was considered to be low or high expression, respectively.

Establishment of NPC SUNE1 cell line with stable expression of ID2 short hairpin RNA

We selected two sequences (ID2−1, sense: 5′-CGCGTCCCCCACGGATATCAGCATCCTGTTCAAGAGACAGGATGCTGATATCCGTGTTTTTGGAAAT-3′; antisense: 5′-CGATTTCCAAAAACACGGATATCAGCATCCTGTCTCTTGAACAGGATGCTGATATCCGTGGGGGA-3′; ID2−2; sense: 5′-CGCGTCCCCGAAGGTGAGCAAGATGGAATTCAAGAGATTCCATCTTGCTCACCTTCTTTTTGGAAAT-3′; antisense: 5′-CGATTTCCAAAAAGAAGGTGAGCAAGATGGAATCTCTTGAATTCCATCTTGCTCACCTTCGGGGA-3′) for targeting *ID2* gene using the BLOCK-It RNAi Designer (Invitrogen). The preparation of lentiviral vectors expressing human *ID2* short hairpin RNA (shRNA) was performed using the pLVTHM-green fluorescence protein (pLVTHM-GFP) lentiviral RNAi expression system [26]. Replication-incompetent lentivirus was produced by co-transfection of the pLVTHM/ID2-shRNA expression vector and ViraPower packaging mix containing an optimized mixture of two packaging plasmids: psPAX2 and pMD2.G into 293FT cells. NPC SUNE1 cells were infected with lentiviral particles containing specific or negative control vectors, and the single colony with strong GFP signals was selected to establish stable silencing cell lines. The total RNA of these cell clones was isolated, and the levels of *ID2* mRNA were measured using real-time PCR analysis.

Western blot analysis

Cells were lysed in radio immunoprecipitation assay buffer [50 mM Tris-HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, 5 mM dithiothreito, 2% sodium dodecyl sulfate (SDS)] and protein concentration was determined using bicinchoninic acid assay assay (Beyotime, Beijing, China). Total protein (30 μg) was resolved using a 10% SDS−polyacrylamide gel electrophoresis (PAGE) and electro-transferred to polyvinylidene fluoride membranes (Invitrogen). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (pH 7.5), followed by immunobloting overnight at 4°C with mouse polyclonal anti-ID2 antibody (1:500; Abcam, Tokyo, Japan), anti-Beta-actin antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, USA), anti-Snail, cadherin-1 (CDH1), and vimentin (VIM) antibody (1:800; Cell signaling technology, Danvers, USA). An HRP-conjugated anti-rabbit IgG antibody was used as the secondary antibody (Zhongshan, Beijing, China). Signals were detected using enhanced chemiluminescence reagents (Pierce, Rockford, USA).

Cell proliferation analysis

Cell proliferation was analyzed using (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 1×10^3 cells were seeded into a 96-well plate with quadruplicate repeat for each condition. After a 24-h incubation, MTT reagent (Sigma, St Louis, USA) was added to each well and incubated for 4 h. The formazan crystals formed by viable cells were then solubilized in DMSO and measured at 490 nm for the absorbance (A) values. Each experiment was performed in triplicate.

Plate colony formation assay

About 100 cells were added to each well of a six-well culture plate, and each cell group contained two wells. After 2 weeks of incubation, cells were washed twice with phosphate-buffered saline and stained with Giemsa solution. The number of colonies containing $\geq$50 cells was counted under a microscope. The colony formation efficiency was calculated as: efficiency = (number of colonies/number of cells inoculated) × 100%. Each experiment was performed in triplicate.

Soft agar colony formation assay

The anchorage-independent growth of NPC cells was monitored by the soft agar colony formation assay. In brief, cells (100/well) were re-suspended in 1.5-ml mixture of 1.2% low-melt agarose and 2 × RPMI 1640 medium (v:v = 1:1), and then loaded in triplicate on the top of the solidified bottom agar comprising equal-volume mixture of 0.7% low-melt agarose and RPMI 1640 in 12-well plates. The cells were finally incubated at 37°C, 5% CO_2 for 13 days. The colonies composed of more than 50 cells were counted.

