Dll4/Notch1 signalling pathway is required in collective invasion of salivary adenoid cystic carcinoma

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Abstract. High expression of δ-like ligand 4 (Dll4) is reportedly related to the invasion, metastasis, and clinical prognosis of various malignant tumours. Our previous study revealed that collective cell invasion was a common pattern in salivary adenoid cystic carcinoma (SACC). However, the roles of the Dll4/Notch1 signalling pathway in the collective invasion of SACC remain unclear. The present study revealed that Dll4 expression was higher at the invasive front of SACC, and that this upregulation was associated with solid tumour type, high TNM grade, and high rates of metastasis and recurrence. Furthermore, the expression levels of Notch1 and Dll4 were positively correlated at the invasive front, and a three-dimensional (3D) culture model revealed that leader cells showed high expression of Dll4, while follower cells showed high expression of Notch1. Moreover, silencing of Dll4 expression using small interfering RNA reduced the migration, invasion, and collective invasion of SACC cells, and these abilities were rescued by Notch1 overexpression. Finally, SACC collective invasion was increased via the Dll4/Notch1 signalling pathway in experiments that involved a stiff 3D gel, hypoxia and co-culture with human endothelial cells. These findings indicated that the Dll4/Notch1 signalling pathway may be involved in the collective invasion of SACC, which may help to provide possible targets for the treatment of SACC.

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Introduction

Salivary adenoid cystic carcinoma (SACC) is one of the most common salivary gland malignancies and is associated with a high local recurrence rate, neurotropic invasion and hematogenous metastasis (1). The standard treatment for SACC involves surgery with or without radiotherapy and chemotherapy, although the recurrence rate is still very high and the long-term survival rate is poor, with 5-year survival rates of 75-80% and 15-year survival rates of 10-30% (2). Thus, there is an urgent need to clarify the molecular mechanisms underlying SACC invasion and metastasis, which may help guide the development of effective therapies.

Collective cell migration is an important process in both normal epithelial development and cancer invasion (3). Previous studies have indicated that the classic mode of single-cell invasion accompanies the epithelial-mesenchymal transition (EMT) in epithelial carcinomas (4-6). Collective invasion is another important process that involves tumour cells maintaining their cell-cell contacts and moving as inter-connected multicellular clumps with or without partial EMT (3,7,8). Therefore, it is important to clarify which features of tumour progression regulate the transition from single-cell invasion to a collective invasive phenotype, and how these features might be related.

Collective invasion is generally speculated to be driven by leader cells, which maintain different cell polarities, extend plate-like and filamentous pseudopods, adhere to the

extracellular matrix, promote actin-mediated cell contraction, and secrete a variety of proteolytic enzymes to degrade the extracellular matrix and guide follower cells (9). Researchers have also demonstrated that breast cancer leader cells express basal epithelial genes [keratin 14 (K14) and p63] and that silencing these genes could inhibit collective invasion (10). Han *et al* (11) also reported that netrin-1 expression was increased in leader cells at the invasion front, which promoted collective migration of hepatoma cells through increased N-cadherin expression in a three-dimensional (3D) culture model. Our previous research also revealed that SACC cells formed cellular masses to invade the surrounding tissues, such as nerves, blood vessels and glands, and that this form of collective invasion was the primary method of SACC invasion (12). However, it is difficult to clarify the relative contributions of specific genetic and microenvironmental changes to carcinoma cell collective invasion, especially as the molecular characteristics of SACC leader and follower cells are not completely understood.

δ-like ligand 4 (Dll4) is the ligand of Notch1 and is mainly expressed in peritumoral vascular endothelial cells (13). Previous studies have indicated that Dll4 upregulation in various cancers can promote invasion and is closely related to a malignant phenotype and poor prognosis in cases of gastric cancer and cervical cancer (14-18). Xu *et al* (19) also reported that blocking Dll4 could inhibit tumour cell proliferation and mammosphere formation in breast cancer. However, there are no data regarding the potential role of the Dll4/Notch1 signalling pathway in the collective invasion of SACC. Therefore, the present study used immunohistochemical staining to evaluate Dll4 and Notch1 expression at the invasion front of SACC. Furthermore, a 3D culture model was used to examine the expression of Dll4 and Notch1 in the leader and follower cells, and it was evaluated whether the expression of these genes changed in differently simulated tumour microenvironments. The results may help guide further research regarding the molecular markers of collective invasion and how the tumour microenvironment influences the collective invasion of carcinoma cells.

