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The new 6q27 tumor suppressor DACT2, frequently silenced by CpG methylation, sensitizes nasopharyngeal cancer cells to paclitaxel and 5-FU toxicity via β -catenin/Cdc25c signaling and G2/M arrest

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Abstract

Background: Nasopharyngeal carcinoma (NPC) is prevalent in South China, including Hong Kong and Southeast Asia, constantly associated with Epstein-Barr virus (EBV) infection. Epigenetic etiology attributed to EBV plays a critical role in NPC pathogenesis. Through previous CpG methylome study, we identified Disheveled-associated binding antagonist of beta-catenin 2 (DACT2) as a methylated target in NPC. Although DACT2 was shown to regulate Wnt signaling in some carcinomas, its functions in NPC pathogenesis remain unclear.

Methods: RT-PCR, qPCR, MSP, and BGS were applied to measure expression levels and promoter methylation of *DACT2* in NPC. Transwell, flow cytometric analysis, colony formation, and BrdU-ELISA assay were used to assess different biological functions affected by DACT2. Immunofluorescence, Western blot, and dual-luciferase reporter assay were used to explore the mechanisms of DACT2 functions. Chemosensitivity assay was used to measure the impact of DACT2 on chemotherapy drugs.

Results: We found that DACT2 is readily expressed in multiple normal adult tissues including upper respiratory tissues. However, it is frequently downregulated in NPC and correlated with promoter methylation. DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine restored its expression in NPC cells. *DACT2* methylation was further detected in 29/32 (91%) NPC tumors but not in any (0/8) normal nasopharyngeal tissue samples. Ectopic expression of DACT2 in NPC cells suppressed their proliferation, migration, and invasion through downregulating matrix metalloproteinases. DACT2 expression also induced G2/M arrest in NPC cells through directly suppressing β -catenin/Cdc25c signaling, which sensitized NPC cells to paclitaxel and 5-FU, but not cisplatin.

Conclusion: Our results demonstrate that DACT2 is frequently inactivated epigenetically by CpG methylation in NPC, while it inhibits NPC cell proliferation and metastasis *via* suppressing β -catenin/Cdc25c signaling. Our study suggests that *DACT2* promoter methylation is a potential epigenetic biomarker for the detection and chemotherapy guidance of NPC.

Keywords: *DACT2*, Nasopharyngeal cancer, Cdc25c, Paclitaxel, 5-FU

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Background

Unlike other malignancies, the incidence of nasopharyngeal carcinoma (NPC) has great ethnic and geographic differences. Its incidence is high in Chinese and Malay populations in Southeast Asia and North Africa [1]. Specific biomarkers would be helpful in populations with a high incidence of NPC, but few are available. Study of NPC pathogenesis should aim to identify diagnostic biomarkers [2]. The Disheveled-associated binding antagonist of β -catenin (DACT) family, also known as Dapper/Frodo, are small intracellular scaffold proteins. There are three family members, DACT1, 2, and 3 [3]. DACT2 is repressed by promoter methylation in various cancers, including breast [3, 4], colon [5], lung [6], and gastric cancers [7], but the mechanisms differ. In breast cancer, our findings demonstrated that DACT2 antagonizes Akt/GSK-3 and Wnt/ β -catenin signaling to suppress epithelial-to-mesenchymal transition (EMT) [3]. In glioma cells, DACT2 interacts with Wnt/ β -catenin signaling to prevent Yes-associated protein translocation to the nucleus, resulting in its sequestration and degradation in the cytoplasm [8]. In esophageal squamous cell cancer, DACT2 suppresses TGF β /SMAD2/3 activity via both the proteasome and lysosomal degradation pathways [9]. Zebrafish DACT2 was reported to inhibit TGF- β /Nodal signaling during mesoderm induction by interacting with type 1 receptors ALK5 and ALK4 and further promoting lysosomal degradation [10, 11].

In our recent study, *DACT2* gene was identified to be a methylated target in NPC [2], but its molecular functions and mechanism were not determined. Here, we intend to investigate the expression and methylation of *DACT2* in NPC cells and tissues. The effect of *DACT2* on the cell cycle was evaluated to explore the influence of *DACT2* overexpression on drug treatment.

Results

DACT2 was downregulated in NPC by promoter methylation

Reverse transcription (RT)-PCR confirmed that *DACT2* was expressed in the majority of normal adult tissues (Fig. 1a). To investigate the expression of *DACT2* in NPC, we analyzed the gene expression data of *DACT2* in Oncomine online database (<https://www.oncomine.org/>), and it clearly shows that its expression is suppressed in the T1 and N0 stage NPC, which means *DACT2* has potential to be an early diagnosed biomarker (Fig. 1b). *DACT2* expression was downregulated in HNE1 and HONE1 NPC cells and was restored by 5-aza-2'-deoxycytidine (Aza) without or with trichostatin A (TSA). Following treatment, quantitative methylation-specific PCR (qMSP) showed a decrease of methylated level and an increase in un-methylated level (Fig. 1c).

Thus, *DACT2* expression was downregulated in these NPC cell lines by promoter methylation.

The methylation status of eight normal nasopharyngeal tissues and 32 NPC tissues was assayed by methylation-specific polymerase chain reaction (MSP), which found that the *DACT2* promoter was not methylated in any of the normal nasopharyngeal tissues but was methylated in 29 of 32 (91%) NPC tissues (Fig. 1d, e). Bisulfite genomic sequencing (BGS) was used to assay methylated *DACT2* promoter alleles in two normal nasopharyngeal tissue and two NPC tissue samples to confirm the result of MSP and found that *DACT2* methylation was more frequent in NPC than in normal nasopharyngeal tissues (Fig. 1f).