In vitro cell migration assay

Cells growing in the log phase were treated with trypsin and re-suspended as single-cell solution. A total of 1×10^5 cells were seeded on a fibronectin-coated polycarbonate membrane insert in a transwell apparatus (Corning, Corning, USA). In the lower chamber, 600 μl of RPMI 1640 medium with 10% NBCS was added as chemoattractant. After the cells were incubated for 12 h, the insert was washed with PBS, and cells on the top surface of the insert were removed by a cotton swab. Cells adhering to the lower surface were fixed with methanol, stained with Giemsa, and counted under a microscope in five predetermined fields (200×). All assays were independently repeated at least three times.

Statistical analysis

All data were analyzed for statistical significance using SPSS 13.0 software. The Mann−Whitney U test was applied to the examination of relationship between ID2

expression levels and clinicopathological characteristics. Survival analysis was performed using Kaplan–Meier method. Multivariate Cox proportional hazards method was used for analyzing the relationship between the variables and patient's survival time. One-way analysis of variance was used to determine the differences between groups for all *in vitro* analyses. A P value of less than 0.05 was considered statistically significant.

Results

ID2 is highly expressed in NPC

From our microarray data, *ID2* was highly expressed in NPC tissues compared with normal nasopharynx tissues with an average of 2.88 folds [**Fig. 1(A)**]. This data was similar to Sengupta *et al.*'s microarray data (GSE12452) between NPC tissues and NP tissues (*ID2* 2.22 folds) [27]. Using real-time PCR to measure the expression of *ID2* transcripts, we found that the *ID2* expression level was significantly increased in eight NPC cell lines in comparison to the immortalized human nasopharyngeal epithelial cell lines NP69 [**Fig. 1(B)**]. Furthermore, we measured subcellular localization and the expression levels of ID2 protein in 154 archived paraffin-embedded NPC samples and 72 paraffin-embedded non-cancerous nasopharynx specimens using immunohistochemical staining [**Fig. 2(A)**]. Specific ID2 protein staining was found in the cytoplasm and nucleus of non-cancerous and malignant nasopharynx tissues. In cytoplasm, we did not observe the differential expression of ID2 in NPC compared with atypical hyperplasia, squamous, and normal nasopharynx tissues ($P = 0.212$) (**Table 1**). However, in nucleus, the expression of ID2 was significantly elevated in NPC compared with normal nasopharynx tissues ($P < 0.001$) (**Table 2**).

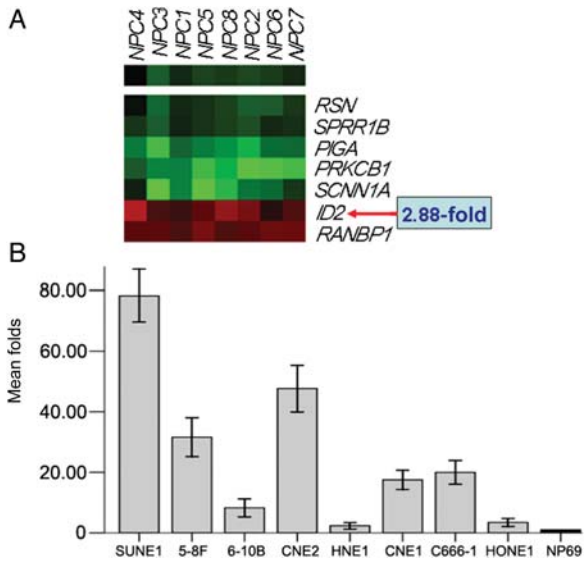

Figure 1 Increased *ID2* mRNA level in NPC tissues and cells (A) Screening differentially expressed genes in eight microarrays between NPC and normal nasopharynx tissues. *ID2* was highly expressed in NPC tissues compared to normal tissues (red color: high expression; green color: low expression). (B) *ID2* was highly expressed in eight NPC cell lines compared with immortalized human nasopharyngeal epithelial cell line NP69.