Materials and methods

Histological analysis. This retrospective study evaluated 84 SACC specimens and 5 normal salivary gland specimens (collected between January 2009 and December 2014) from the Department of Oral Pathology database (West China Hospital of Stomatology, Sichuan University, Chengdu, China). The patients had provided informed consent for the use of their surgical specimens and clinical data in research. The study protocol was approved by the Institutional Ethics Committee of the West China Medical Center, Sichuan University (approval no. WCHSIRB-D-2017-208). All specimens had been fixed with 10% buffered formalin at 4˚C for 24 h, and then embedded in paraffin.

Cell culture and transfection. Commonly used cell lines (SACC-83, SACC-LM and HUVECs) were obtained from the State Key Laboratory of Oral Diseases (Sichuan University, Chengdu, China). All cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin (HyClone; Cytiva) and 100 μg/ml streptomycin (HyClone; Cytiva) and maintained in an incubator at 37˚C with 5% CO_2. The experimental conditions for hypoxia were conducted by incubating cells at 1% O_2 in a hypoxia workstation (Ruskinn Invivo 400 Hypoxic Workstation). Three small interfering (si)RNAs against Dll4 and a negative control siRNA were designed by Shanghai GeneChem Co., Ltd. SACC-83 and SACC-LM cells were seeded at a density of $2x10^5$ cell/ml and were transfected with the siRNA (20 pM) using EndoFectin™ reagent (GeneCopoeia, Inc.), according to the manufacturer's instructions. The sequences of the siRNA were as follows: Duplex-1, GCGUCUGCCUUAAGCACUUTTAAG UGCUUAAGGCAGACGCTT; duplex-2, CCAGAAGGACAA CCUGAUUTTAAUCAGGUUGUCCUUCUGGTT; duplex-3, GCAACUGCCCUUAUGGCUUTTAAGCCAUAAGGGCAG UUGCTT; and negative control, UUCUCCGAACGUGUCACG UTTACGUGACACGUUCGGAGAATT. The transfected cells were then used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR), migration and invasion assays, 3D cultures, and immunofluorescence (IFC) staining 24 h after transfection.

Haematoxylin and eosin (H&E) and immunohistochemical (IHC) staining. The sections of SACC specimens and normal glands were deparaffinized using xylene, and then rehydrated using a series of ethanol and water solutions. For H&E staining, slices were stained with hematoxylin solution for 5 min, dipped in acid ethanol, and then washed with distilled water in turn, all at room temperature. The slices were subsequently stained with eosin solution for 3 min and re-immersed in alcohol and xylene at room temperature. For IHC staining, the sections were autoclaved for antigen retrieval, blocked with 3% hydrogen peroxide for 2 min and 10% goat serum albumin (OriGene Technologies, Inc.) for 30 min at room temperature, and then subjected to routine IHC staining procedures as previously described (16,17). The primary antibodies were mouse anti-Dll4 (1:500; cat. no. 220726; ZenBio, Inc.) and rabbit anti-Notch1 (1:300; cat. no. 516673; ZenBio, Inc.). Then, sections were incubated with a commercial goat anti-mouse/rabbit IgG secondary antibody (cat. no. SA1020; Wuhan Boster Biological Technology, Ltd.) for 15 min, and detected with a DAB Staining Kit (Wuhan Boster Biological Technology, Ltd.) at room temperature. The IHC staining was independently scored by two researchers using the average percentage of stained cells: i) 0 points, <5% of cells (no staining); ii) 1 point, 5-25% of cells (mild staining); iii) 2 points, 25-50% of cells (moderate staining); and iv) or 3 points, >50% of cells (strong staining).

RT-qPCR. Total RNA was extracted using a RNeasy Mini kit (Qiagen China Co., Ltd.) and reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The primer sequences used were as follows: Dll4 forward, 5'-AGCTGT AAGGACCAGGAG-3' and reverse, 5'-ACATTCACAAGC ATAGTTGG-3'; Notch1 forward, 5'-GAGGCGTGGCAGACT ATGC-3' and reverse, 5'-CTTGTACTCCGTCAGCGTGA-3'; transcription factor HES-1 (Hes1) forward, 5'-ACGTGCGAG GGCGTTAATAC-3' and reverse, 5'-GGGGTAGGTCAT GGCATTGA-3'; hairy/enhancer-of-split related with YRPW