Overexpression of DACT2 inhibited NPC cell proliferation, viability, and colony formation

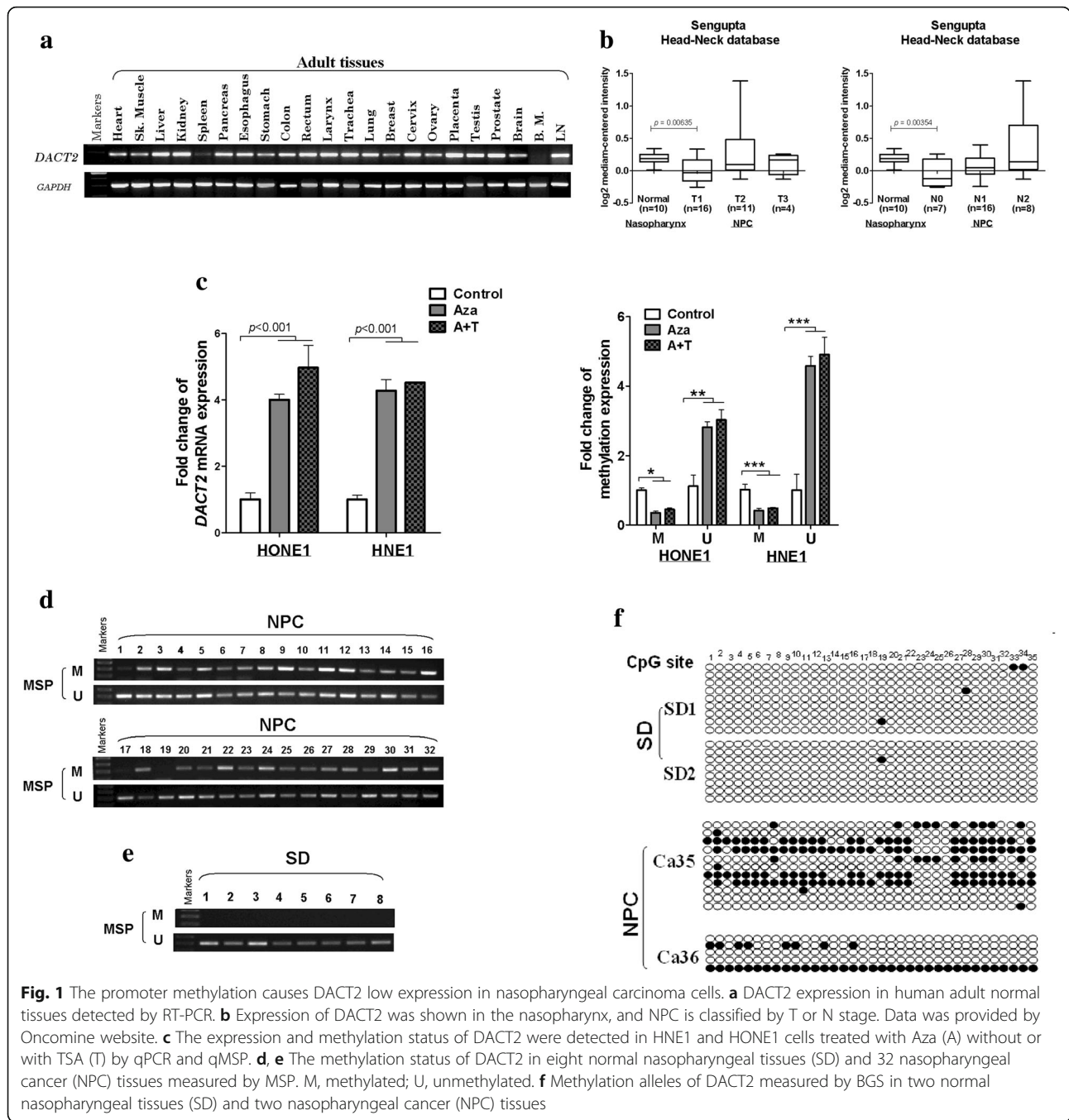
The overexpression of *DACT2* after *DACT2* plasmid transfection was confirmed using RT-PCR and Western blot by comparing to empty control (Fig. 2a, b). The MTS assay (Fig. 2c) showed that cell viability was significantly reduced in *DACT2*-expressing cells. Colony formation (Fig. 2d) was also significantly suppressed compared with the control cells. These results indicated that *DACT2* suppressed both viability and growth of NPC cells.

DACT2 induced G2/M cell cycle arrest and apoptosis in NPC cells

The influence of *DACT2* on tumor cell proliferation might be mediated by its effects on the cell cycle and apoptosis. Flow cytometry of HONE1 and HNE1 cells found that the percentage of cells in the G2/M phase was increased in those that overexpressed *DACT2* compared with controls transfected with an empty vector (Fig. 3a, b), accompanied by the increased cell population of S phase. Furthermore, the BrdU-ELISA assay, which reflects active DNA synthesis, revealed that the cell proliferation rate was decreased in *DACT2*-expressing cells (Fig. 3c). *DACT2* overexpression also promoted cell apoptosis compared with controls (Fig. 3d, e). These results indicated that *DACT2* inhibited cell proliferation by blocking the cell cycle in G2/M and by inducing cell apoptosis.

DACT2 inhibited NPC cell migration and invasion

Wound healing and Transwell assays were used to investigate the influence of *DACT2* expression on NPC cell migration and invasiveness. In the Transwell assay, significantly fewer *DACT2*-overexpressing cells passed through the membrane than control cells ($p < 0.001$) (Fig. 4a). Further wound healing assay revealed that scratches made in confluent layers of *DACT2*-overexpressing cells healed significantly slower than control cell layers over 24 h for HNE1 ($p < 0.001$) or 33 h for



HONE1 ($p < 0.01$, Fig. 4b), which showed that DACT2 inhibited NPC cell migration. In the Transwell assay including a Matrigel barrier, DACT2 overexpression was associated with significant inhibition of NPC cancer cell invasion through the Matrigel before traversing the Transwell chamber membrane ($p < 0.01$, $p < 0.001$ at 24 h, Fig. 4c). qPCR and Western blot assays assessed the effect of DACT2 on expression of matrix metalloproteinases (MMPs) 2 and 9 (Fig. 4d, e), which are essential for cell migration and invasion [12, 13]. Overall,

the results indicate that DACT2 suppressed cell migration and invasion in NPC cells.