Relationship between clinicopathological characteristics and nuclear expression of *ID2* in NPC patients

The relationship between clinicopathological characteristics and *ID2* nucleus expression levels in individuals with NPC

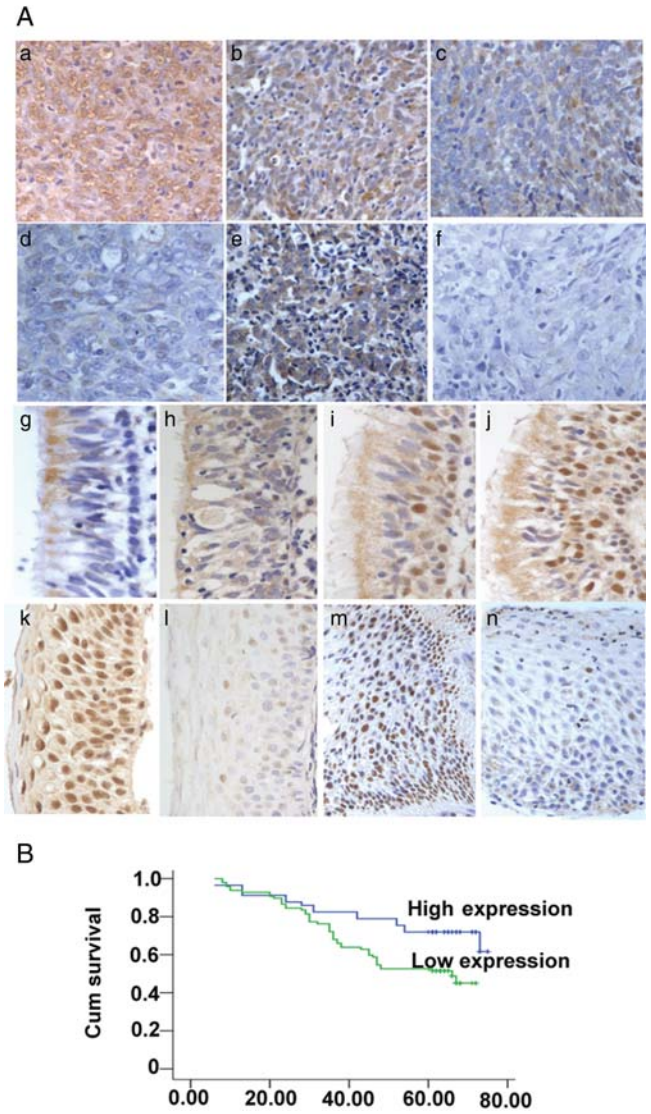

Figure 2 Accumulation expression of *ID2* in cell nucleus was unfavorable for NPC prognosis (A) a–d, co-expression in nucleus and cytoplasm in NPC; e–f, expression in cytoplasm in NPC; g, expression in cytoplasm of normal nasopharynx tissues; h–j, co-expression in nucleus and cytoplasm of normal nasopharynx tissues; k–l, co-expression in nucleus and cytoplasm of squamous nasopharynx tissues; m–n, co-expression in nucleus and cytoplasm of atypical hyperplasia nasopharynx tissues. (B) Kaplan–Meier survival analysis of overall survival duration in 154 NPC patients according to ID2 protein expression in cell nucleus. Accumulation expression of ID2 was unfavorable for NPC prognosis.

Table 1 Similar cytoplasm expression level of ID2 protein in NPC samples, normal nasopharynx, squamous epithelium, and atypical hyperplasia tissues

Group	Protein expression (*n*)			*P*
	Total	High	Low	C/A/S/N
Cancer cells	154	120	34	0.212[a]
Atypical hyperplasia	16	12	4	
Squamous epithelium	14	10	4	
Normal epithelium	42	38	4	

[a]Kruskal Wallis test; S: squamous; N: normal; A: atypical; C: cancer.