motif protein 1 (Heyl) forward, 5'-GAAGTTGCGCGTTAT CTGAGC-3' and reverse, 5'-ATGCGAAACCAGTCGAAC TCG-3'; MMP9 forward, 5'-GGGACGCAGACATCGTCA TC-3' and reverse, 5'-TCGTCATCGTCGAAATGGGC-3'; C-X-C chemokine receptor type 4 (CXCR4) forward, 5'-GGG CAATGGATTGGTCATCCT-3' and reverse, 5'-TGCAGCCTG TACTTGTCCG-3'; Twist-related protein 1 (Twist1) forward, 5'-GCCTAGAGTTGCCGACTTATG-3' and reverse, 5'-TGC GTTTCCTGTTAAGGTAGC-3'; vascular endothelial growth factor A (VEGFA) forward, 5'-GGACCCTGGCTTTACTGC TGTACC-3' and reverse, 5'-TCACCGCCTTGGCTTGTC ACA-3'; hypoxia-inducible factor 1-α (HIF-1α) forward, 5'-TGC TCATCAGTTGCCACTTCC-3' and reverse, 5'-CGCTGTGTG TTTTGTTCTTTACCC-3'; GADPH forward, 5'-ACAACTTTG GTATCGTGGAAGG-3' and reverse, 5'-GCCATCACGCCA CAGTTTC-3'. The RT-qPCR reactions were performed using SYBR-Green (Roche Diagnostics) at the following thermocycling conditions: Preincubation at 95˚C for 30 sec, followed by 45 cycles of 5 sec at 95˚C, 10 sec at 55˚C, and 25 sec at 72˚C. The sample values were normalized to the housekeeping gene (GAPDH) and calculated using the $2^{-\Delta\Delta Cq}$ method (20).

Migration and invasion assays. The cells were seeded in 6-well plates at a density of $1.5x10^5$ cell/well (at 50% confluence), and then transfected with Dll4 siRNA or control siRNA. After 24 h, the cells were cultured in serum-free medium, and wounds were created by scraping each plate with a 200-μl pipette tip. Images of identical scratch regions were captured at various time points using a phase contrast microscope (Olympus IX 71; Olympus Corporation). The wound healing area of five random scopes was quantified using ImageJ software (version 1.52a; National Institutes of Health). Invasion assays were performed using Transwell chambers (8-μm pore size; Costar; Corning Inc.) that were coated with 100 μl Matrigel for 30 min at 37˚C. A total of $5-6x10^4$ cells were seeded into the upper compartment with 200 ml serum-free medium and the chambers were then placed in 24-well dishes containing 700 μl DMEM with 10% FBS in the lower chamber. After 24 h or 48 h of co-culture in an incubator at 37˚C, the membranes were fixed with 4% formaldehyde for 20 min at room temperature, and stained with 10% Giemsa staining solution for 30 min at room temperature. After removing the cells from the membrane's upper surface, the cells on the lower membrane surface of five random scopes (magnification, x10) were counted under a microscope (Olympus IX 71; Olympus Corporation).

Tube formation assay. Conditioned medium (CM) was harvested 48 h after SACC cells were transfected with Dll4 siRNA. Then, the 48-well plates were coated with Matrigel (150 μl per well) and HUVECs were added ($4x10^4$ cells/well). After incubation at 37˚C for 6 h, tube formation was observed under a microscope (Olympus IX 71; Olympus Corporation), and the number of nodes as well as meshes of five random scopes were quantified (magnification, x10) by ImageJ software (version 1.52a; National Institutes of Health).

Cell co-culture. The SACC-83 or SACC-LM cells were seeded in 6-well plates at a density of $1.5x10^5$ cells/well and transfected with Dll4 siRNA or Control siRNA. HUVECs were seeded into the upper chamber of the Transwell (0.4 μm; EMD Millipore) co-culture system at the same density of SACC cells. Then, the upper and lower compartment were combined for 24-48 h, followed by subsequent assays.

3D culture. Multicellular spheroids were generated using the hanging drop technique or by plating the cells on 96-well ultra-low attachment plates (Corning, Inc.) or soft agar-coated 96-well plates. For the hanging drop method, the cells were re-suspended in DMEM supplemented with 10% methylcellulose at a density of $5x10^4$ cells/ml, and incubated overnight in 25 μl droplets as previously described (21). For the other methods, 1% agar was used to coat 96-well plates, the cells were re-suspended in 200 μl DMEM at a density of $5x10^4$ cells/ml, and then the cells were seeded into 96-well ultra-low attachment plates or soft agar-coated plates. The cells were cultured for 48-72 h to ensure multicellular aggregation, and then the spheroids were incorporated into a rat tail collagen solution (cat. no. C8062; Beijing Solarbio Science & Technology Co., Ltd.) before collagen polymerization occurred. The DMEM was then added and the plates were incubated at 37˚C for 24-48 h. Leader and follower cells were defined based on their positions in the cell cluster (at the front of the cluster and at the back of the cluster) (3). Leader cells appeared as protrusive cells at the front of invasive strands. After 4 days, the plates were scanned and the relative diameters of the well and the gel were measured using ImageJ software (version 1.52a; National Institutes of Health).