DACT2 induced G2/M cell cycle arrest through the β -catenin/Cdc25c signaling pathway in nasopharyngeal cancer cells

Investigation of the mechanism of G2/M cell cycle arrest was based on a previous report that DACT2 suppressed β -catenin activity in colon cancer by competition for LEF1 binding [5]. In addition, the gene coding for cell

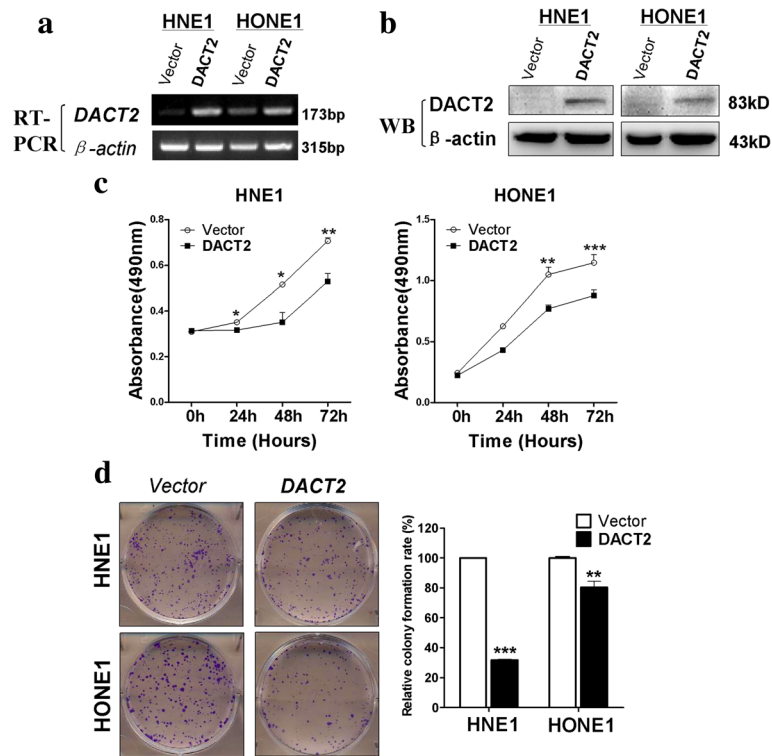


Fig. 2 Overexpressed DACT2 suppress nasopharyngeal carcinoma cell proliferation ability. **a, b** The expression of DACT2 was detected in HNE1 and HONE1 cells stably transfected with vector (pcDNA3.1) or DACT2 by RT-PCR and Western blot. **c** The ability of cell proliferation was measured in vector- and DACT2-expressing HNE1 and HONE1 cells by MTS. **d** Representative images and the histogram statistics of the colony-formation assay in vector- and DACT2-expressed HNE1 and HONE1 cells. Mean \pm SD, * p < 0.05, ** p < 0.01, *** p < 0.001. All experiments were performed in triplicate, respectively

division control protein 25C (Cdc25c), a regulator of G2/M cell cycle progression, has a LEF1 binding site on its promoter region. The function of that protein may thus be responsive to Wnt/ β -catenin signaling activity [14, 15]. It is thus reasonable that DACT2 inhibits the activity of β -catenin/LEF1 complex by competitively binding with β -catenin followed by downregulation of Cdc25c, which ultimately blocks cell division in the G2/M phase. DACT2 regulation of the β -catenin/Cdc25c pathway to produce G2/M cell cycle arrest was assessed by immunofluorescent staining of β -catenin. As shown in Fig. 5a, the morphology of HNE1 cells overexpressing DACT2 differed from that of control cells (Fig. 5a), and the expression of total β -catenin did not significantly change but active β -catenin decreased in the cell nuclear location (Fig. 5b, c). qRT-PCR showed that DACT2 overexpression was associated with decreased expression of Cdc25c and cyclin B1 (Fig. 6a). Western blots revealed that DACT2 suppressed the expression of active β -catenin, Cdc25c, and the downstream target genes of β -catenin/Cdc25c signaling (Fig. 6b). A dual-luciferase reporter assay was performed to confirm whether DACT2 inhibited the activity of the β -catenin/LEF1 complex. It showed that DACT2 downregulated the

induced TOPflash luciferase activities (Fig. 6c). Overall, the results indicate that DACT2 led to G2/M cell cycle arrest by inhibiting the β -catenin/Cdc25c signaling pathway.

DACT2 induced sensitivity of NPC cells to paclitaxel and 5-FU but not cisplatin

As DACT2 expression led to G2/M cell cycle arrest, the impact of DACT2 overexpression on the sensitivity of NPC cells to cell cycle phase-specific and phase-nonspecific chemotherapy drugs was tested. Paclitaxel, 5-fluorouracil (5-FU), and cisplatin were selected. Paclitaxel acts by arrest of the cell cycle in G2/M [16]. 5-FU is an atypical periodic chemotherapy drug targeting on the S and other phases [17]. Cisplatin is cell cycle phase-nonspecific chemotherapy drug [18]. As shown in Fig. 7, DACT2 expression increased the sensitivity of HNE1 and HONE1 cells to paclitaxel and 5-FU compared with controls, but had no effect on cisplatin sensitivity (Fig. 7).

Discussion and conclusion

Promoter CpG methylation, which downregulates the expression of tumor suppressor genes, is essential to the