Table 2 Elevated nuclear expression of ID2 protein in NPC samples compared with normal nasopharynx, and squamous epithelium tissues and atypical hyperplasia

Group	Protein expression (*n*)			*P*			
	Total	High	Low	C/A/S/N	C/A	C/S	C/N
Cancer cells	154	97	57	0.000[a]	0.418[b]	0.159[b]	0.000[b]
Atypical hyperplasia	16	8	8				
Squamous epithelium	14	6	8				
Normal epithelium	42	10	32				

[a]Kruskal Wallis test.
[b]Mann–Whitney U test; S: squamous; N: normal; A: atypical; C: cancer.

Table 3 Correlation between the clinicopathological characteristics and expression of nuclear protein of ID2 in NPC

Characteristics	*n*	ID2 (%)		*P*
		High	Low	
Gender				
Male	111	66 (81.0)	45 (19.0)	
Female	43	31 (43.6)	12 (56.4)	0.193
Age (year)				
≥50	80	52 (75.3)	28 (24.7)	
<50	74	44 (47.7)	30 (52.3)	0.509
Smoking				
Yes	38	19 (79.5)	19 (20.5)	
No	116	78 (76.8)	38 (23.2)	0.081
T classification				
T_1–T_2	116	66 (73.3)	50 (26.7)	
T_3–T_4	38	31 (87.5)	7 (12.5)	0.007
N classification				
N_0–N_1	85	46 (66.2)	39 (33.8)	
N_2–N_3	69	51 (86.7)	18 (13.3)	0.012
Distant metastasis				
Yes	10	7 (87.5)	3 (12.5)	
No	144	90 (76.9)	54 (23.1)	0.746
Clinical stage				
I∼II	62	25 (54.5)	37 (45.5)	
III∼IV	92	72 (89)	20 (11)	0.000
Family tumor history				
Yes	12	8 (72.7)	4 (27.3)	
No	25	89 (42.5)	43 (57.5)	1.000
Patients from area				
Zhongshan	110	30 (38.2)	14 (61.8)	
Non-Zhongshan	44	67 (45.9)	43 (54.1)	0.462

are summarized in Table 3. We did not find any significant association of *ID2* expression levels with patient's age, sex, smoking, family tumor history, place of origin, and status of distant metastases (M classification). However, we observed that the nuclear expression level of *ID2* was positively correlated with tumor size (T classification) (T_1–T_2 vs. T_3–T_4, $P = 0.007$), lymph node metastasis (N classification) (N_0–N_1 vs. N_2–N_3, $P = 0.012$), and clinical stage (I–II vs. III–IV, $P < 0.001$) in NPC (Table 3).

Survival analysis

To investigate the prognostic value of ID2 expression for NPC, we assessed the association between the nuclear expression levels of ID2 and patient survival using Kaplan–Meier analysis with the log-rank test. In 154 NPC cases with prognosis information, we observed that the nuclear expression level of ID2 protein was significantly correlated with the overall survival of NPC patients. Patients with higher levels of ID2 expression had poorer survival rates than those with lower ID2 expression levels [**Fig. 2(B)**]. Univariate analysis indicated that ID2 expression level, T/N/M classifications and clinical stages were significantly correlated with patients' survival ($P = 0.01$, $P < 0.001$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively). To determine whether ID2 is an independent prognostic factor for NPC, we performed multivariate analyses using the Cox proportional hazards model. The results indicated that the level of high nuclear expression of ID2 was not an independent prognostic factor for NPC ($P = 0.144$, Table 4).

Reduced *ID2* expression suppresses the proliferation of NPC cells *in vitro*

Among NPC cell lines, SUNE1 cells had the highest expression levels of *ID2* [**Fig. 1(B)**], suggesting that this cell line might be a good model system for studying the

Table 4 Summary of univariate and multivariate Cox regression analysis of overall survival duration