Statistical analysis. All statistical analyses were performed using SPSS software (version 22.0; IBM Corp.) and GraphPad Prism software (version 5.03; GraphPad Software, Inc.). Continuous data were reported as the mean ± standard deviation and analysed using unpaired Student's t-test (comparisons between 2 groups) or using one-way analysis of variance with Dunnett's post hoc multiple comparisons (comparisons between ≥3 groups). The correlation between the expression of Dll4 and Notch1 was assessed with a Spearman's correlation coefficient. A Chi-squared test was used to evaluate whether expression of Dll4 or Notch1 was associated with the patients' clinicopathological characteristics. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Increased expression of Dll4 at the invasion front is associated with metastasis and recurrence of SACC. The significance of Dll4 and Notch1 expression in human SACC tissues was evaluated using IHC. The results revealed that Dll4 staining was predominantly observed in the cytoplasm and membrane and Notch1 staining was observed in the cytoplasm, membrane and nucleus (Fig. 1A). Furthermore, Dll4-positive staining was observed in 46 of the 84 patients with SACC (54.76%) and Notch1-positive staining was observed in 37 patients (44.04%). Of note, Dll4 expression was much higher in tumour cells at the invasive front (vs. at the centre of the SACC mass; $P=0.001$; Fig. 1B) and there was a close correlation between Dll4 and Notch1 expression at the invasive front ($r=0.74$, $P<0.001$; Fig. 1C).

Tables I and II show the relationships between Dll4/Notch1 expression and the patients' clinicopathological characteristics.