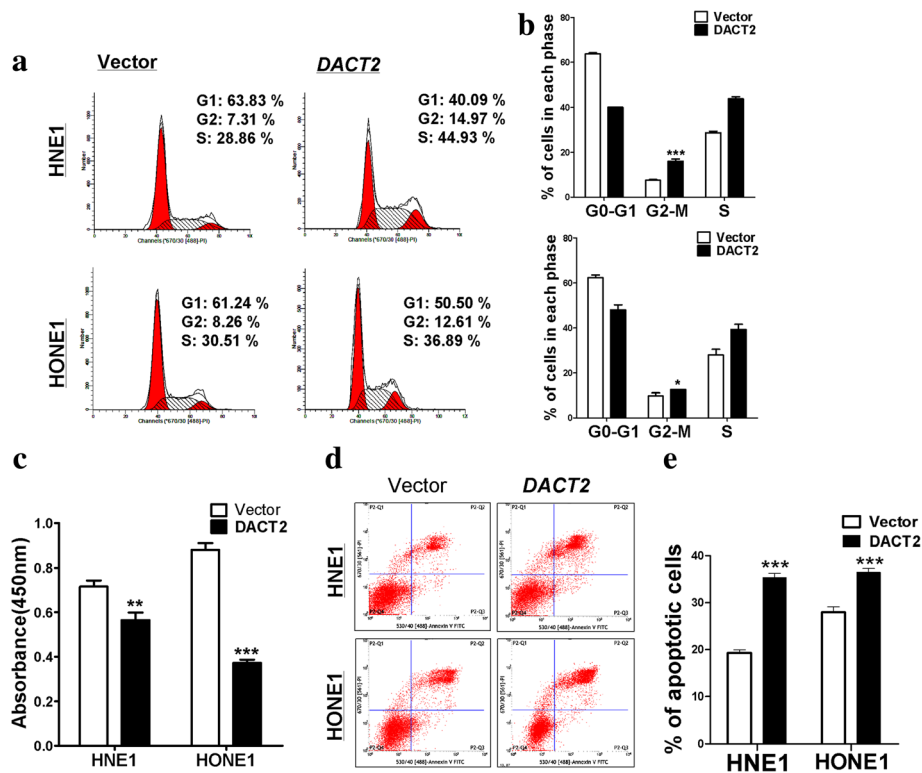


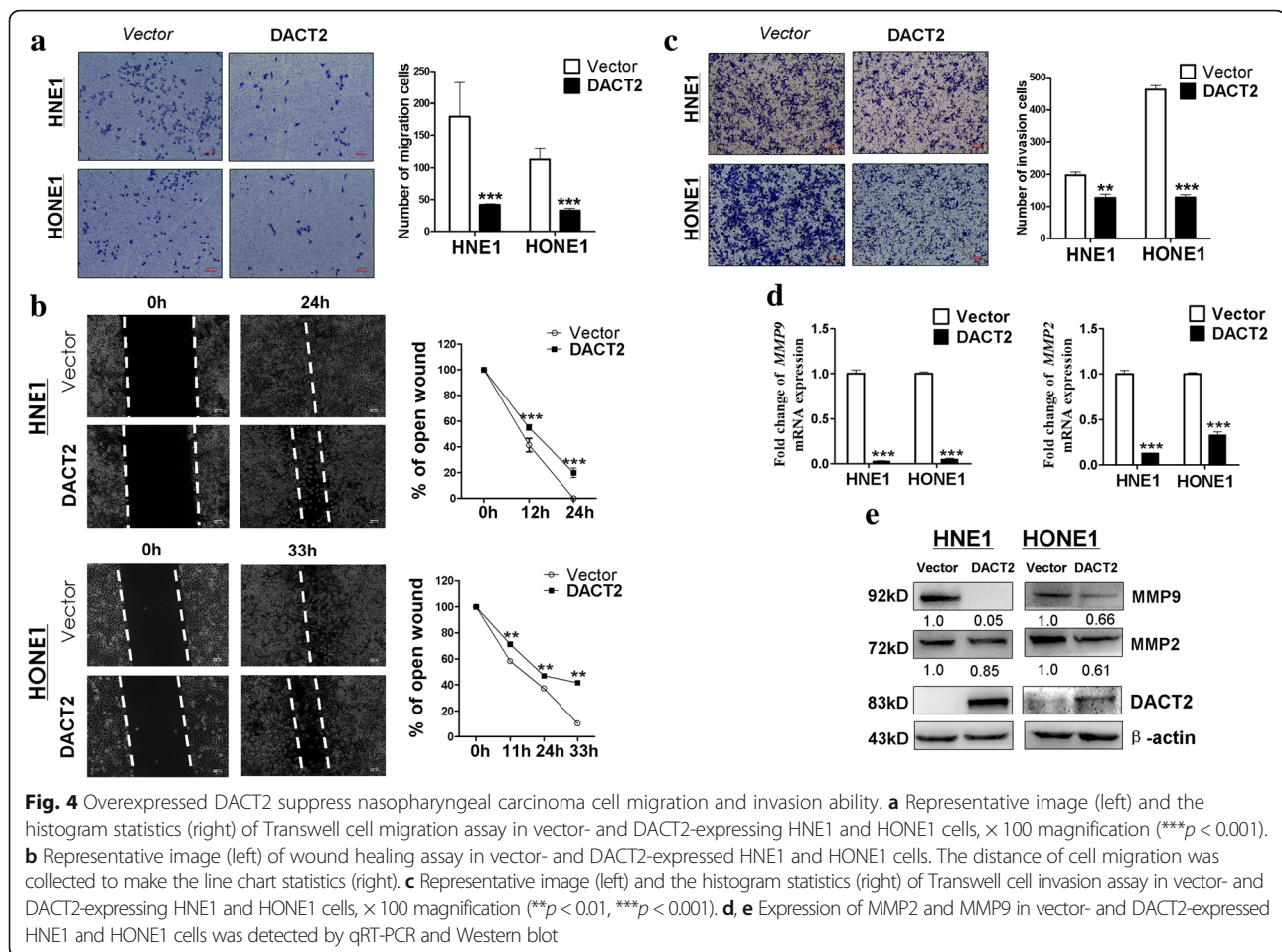
Fig. 3 Overexpressed DACT2 induced G2/M cell cycle arrest and promote cell apoptosis in nasopharyngeal carcinoma cells. **a, b** The effect of DACT2 on cell cycle in vector- and DACT2-expressing HNE1 and HONE1 cells was detected by flow cytometry analysis. Representative flow cytometry plots (**a**) and the histogram statistics of cell cycle changes (**b**). **c**, BrdU-ELISA assay at 24 h in vector- or DACT2-transfected HNE1 and HONE1 cells. **d, e** The proportion of apoptotic cells was detected in vector- and DACT2-expressing HNE1 and HONE1 cells by flow cytometry analysis. Representative flow cytometry plots (**d**) and the histogram statistics of apoptosis changes (**e**)

pathogenesis of malignancies including NPC [19, 20]. Specific epigenetic therapy may increase the effectiveness of NPC treatment [21]. In papillary thyroid cancer [22] and hepatocellular cancer [23, 24], the expression of *DACT2* is downregulated by promoter methylation. In this study, *DACT2* was strongly expressed in normal adult tissues but weakly expressed and hyper-methylated in NPC cell lines. *DACT2* expression was restored in NPC cell lines by Aza and TSA demethylation. Promoter methylation was detected in 29 of 32 (91%) NPC tissue samples but was not detected in any of the normal nasopharyngeal tissue samples. The results indicated that the low expression of *DACT2* in NPC was caused by promoter CpG methylation. The function of *DACT2* was investigated in HONE1 and HNE1 cells transfected with *DACT2* gene. The restoration of *DACT2* expression inhibited NPC cell proliferation, migration, and invasiveness and induced G2/M cell cycle arrest.

The Wnt signaling pathway is active in tumorigenesis, cell differentiation, and cell proliferation [25]. β -catenin is a transcription cofactor that induces target gene expression by binding to T cell factor/lymphoid enhancer factor (TCF/LEF) in the activated Wnt pathway [26].