Parameter	Univariate analysis			Multivariate analysis		
	P	HR	95%CI	*P*	HR	95%CI
Gender						
Male vs. female	0.975	1.009	0.592–1.719			
Age						
≥50 vs. <50 years	0.320	1.281	0.787–2.084			
Family tumor history						
Yes vs. no	0.180	0.453	0.142–1.443			
Smoking						
Yes vs. no	0.284	1.342	0.783–2.299			
Area						
Zhongshan vs. non-Zhongshan	0.137	1.471	0.885–2.446			
T classification						
T_1–T_2 vs. T_3–T_4	0.000	3.797	2.306–6.254	0.000	4.447	2.377–8.320
N classification						
N_0–N_1 vs. N_2–N_3	0.000	2.873	1.733–4.763	0.010	2.578	1.253–5.304
M classification						
M_0 vs. M_1	0.000	7.525	3.680–15.385	0.000	7.593	3.359–17.160
Clinical stage						
I∼II vs. III∼IV	0.000	3.655	1.977–6.756	0.440	0.672	0.245–1.841
NESG1 expression						
High expression vs. low expression[a]	0.010	2.110	0.198–3.719	0.144	1.564	0.859–2.846

[a]Kruskal Wallis test. CI, confidence interval.

functions of endogenous ID2 by the loss-of-function approach. We used a lentiviral shRNA vector specifically targeting *ID2* to stably knockdown the expression of *ID2* in SUNE1 cell line. Seven stably transfected cell clones were obtained (1–7) [**Fig. 3(A)**]. Real-time PCR analysis showed that *ID2* mRNA expression in clone-6 (C6) and 7 (C7) cells was markedly reduced compared to empty vector control SUNE1 cells [pLVTHM-GFP-Control (PLV-Ctr)]. In addition, decreased expression of ID2 protein was further confirmed by western blot in these two clones compared with PLV-Ctr cells [**Fig. 4(B)**]. Subsequently, we examined the effect of decreased *ID2* expression on NPC cell growth *in vitro*. Using MTT assay, we found that the parental NPC SUNE1 cell line had a similar growth rate as PLV-Ctr cells over a 7-day period, the growth of shRNA-ID2 cells was significantly slower than the former two lines from day 1 [$P < 0.05$, **Fig. 3(B)**]. Interestingly, this result was also consistent with the plate clone formation test. Both the parental SUNE1 cell line (0.73 ± 0.03) and the PLV-Ctr cells (0.72 ± 0.02) formed a similar number of colonies on plate over a 2-week period. In contrast, knocking down endogenous *ID2* could dramatically reduce the number of colonies in C6 cells (0.35 ± 0.03) and C7 cells (0.35 ± 0.02) [$P < 0.05$, **Fig. 3(C)**].

ID2 knockdown inhibits cellular transformation

We next explored the effect of ID2 on cellular transformation. Since anchorage-independent growth is a hallmark for transformed cells, we measured the growth of different cells on soft agar. Both the parental SUNE1 and shCtrl cells formed similar number of colonies on soft agar over a 13day period (34 ± 3.61 vs. 40 ± 2.00). In contrast, knocking down endogenous *ID2* significantly reduced the amount of colonies in shRNA-ID2–1 (19 ± 5.03) and shRNA-ID2–2 (16 ± 1.53) ($P < 0.05$), implying an essential role of *ID2* in regulating cellular transformation [**Fig. 3(D)**].

Knockdown of *ID2* does not suppress cell migration

Cell migration is a key step during tumor development and metastasis. We tested the ability of NPC cells to migrate through the 8 μm pores on the polycarbonate membrane, and found that the knockdown of endogenous *ID2* expression could not decrease cell migration of C6 cells (237.40 ± 31.58) and C7 cells (255.00 ± 36.69) compared with the parental cells (227.20 ± 21.39) or PLV-Ctr cells (243.60 ± 39.22) [$P = 0.61$, **Fig. 3(E)**].