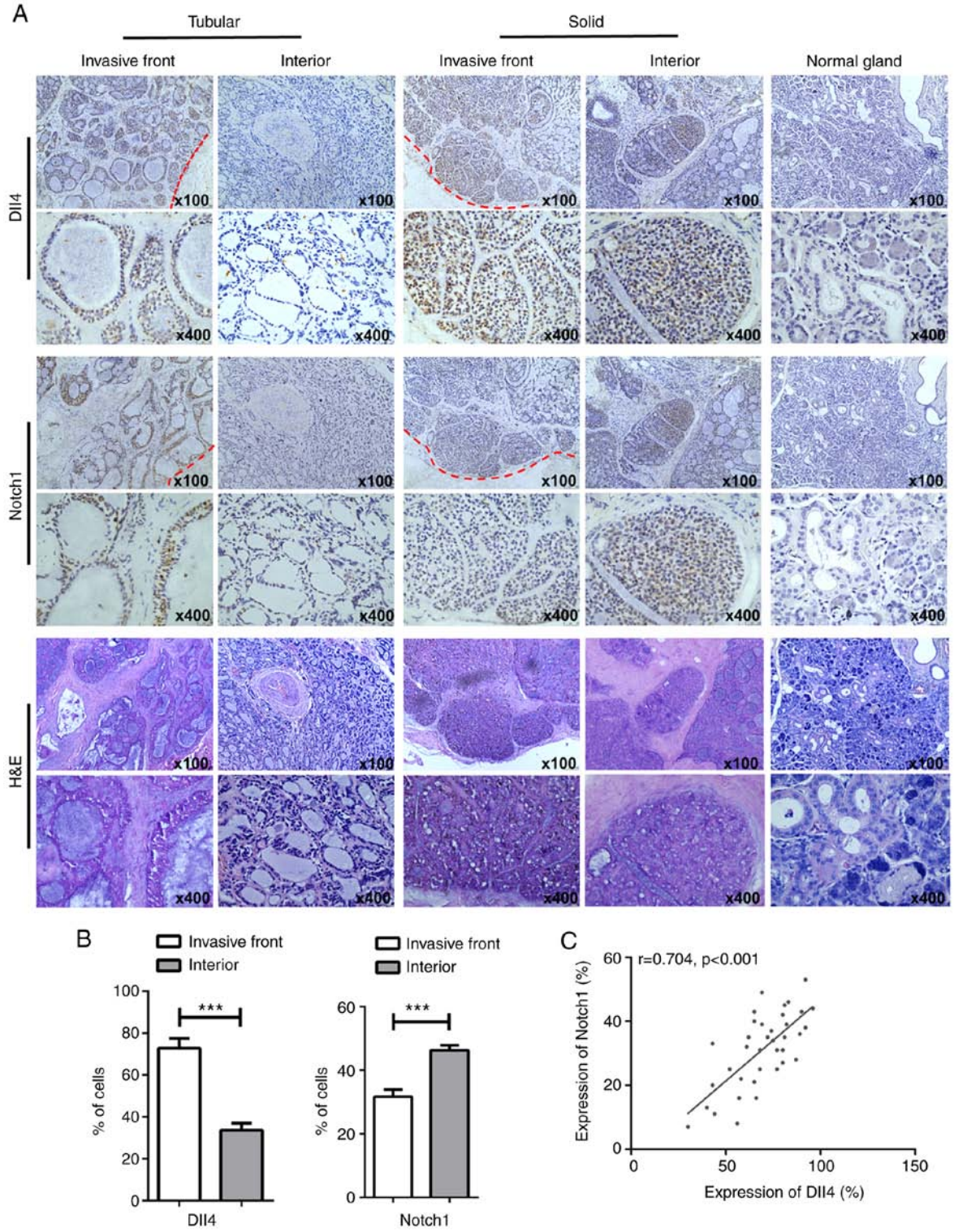

Figure 1. Immunohistochemical expression of Dll4 and Notch1 in the central and invasive areas of SACC tissues. (A) Representative images of Dll4 and Notch1 expression at the invasive front and interior of SACC tissues, with a normal gland used as a negative control. The red dotted line indicates the boundary between the tumour invasive front and the peri-carcinomatous tissue. (B) The percentage of tumour cells with Dll4 expression and the percentage of tumour cells with Notch1 expression at the invasive front and interior of SACC tissues. (C) Spearman's correlation coefficient was performed to analyse the correlation between Dll4 and Notch1 expression at the collective invasive front of SACC (r=0.704, P<0.001). ***P<0.001. SACC, salivary adenoid cystic carcinoma; Dll4, δ-like ligand 4; H&E, haematoxylin and eosin.

High expression of Dll4 and Notch1 at the invasive front was significantly related to solid tumour type, high TNM grade, and high rates of metastasis and recurrence (all P<0.05), but not with age (P=0.794 for Dll4 and P=0.922 for Notch1), sex (P=0.752 for Dll4 and P=0.703 for Notch1) or tumour site (P=0.875 for Dll4 and P=0.603 for Notch1). Thus, Dll4 and Notch1 expression appears to play important roles at the invasive front of SACC.

Dll4 is primarily expressed in leader cells and Notch1 is mainly expressed in follower cells. Multicellular spheroids

Table I. Association between Dll4 expression and clinicopathological characteristics of patients with SACC.

Parameter	N (%)	Dll4 expression, n (%)		P-value
		Low	High	
Sex				0.752
Male	36 (42.86)	17 (44.70)	19 (41.30)	
Female	48 (57.14)	21 (55.30)	27 (58.70)	
Age, years				0.794
≥60	54 (65.79)	25 (65.79)	29 (63.04)	
<60	30 (34.21)	13 (34.21)	17 (36.96)	
Tumour location				0.875
Major salivary	39 (46.43)	18 (47.37)	21 (45.65)	
Minor salivary	45 (53.57)	20 (52.63)	25 (54.35)	
Pathological type				0.030[a]
Cribriform/tubular	61 (72.62)	32 (84.21)	29 (63.04)	
Solid	23 (27.38)	6 (15.79)	17 (36.96)	
TNM stage				0.007[a]
I-II	27 (32.14)	18 (47.37)	9 (19.57)	
III-IV	57 (67.86)	20 (52.63)	37 (80.43)	
Distant metastasis				0.027[a]
Yes	18 (21.43)	4 (10.53)	14 (30.43)	
No	66 (78.57)	34 (89.47)	32 (69.57)	
Recurrence				0.030[a]
Yes	15 (17.86)	3 (7.89)	12 (26.09)	
No	69 (82.14)	35 (92.11)	34 (73.91)	

[a]P<0.05. SACC, salivary adenoid cystic carcinoma; Dll4, δ-like ligand 4; TNM, Tumour Node Metastasis.

were generated using the hanging drop technique, agar-coated suspension technique, and ultra-low 96-well attachment plates, and then embedded in extracellular matrix or collagen I. After 24-72 h, several invasive multicellular strands had extended from the spheroid margin, which simulated *in vitro* collective invasion (Fig. 2A), similar to that also observed in our previous study (12). IFC staining revealed that leader cells at the invasive front expressed Dll4, while follower cells within the multicellular spheroids expressed Notch1 (Fig. 2B). Thus, Dll4 may be a marker for leader cells and Notch1 may be a marker for follower cells in the collective invasion of SACC.