The *DACT* family members are inhibitors of Disheveled, an important Wnt pathway component that suppresses c-Jun N-terminal kinase (JNK) signaling and the β -catenin cascades [27, 28]. *DACT2* has been reported to decrease LEF1- β -catenin binding in colon cancer by competing with β -catenin [5]. In this study, *DACT2* decreased the expression of active β -catenin and its downstream genes in NPC cells and suppressed the activity of the β -catenin/LEF1 complex. *Cdc25c*, which has been shown to regulate the G2/M checkpoint, has also been reported to have a functional LEF binding site [14, 15, 29, 30]. In NPC cells, *DACT2* was found to suppress the expression of *Cdc25c* and its downstream genes related to G2/M arrest. We conclude that *DACT2* downregulated the activity of the β -catenin/LEF1 complex by binding to β -catenin and that the decreased expression of *Cdc25c* induced G2/M arrest (Fig. 8). *DACT2* overexpression increased the sensitivity of NPC cells to the cell cycle phase-specific chemotherapy drugs, paclitaxel and 5-FU.

In summary, *DACT2* was silenced by promoter methylation as a tumor suppressor in NPC cells and induced G2/M phase arrest by the regulating β -catenin/*Cdc25c*



signaling pathway. The results show that DACT2 is a tumor suppressor in nasopharyngeal cancer and support continuing evaluation of its value for early diagnosis and for targeted therapy of nasopharyngeal cancer.

Methods

Tumor cell lines and tissues

HNE1 and HONE1, two poorly differentiated nasopharyngeal squamous carcinoma cell lines, were used [19, 31]. Cells were maintained in RPMI 1640 (Gibco BRL, MD, USA) with 10% fetal bovine serum (Gibco, CA, USA) and 1% penicillin and streptomycin (Gibco BRL). Thirty-two nasopharyngeal cancer and eight normal nasopharyngeal tissue samples were obtained from Department of Otolaryngology of the First Affiliated Hospital of Chongqing Medical University between December 2010 and July 2013 [32–34]. All samples were verified by histology. All patients gave written informed consent.

RNA isolation, reverse transcription-PCR, and quantitative real-time PCR

Total RNA was extracted from cell lines and tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA)

following the manufacturer's instructions, and aliquots containing 1 μ g of total RNA were reverse-transcribed to 20 μ l cDNA. PCR was performed using Go-Taq (Promega, Madison, WI, USA) with initial denaturation at 95 $^{\circ}$ C for 2 min, followed by 32 cycles (95 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 30 s) of amplification, with a final extension at 72 $^{\circ}$ C for 3 min [35] with β -actin used as a control. Twenty-three cycles of amplification were performed. The primer sequences are shown in Table 1. qPCR was performed using SYBR Green (Thermo Fisher) following the manufacturer's instructions (7500 Real-Time PCR System, Applied Biosystems, Foster City, CA, USA). Each sample was tested in triplicate. Gene expression level was calculated by the $2^{-\Delta\Delta Ct}$ method.

5-Aza-2'-deoxycytidine and trichostatin A treatment

Cell lines were treated with 10 μ mol/L 5-aza-2'-deoxycytidine (Aza, Sigma-Aldrich, Steinheim, Germany), a DNA methyltransferase (DNMT) inhibitor, for 3 days and then without or with 100 nmol/L trichostatin A (TSA, Sigma-Aldrich) for 24 h as previously described [36].