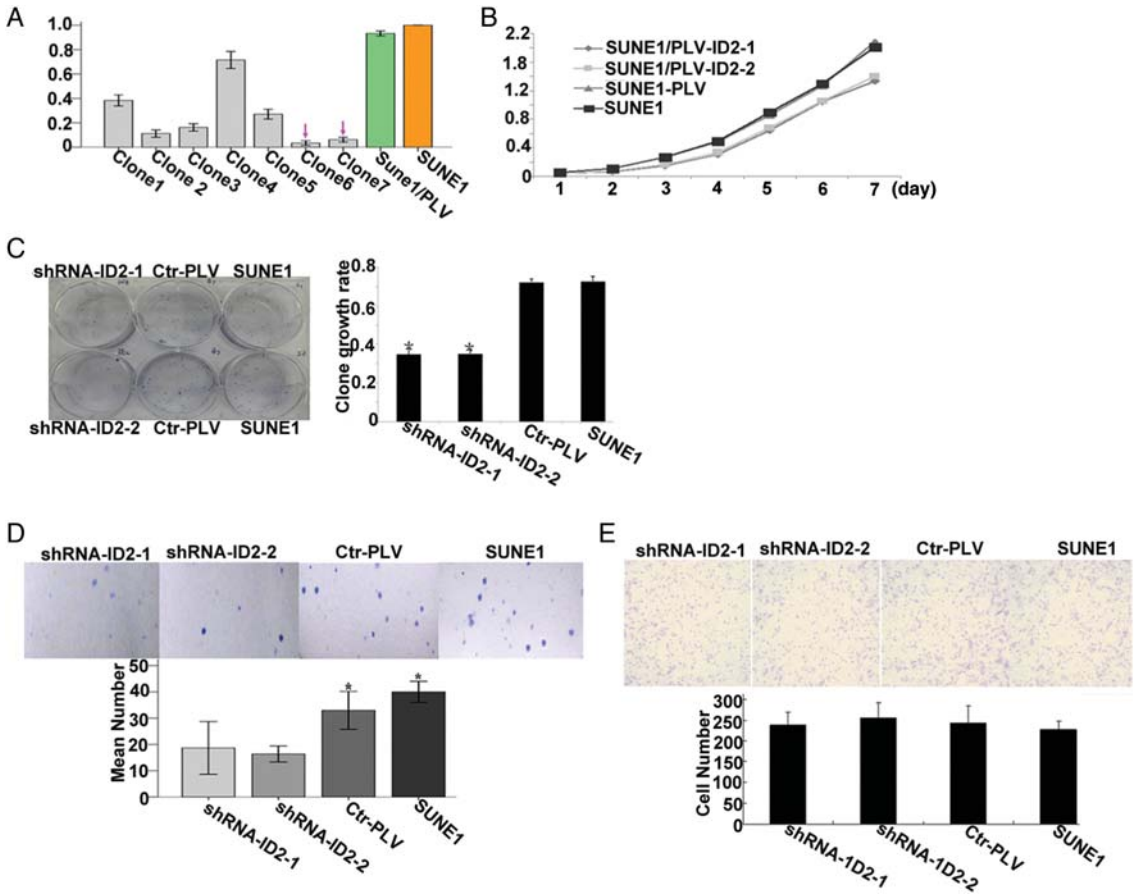

Figure 3 Down-regulation of *ID2* inhibited cell growth, but did not change the potential of cell migration *in vitro* (A) Markedly reduced mRNA expression of *ID2* after shRNA-ID2. Seven single clone cells (C1−7) compared with PLV-Ctr and the parental cells SUNE1 by real-time polymerase chain reaction. (B) The cell growth of parental SUNE1 cells and their stable derivatives, PLV-Ctr and shRNA-ID2, was examined by MTT assay over a 7-day period. $*P < 0.05$, as compared with SUNE1 and PLV-Ctr cells.(C) The anchorage-dependent growth of parental SUNE1 cells and their stable derivatives, PLV-Ctr and shRNA-ID2, was examined by plate colony formation assay. (D) The anchorage-independent growth of parental SUNE cells and their stable derivatives, shCtrl and shRNA-ID2-1,2 were examined by soft agar colony formation assay. (E) The migrating capability of parental SUNE1 cells and their stable derivatives, PLV-Ctr and shRNA-ID2, was examined by transwell test. $*P < 0.05$, as compared with SUNE1 and PLV-Ctr cells.