Increased extracellular matrix stiffness may promote SACC collective invasion and enhance Dll4/Notch1 expression. The stiffness of the 3D culture model was gradually increased by decreasing the proportion of Matrigel and increasing the proportion of collagen I. Greater stiffness was associated with an increased number and range of invasive branches, as well as increased expression of Dll4 and Notch1 based on IFC staining (Fig. 3). These findings indicated that increased extracellular matrix stiffness may promote collective invasion and increase Dll4/Notch1 expression in SACC cells.

Knockdown of Dll4 inhibits the migratory, invasive and pro-angiogenic abilities of SACC cells. The three siRNA duplexes against Dll4 were characterized and the siRNA with the highest efficiency was used for subsequent experiments (Fig. 4A). Wound healing and Transwell assays revealed that transfection with Dll4 siRNA-3 reduced the migratory and invasive abilities of the SACC cells, relative to cells that were transfected with control siRNA (Fig. 4B and C). Additionally, the tube formation assay indicated that Dll4 siRNA-3-transfected SACC cells induced less microvessel formation of HUVECs (Fig. 4D). Moreover, Dll4 siRNA-3-transfected SACC cells had decreased mRNA expression levels of Notch1, Hey1, Hes1, Twist1, MMP9 and CXCR4 (Fig. 4E). Therefore, Dll4 knockdown appeared to inhibit the migratory, invasive and pro-angiogenic capabilities of SACC cells.

Hypoxia promotes SACC collective invasion by modulating the Dll4/Notch1 signalling pathway. A hypoxic environment is a common characteristic of solid tumours and contributes to the invasion and metastasis of tumour cells (22). Thus, whether hypoxia could regulate SACC collective invasion by modulating the Dll4/Notch1 signalling pathway was analysed. Under hypoxic conditions, it was observed that the spheroids developed multicellular strands from their margins, which exhibited mesenchymal features. IFC staining suggested that Dll4 and Notch1 were highly expressed in the invading cells, especially in the leader cells. However, siRNA silencing of Dll4 reduced the crab-like invasive processes of the leader cells and decreased the expression of Dll4 and Notch1 (Fig. 5A).

Table II. Association between Notch1 expression and clinicopathological characteristics of patients with SACC.

Parameter	N (%)	Notch1 expression, n (%)		P-value
		Low	High	
Sex				0.703
Male	36 (42.86)	21 (44.68)	15 (40.54)	
Female	48 (57.14)	26 (55.32)	22 (59.46)	
Age, years				0.922
≥60	54 (65.79)	30 (63.83)	24 (64.86)	
<60	30 (34.21)	17 (36.17)	13 (35.14)	
Tumour location				0.603
Major salivary	39 (46.43)	23 (48.93)	16 (43.24)	
Minor salivary	45 (53.57)	24 (51.07)	21 (54.35)	
Pathological type				0.016[a]
Cribriform/tubular	61 (72.62)	39 (82.98)	22 (59.46)	
Solid	23 (27.38)	8 (17.02)	15 (40.54)	
TNM stage				0.021[a]
I-II	27 (32.14)	20 (42.55)	7 (18.92)	
III-IV	57 (67.86)	27 (57.45)	30 (81.08)	
Distant metastasis				0.029[a]
Yes	18 (21.43)	6 (12.77)	12 (32.43)	
No	66 (78.57)	41 (87.23)	25 (68.57)	
Recurrence				0.012[a]
Yes	15 (17.86)	4 (8.51)	11 (29.73)	
No	69 (82.14)	43 (91.49)	26 (70.27)	

[a]P<0.05. SACC, salivary adenoid cystic carcinoma; TNM, Tumour Node Metastasis.

The hypoxic conditions appeared to enhance the migratory and invasive abilities of SACC cells, which could be partially inhibited by silencing Dll4 (Fig. 5B and C). Furthermore, under hypoxic conditions, Dll4/Notch1 and their downstream molecules (Hey1, Hes1, HIF-1α, MMP9 and Twist1) expression levels were increased, but lower expression levels were observed in SACC cells transfected with Dll4 siRNA (Fig. 5D). These results implied that the Dll4/Notch1 signalling pathway was modulated by hypoxia to contribute to the collective invasion of SACC.