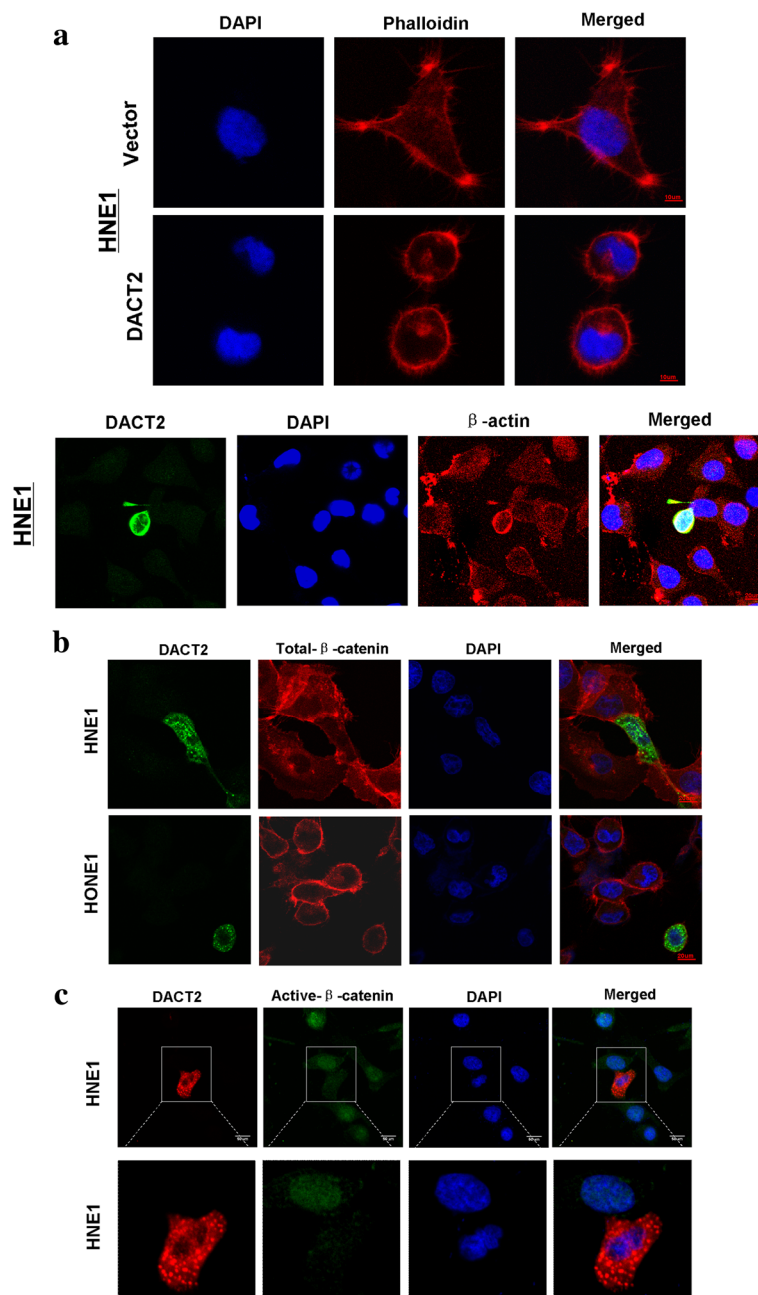


Fig. 5 Overexpressed DACT2 led HNE1 cell morphological alteration and decreased the expression of active- β -catenin. **a** HNE1 cell morphological alteration caused by DACT2 overexpression was observed by immunofluorescence. Upper—the cell shape became round and fewer thin fibers in DACT2-expressing cells but longer cell processes in control cells. Lower—double immunofluorescent staining also showed that DACT2-expressing cell became rounder and its pseudopods decreased. Photos were taken under $\times 400$ magnification. **b** Localization and expression of endogenous total β -catenin was showed in DACT2-expressing HNE1 and HONE1 by immunofluorescence. **c** Immunofluorescence showed the localization and expression of active β -catenin in HNE1 transfected with DACT2 plasmid

DNA isolation, bisulfite modification of DNA, methylation-specific PCR, and quantitative methylation-specific PCR

Genome DNA was extracted from tissues using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Bisulfite modification of DNA was performed as previously described

[37, 38]. The MSP primers (annealing temperature of 60°C , 40 cycles) are shown in Table 1, and have been confirmed not amplify any nonbisulfited DNA [3]. MSP was performed using AmpliTaq-Gold DNA Polymerase (Applied Biosystems). The PCR products were identified on 2% agarose gels. qMSP was performed with the 7500

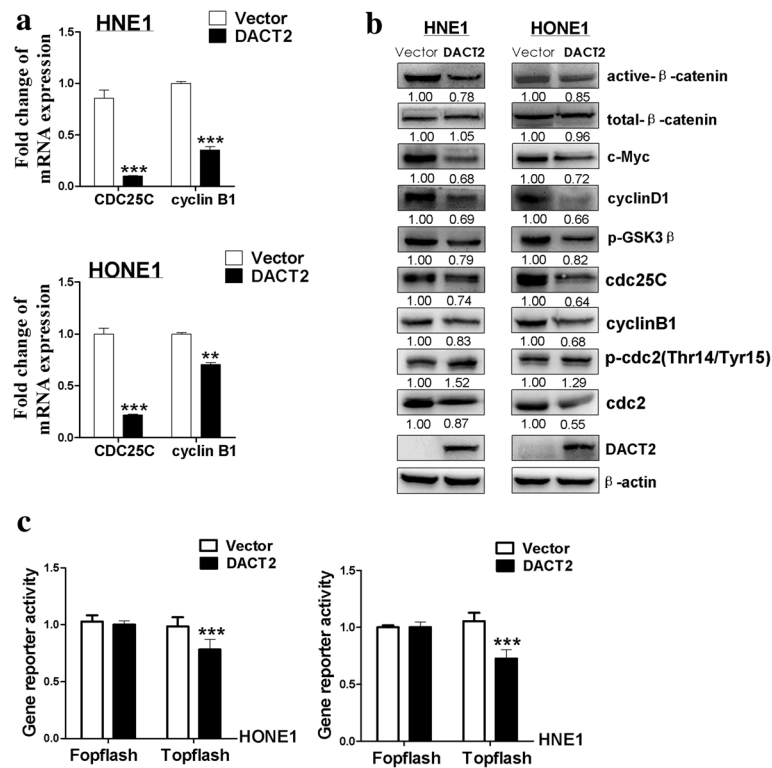


Fig. 6 Overexpressed DACT2 suppressed β -catenin/Cdc25c pathway in nasopharyngeal carcinoma cells. **a** Expression of CDC25C and CyclinB1 in vector- and DACT2-expressed HNE1 and HONE1 cells was detected by qPCR. **b** Expression of β -catenin and its target genes was measured by Western blot in vector- and DACT2-expressing HNE1 and HONE1 cells. **c** The activity of β -catenin/LEF1 complex was examined by dual-luciferase reporter assay in vector- and DACT2-expressed HNE1 and HONE1 cells

Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) [39].

Bisulfite genomic sequencing

BGS primers (Table 1) were used to amplify bisulfite-treated DNA, and the PCR products were cloned into a pCR4-Top vector (Invitrogen). Eight to 12 colonies were randomly chosen and sequenced.

Construction of vector- and DACT2-expressed stable cell lines

Stable cell lines were constructed by transfecting cell lines with plasmids using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. pcDNA3.1 and pcDNA3.1-DACT2 plasmids were transfected at a concentration of 4 μ g and selection by geneticin at 48 h after transfection [3]. Ectopic expression of DACT2 was assayed by RT-PCR and Western blotting prior to the other experimental procedures.

Cell viability assay

Cell viability was evaluated with a CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS, Promega) following the manufacturer's instructions. HONE1 and

HNE1 cells were cultured in 96-well plates after transient transfection with DACT2 and vector (pcDNA3.1) plasmids. Cell viability was measured at 0, 24, 48, and 72 h. Absorbance was read in a microplate reader at 490 nm. All experiments were performed in triplicate.

Colony formation assay

Cell proliferation was assayed by colony formation assay [40]. DACT2- and vector-expressing cells were plated in 6-well plates at densities of 200, 400, or 600 cells/well with geneticin. Surviving colonies (≥ 50 cells/colony) were counted on day 10 of culture after fixation and staining with Gentian violet. All experiments were performed in triplicate.

BrdU cell proliferation enzyme-linked immunosorbent assay

Cells were seeded in 96-well plates at 1×10^4 cells per well after transfection with DACT2 and vector (pcDNA3.1) plasmids for 48 h. After 24-h culture, BrdU (bromodeoxyuridine) was added to the wells to incorporate into proliferating cells for 4 h. The BrdU-ELISA assay was performed by BrdU Cell Proliferation ELISA Kit (colorimetric) (Abcam, Cambridge, UK) following