ID2 regulates the expression of EMT-associated gene snail in NPC

We examined the effect of *ID2* on the expression of key regulators of EMT including tumor necrosis factor β (TGF-β), CDH1, smad interacting protein 1 (SIP1), and VIM. Real-time PCR analysis indicated that reduced *ID2* only significantly decreased the expression of snail, a gene that could promote cell proliferation [**Fig. 4(A)**]. Accordingly, using western blot analysis, we also found that snail protein level decreased in ID2-deficient cells compared with Ctr-vector cells [**Fig. 4(B)**]. However, epithelial biomarker gene *CDH1* and mesenchymal biomarker gene *VIM* did not show the significant expression change in shRNA-ID2 cells compared with in PLV-Ctr cells.

Discussion

ID2, a transcriptional regulator, plays dual roles as oncogene and tumor suppressor in different cancer types [8,13,16−24], which may attribute to tissue-specific patterns of expression in different tissues and organs in tumourigenesis. However, its roles and molecular mechanisms linking the initiation and development of NPC are seldom reported [27].

In this study, we first compared gene expression profiles in NPC tissues and NP tissues, and found that ID2 was more highly expressed in NPC than in normal tissue. Our result strongly supported Sengupta *et al.*'s [28] microarray data (GSE12452), suggesting that over-expressed ID2 was involved in promoting the NPC pathogenesis. Furthermore, we confirmed the differential mRNA expression in eight NPC cell lines and immortalized nasopharyngeal epithelial cell line NP69 by real-time PCR, and found that *ID2* mRNA was highly expressed in NPC cell lines compared with NP69 cell line, which is in consistence with our microarray data.

To confirm the reliability of ID2 over-expression in NPC, we used immunohistochemistry to examine the

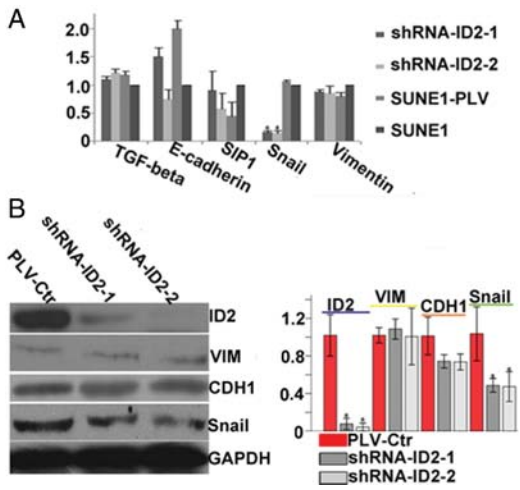

Figure 4 Down-regulation of *ID2* decreased the expression of Snail, but did not change the expression of CDH1, VIM, TGF-β, Slug, and SIP1 (A) mRNA expression of *Snail* was inhibited in shRNA-ID2 cells compared with PLV-Ctr cells and parental SUNE1 cell line, but did not change the expression of *CDH1*, *VIM*, *TGF-β*, *Slug*, *and SIP1*. (B) Significantly decreased protein expression of ID2 was found in shRNA-ID2 cells (C6, C7) compared with PLV-Ctr and SUNE1 cells by western blot. ACTB was used as internal control. Snail protein expression was suppressed in shRNA-ID2 cells compared with PLV-Ctr cells. Epithelial biomarker gene *CDH1* and mesenchymal biomarker gene *VIM* did not show the expression change in shRNA-ID2 cells compared to PLV-Ctr cells. Data was presented as the mean ± SD from three independent experiments. $*P < 0.05$.

expression level of ID2 protein in NPC tissues, atypical hyperplasia, squamous, and normal nasopharynx tissues. We observed that ID2 was a co-expressed factor of nucleus and cytoplasm in NPC and non-cancerous nasopharynx tissues. In cytoplasm, we did not find significantly differential expression of ID2 between NPC and non-cancerous nasopharynx tissues. However, it was in nucleus that the expression of ID2 was significantly elevated in NPC compared with normal nasopharynx tissues. This result was consistent with the Chow's report [27], revealing that the accumulation of ID2 protein in nucleus might play an important role in NPC.