Co-culture with HUVECs promotes collective invasion in SACC by regulating the Dll4/Notch1 signalling pathway. Previous research has suggested that Dll4 could mediate the HUVEC phenotype and promote mature vessel formation (23). Thus, whether HUVECs could also modulate Dll4/Notch1 expression and SACC collective invasion was investigated. Co-culture with HUVECs upregulated the expression of Dll4 and Notch1 in SACC cells, and led to enhanced migratory and invasive abilities. Furthermore, increased expression levels of Dll4/Notch1 and their downstream molecules (Hey1, Hes1, VEGFA, MMP9 and Twist1) were also observed in SACC cells co-cultured with HUVECs (Fig. 6B-D). Silencing of Dll4 partially inhibited the crab-like invasive processes of the leader cells during co-culture with HUVECs, reduced the migratory and invasive abilities of cells, and downregulated the expression of Hey1, Hes1, VEGFA, MMP9 and Twist1 (Fig. 6A-D). These results implied that the Dll4/Notch1 signalling pathway was important in HUVEC-related induction of SACC collective invasion.

Discussion

Unlike the classic single-cell invasion that accompanies the EMT, collective invasion plays an important role in the invasion and metastasis of various epithelial tumours (24,25). Early research regarding this topic used H&E staining of SACC specimens and IHC staining for EMT-related molecules, which revealed that collective invasion was an important mechanism for SACC invasion (12,26). The present study revealed that Dll4 and Notch1 were highly expressed at the invasive front of SACC, while knockdown of Dll4 inhibited SACC collective invasion and reduced the *in vitro* migratory and invasive abilities of SACC cells. Furthermore, greater extracellular matrix stiffness, hypoxia and co-culture with HUVECs appeared to promote SACC collective invasion by upregulating Dll4. Therefore, the present results suggested that Dll4 and Notch1 facilitated the collective invasion of SACC cells.

Initially, the expression of Dll4 and Notch1 in SACC tissues was evaluated, which revealed that Dll4 and Notch1 were highly expressed at the invasive front of SACC specimens. In addition, higher expression of Dll4 and Notch1 at the invasive