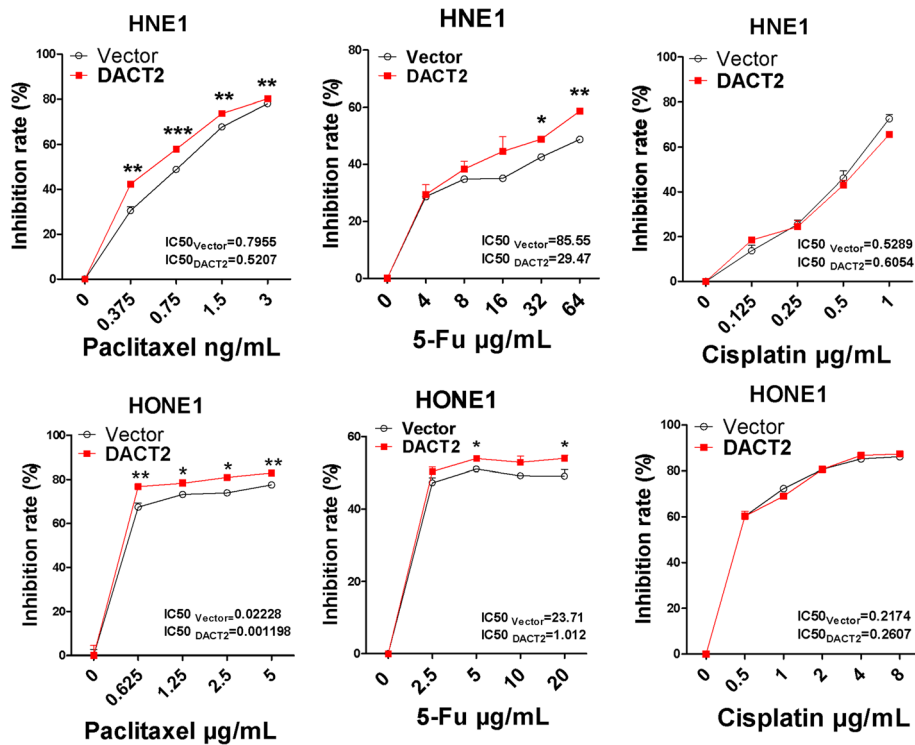


Fig. 7 Overexpressed DACT2 sensitized nasopharyngeal carcinoma cells to paclitaxel, 5-FU rather than cisplatin. The cell viability was detected by CCK8 in vector- and DACT2-expressed HNE1 and HONE1 cells after the treatment of chemotherapy drugs paclitaxel or cisplatin for 48 h or 5-FU for 24 h. All experiments were performed in triplicate, respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

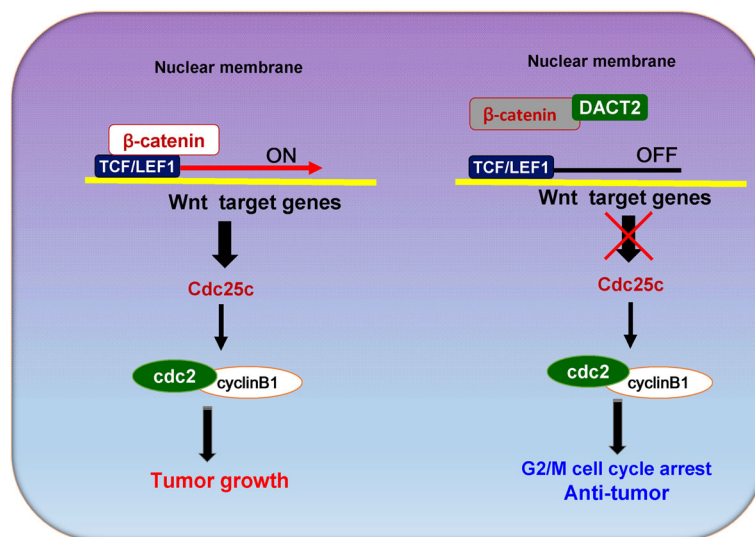


Fig. 8 Proposed models how DACT2 affects the β -catenin/Cdc25c pathway in nasopharyngeal carcinoma. When β -catenin/LEF1 complex is activated, it promotes the development of nasopharyngeal carcinoma by targeting its downstream genes including Cdc25c, which plays a crucial role in regulating cell cycle G2/M stage. DACT2 inhibits the activity of β -catenin/LEF1 complex by competitively binding to β -catenin and downregulates the expression of Cdc25c and its downstream genes, which can suppress nasopharyngeal carcinoma growth

Table 1 List of primers used in this study

PCR	Primer	Sequence (5'-3')	Product size (bp)	PCR cycles	Annealing temperature (°C)
RT-PCR	DACT2F	AGCCGTGGGGCACATTCTG	173	32	55
	DACT2R	CCAGGTCCTGCCGATACTTG			
	β-actinF	TCCTGTGGCATTCCACGAAACT	315	23	55
	β-actinR	GAAGCATTTCGGTGGACGAT			
qRT-PCR	CyclinB1F	TCTGGATAATGGTGAATGGACA	157		60
	CyclinB1R	CGATGTGGCATACTTGTCTTG			
	MMP2F	CATACAGGATCATTGGCTACACAC	99		60
	MMP2R	GCAGTGGGGTCACATCGCT			
	MMP9F	CCTGGAGACCTGAGAACCAATC	79		60
	MMP9R	CCACCCGAGTGTAAACCATAGC			
	Cdc25cF	GATGTCCCTAGAACTCCAGTG	120		60
	Cdc25cR	AGTTATCTCCCCACTGCTAAGA			
	β-actinF1	GTCTCCCTCCATCGTG	113		60
	β-actinR1	AGGGTGAGGATGCCTCTCTT			
	MSP	DACT2m1	CGTGTAGATTTTCGTTTTTCGC	200	40
DACT2m2		CCGAAAATCCGCCGACG			
DACT2u1		TGTGTGTAGATTTTGTTTTTTGT	203	40	58
DACT2U2		CCCCAAAATCCACCCAACA			
BGS	DACT2BGS1	GGTTATAGATTTTAGTTTATTTTGG	249	40	60
	DACT2BGS2	CTACAACCTCTACAACCCC			

the kit manufacturer's instructions. The results were read at 450 nm using a microplate reader.

Flow cytometry

Flow cytometry was used for cell cycle analysis and to assay apoptosis [36, 41]. To assess cell cycle status, cells were stained with propidium iodide (PI) following transfection and fixation. For apoptosis, cells were double-stained with annexin V-fluorescein isothiocyanate and PI. The flow cytometry results were evaluated using a Cell Quest kit (BD Biosciences, CA, USA) and were performed in triplicate.