In the past few years, different expression pattern of ID2 in tumor cells had been shown to be associated with tumor progression, which displayed a favorable or unfavorable prognostic significance in various tumor types. In 45 hepatocellular carcinoma (HCC) samples, well-differentiated tumors mostly exhibited strong or moderate immunostaining for ID2 protein, while proportion of the samples with weak or no expression of ID2 increased with tumor dedifferentiation and frequently observed in poorly (93.3%) or undifferentiated (100%) HCCs [29]. Furthermore, the decreased *ID2* mRNA level correlated inversely with tumor invasion, tumor-node-metastasis stage, tumor size, and early intrahepatic recurrence. When limited to a cohort of hepatitis C virus-related HCCs, patients with low levels of ID2 had significantly shorter disease-free survival time than those with high levels of ID2 [22]. Interestingly, similar results were reported in breast cancer. Stighall *et al.* [23] observed that a high cytoplasmic ID2 protein level correlated with a favorable prognosis, which was consistent with Kamalian *et al.*'s [30] report in small cell lung cancer. On the contrary, there was more evidence indicating that nuclear over-expression of ID2 in cancer cell could promote tumor progression and was not a favorable prognosis factor in prostate cancer [8], non-small cell lung cancer [19], and ovarian cancer, etc. [20]. In this study, attributing to the similar expression level of ID2 in cytoplasm among NPC, atypical hyperplasia, squamous, and normal nasopharynx tissues, we did not evaluate the correlation of cytoplasm ID2 expression with clinical parameters of NPC patients, whereas in nucleus we found that ID2 over-expression was significantly associated with T classification, N classification, and clinical stages of NPC patients. Over-expressed ID2 in NPC may accelerate tumor progression by promoting cell growth.

Subsequently, we presented the evidence that nuclear ID2 protein expression in NPC was inversely correlated with patient's overall survival. Patients with higher expression of ID2 protein had a shorter overall survival time. According to univariate analysis, patient's overall survival was also inversely proportional to T/N/M classifications, TNM stage, and ID2 expression. However, multivariate analyses showed that increased expression of ID2 protein was not a significant predictor of poor prognosis for NPC patients.

ID2 is thought as an oncogene promoting cell proliferation and invasion in some tumors. Furthermore, some recent investigations also demonstrate that ID2 participated in EMT regulation. Ectopic ID2 expression renders epithelial cells refractory to growth inhibition and EMT induced by TGF-β, phenocopying the bone morphogenetic protein (BMP) response. Knockdown of endogenous ID2 sensitizes epithelial cells to BMP, leading to robust growth inhibition and induction of trans-differentiation [31]. To specifically determine the contributions of ID2 protein in the regulation of NPC cell phenotypes, we modulated its expression *in vitro*, using SUNE1 cell lines, and found that decreased expression of ID2 converted SUNE1 cells into less aggressive cells, with lower cell growth and transformation capability. However, the alteration of cell migration capability was not observed in ID2-shRNA cells compared with PLV-Ctr and SUNE1 cells. In accordance with our findings, the activity of Snail, an EMT-related gene that promoted cell growth [32,33], was significantly suppressed in ID2 knockdown SUNE1 cells, which suggested that ID2 could promote cell growth partly attributing to the regulation of Snail activity in NPC. However, the expression of other several biomarkers for EMT including CDH1, VIM, TGF-β, and SIP1 [34–37]

was not changed after down-regulation of ID2, implying that ID2 might be not associated with the regulation of EMT in NPC.

In summary, our study provided evidences that ID2 is over-expressed in NPC and its accumulation expression in cellular nucleus may be involved in the development of NPC. Owing to the limited sample size of patients in our investigation, further studies will be needed to verify these findings and establish the role of ID2 as a reliable clinical predictor for NPC outcome. Finally, our work is the first to present that ID2 mediates cell proliferation by regulating the expression of Snail in NPC.

Supplementary Data

Supplementary data are available at *ABBS* online.

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