Wound healing, Transwell, and Matrigel assays

Cell migration was evaluated by wound healing and Transwell assays [42]. Stably transfected DACT2- and vector-(pcDNA3.1) HONE1 and HNE1 cells were plated in 6-well plates and were wounded when confluent by scratching with a sterile pipette tip. Migration was measured on phase-contrast micrographs (Leica DMI4000B, Milton Keynes, Buckinghamshire, UK) at 0, 12, and 24 h for HNE1 and 0, 11, 24, and 33 h for HONE1. Transwell chambers (Corning Life Sciences, Corning, NY, USA) with a pore size of 8 μm were used to evaluate cell migration and cell invasion. To assay cell invasiveness, a Matrigel (BD Biosciences) barrier was added on top of the Transwell membrane. Cells on the lower surface of

the chamber at 24 h were photographed using a phase-contrast microscope (Leica) after fixation and staining and were then counted. All experiments were performed in triplicate.

Chemosensitivity assay

The effect of DACT2 on the cytotoxicity of paclitaxel, cisplatin, and 5-fluorouracil (5-FU) was assayed using a Cell Counting Kit-8 (CCK-8). Briefly, HONE1 and HNE1 cells transfected with DACT2 or vector (pcDNA3.1) plasmids were plated at 5000/well in 96-well plates. After cell attachment, culture media containing different concentrations of the tested drugs were added. Cells were then counted at 24 or 48 h with CCK-8 (Dojindo, Shanghai, China) following the manufacturer's instructions. Absorbance was read with a microplate reader at 450 nm. The half-maximal inhibitory concentration (IC50) was calculated for each drug concentration. All experiments were performed in triplicate.

Dual-luciferase reporter assay

The effect of DACT2 on TCF/LEF transcriptional activities was investigated by a dual-luciferase reporter assay. pTopflash and pFopflash were used in our previous work [41]. pTopflash was constructed with TCF/LEF binding sites but pFopflash containing a mutant TCF/

LEF binding sites as a control. HONE1 and HNE1 cells were transiently co-transfected with a pTOPflash or pFOPflash and DACT2 or vector (pcDNA3.1) with a Renilla luciferase reporter pRL-TK (Promega) as an internal control. Luciferase activity was measured after 48 h transfection using a dual-luciferase reporter assay kit (Promega) following the manufacturer's instructions. All experiments were performed in triplicate.

Immunofluorescence staining

Cells were seeded in 24-well plates containing glass coverslips and then transfected with pcDNA3.1-DACT2 or pcDNA3.1 plasmid for 48 h. After transfection, cells were fixed with 4% paraformaldehyde in pH 7.4 PBS for 10 min, permeabilized for 10 min in 0.5% Triton X-100, and blocked with blocking buffer for 1 h at room temperature. Cells were incubated with primary antibodies against DACT2 (TA306668, Origene) and β -catenin (#2677, Cell Signaling Technology, Danvers, MA, USA) or β -actin (sc-8432, Santa Cruz Biotechnology, CA, USA), Flag-M2 (F3165, Sigma-Aldrich, Darmstadt, Germany) and non-p- β -catenin (#19807, Cell Signaling Technology) overnight at 4 °C. After primary antibody binding, cells were incubated with Alexa Fluor 594- or 488-conjugated goat anti-rabbit or anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) for an additional 30 min. Nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI, Roche, Palo Alto, CA, USA). The slides were observed with a confocal laser scanning microscope and photographed. Phalloidin staining was performed as our previous work [43]. Stable cells were used. Phalloidin-iFluor™ 594 Conjugate (23122, AAT Bioquest, CA, USA) at room temperature for 1 h.

Western blot assay

Western blotting was performed as previously described [31]. Aliquots of 40 μ g of protein lysate were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Membranes were incubated with DACT2 (TA306668, Origene), active β -catenin (#4270; Cell Signaling Technology), total β -catenin (#9562; Cell Signaling Technology), MMP9 (ab76003, Abcam), MMP2 (ab86607, Abcam), c-Myc (#13987, Cell Signaling Technology), Cyclin D1(sc-450), p-GSK3 β (sc-373800), Cdc25c (sc-13138), Cdc2 (sc-54), p-Cdc2 (pY15.44) (sc-136014), β -actin (sc-8432) (all from Santa Cruz Biotechnology, CA, USA), or CyclinB1 (ab32053, Abcam) primary antibodies. Proteins were visualized using an Immobilon Western Chemiluminescent HRP Substrate kit (Millipore Corporation, Billerica, MA, USA).

Statistical analysis

SPSS16 (SPSS, Chicago, IL, USA) was used to perform the statistical analysis. Differences were evaluated for significance with the χ^2 test and Fisher's exact test. *p* values < 0.05 were considered statistically significant.

Abbreviations

5-FU: 5-Fluorouracil; Aza: 5-Aza-2-deoxycytidine; BGS: Bisulfite genomic sequencing; BrdU: Bromodeoxyuridine; CCK-8: Cell Counting Kit-8; Cdc25c: Cell division control protein 25C; DACT2: Disheveled-associated binding antagonist of beta-catenin 2; DAPI: 4, 6-diamidino-2-phenylindole; DNMT: DNA methyltransferase; EBV: Epstein-Barr virus; ELISA: Enzyme-linked immunosorbent assay; EMT: Epithelial-to-mesenchymal transition; IC50: Half-maximal inhibitory concentration; JNK: c-Jun N-terminal kinase; MMPs: Matrix metalloproteinases; MSP: Methylation-specific polymerase chain reaction; NPC: Nasopharyngeal carcinoma; PI: Propidium iodide; PVDF: Polyvinylidene difluoride; qMSP: Quantitative methylation-specific polymerase chain reaction; qPCR: Quantitative real-time polymerase chain reaction; RT-PCR: Reverse transcription polymerase chain reaction; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCF/LEF: T Cell factor/lymphoid enhancer factor; TSA: Trichostatin A

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Availability of data and materials

The datasets analyzed during the current study are available in the Oncomine repository (<https://www.oncomine.org/>).

Authors' contributions

YZ, TX, and QT contributed to the conception and design of the study. YZ, JF, DZ, XS, and JM performed the experiments and analyzed the data. XH and QX contributed to the RNA and DNA extraction. YY collected the samples. YZ and TX prepared the figures and drafted the manuscript. GR, LL, and YF reviewed the manuscript. TX and QT finalized the manuscript. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

This research was approved by the Institutional Ethics Committees of the First Affiliated Hospital of Chongqing Medical University (#20130306) and conformed to the tenets of the Declaration of Helsinki.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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