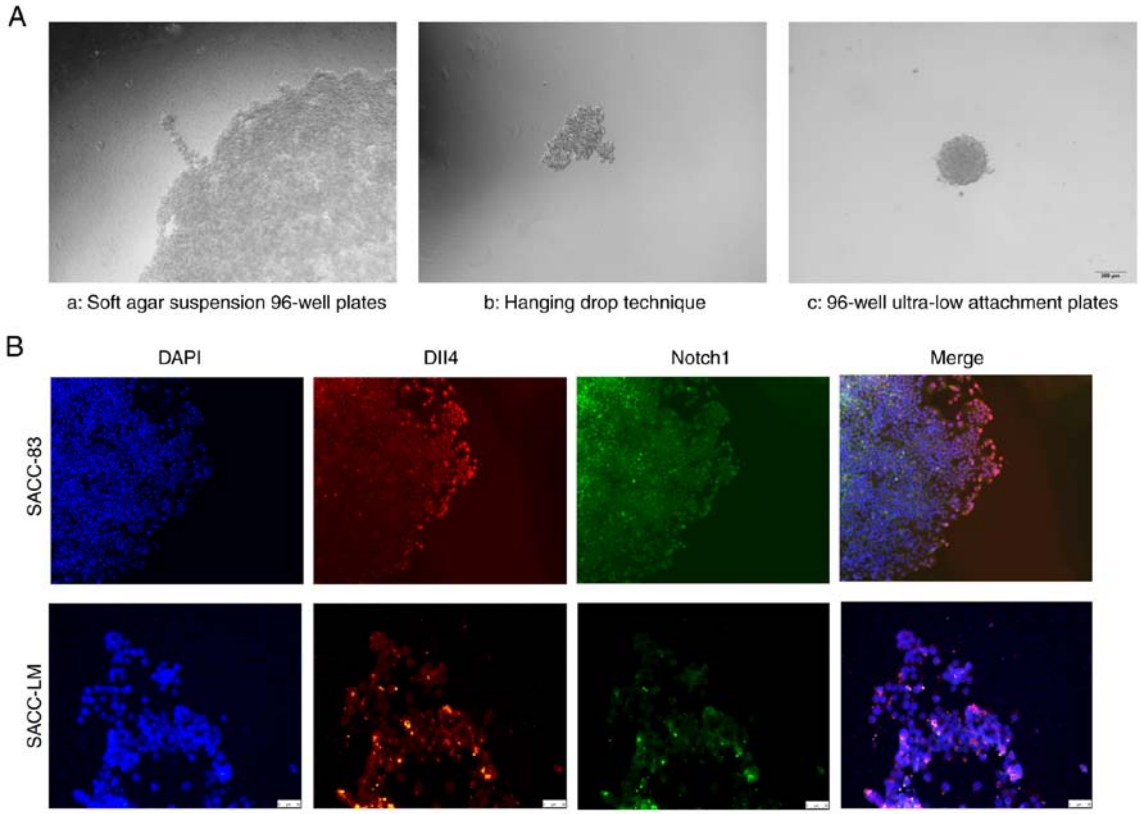

Figure 2. Dll4 and Notch1 expression can be used as markers of leader and follower SACC cells as determined using a 3D culture. (A) SACC cells were subjected to three methods of 3D culture: (Aa) Soft agar suspension in 96-well plates (magnification, x200), (Ab) the hanging drop technique (magnification, x40) and (Ac) using 96-well ultra-low attachment plates (magnification, x40). (B) Immunofluorescence results are shown for Dll4 expression (red) and Notch1 expression (green) in the 3D cultures (magnification, x200). SACC, salivary adenoid cystic carcinoma; Dll4, δ-like ligand 4; 3D, three-dimensional.

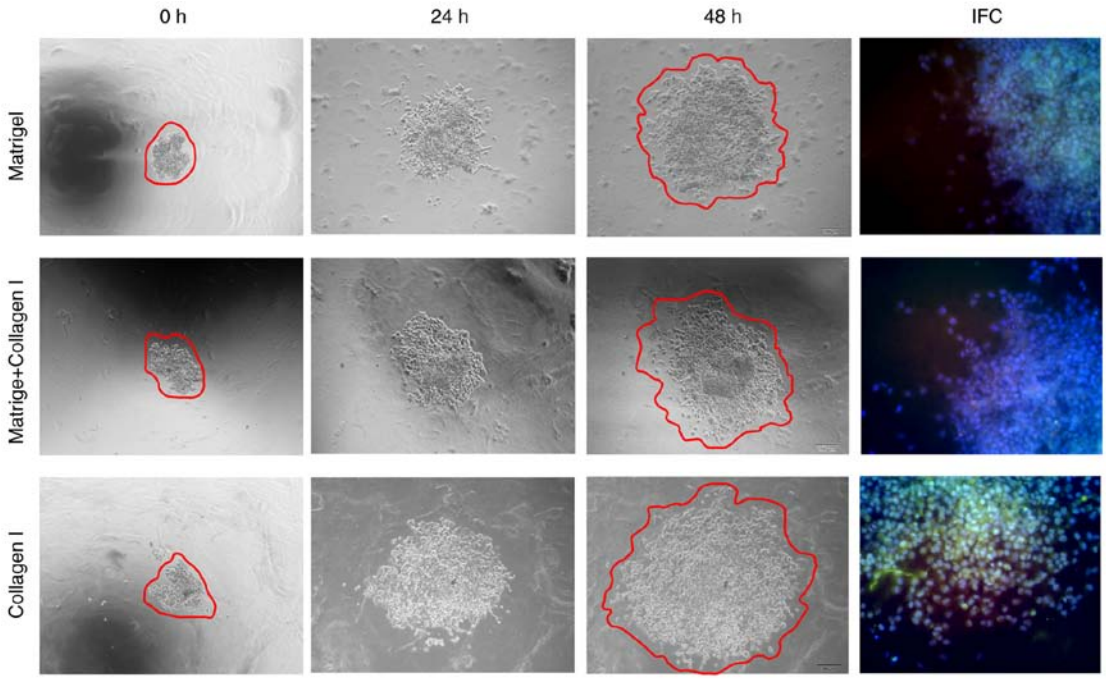

Figure 3. Effects of gel stiffness on SACC collective invasion and Dll4/Notch1 expression. The SACC-83 sphere in three-dimensional culture was examined at various gel stiffness levels, and the invasion area is indicated by the red line. IFC results are shown for Dll4 and Notch1 expression according to the different gel stiffness levels (magnification, x40). SACC, salivary adenoid cystic carcinoma; Dll4, δ-like ligand 4; IFC, immunofluorescence.

front was associated with higher TNM staging and higher rates of metastasis and recurrence. Similarly, Torab *et al* (27) reported that Dll4 was upregulated in leader cells using a 3D microtumour invasion model and specimens from patients with

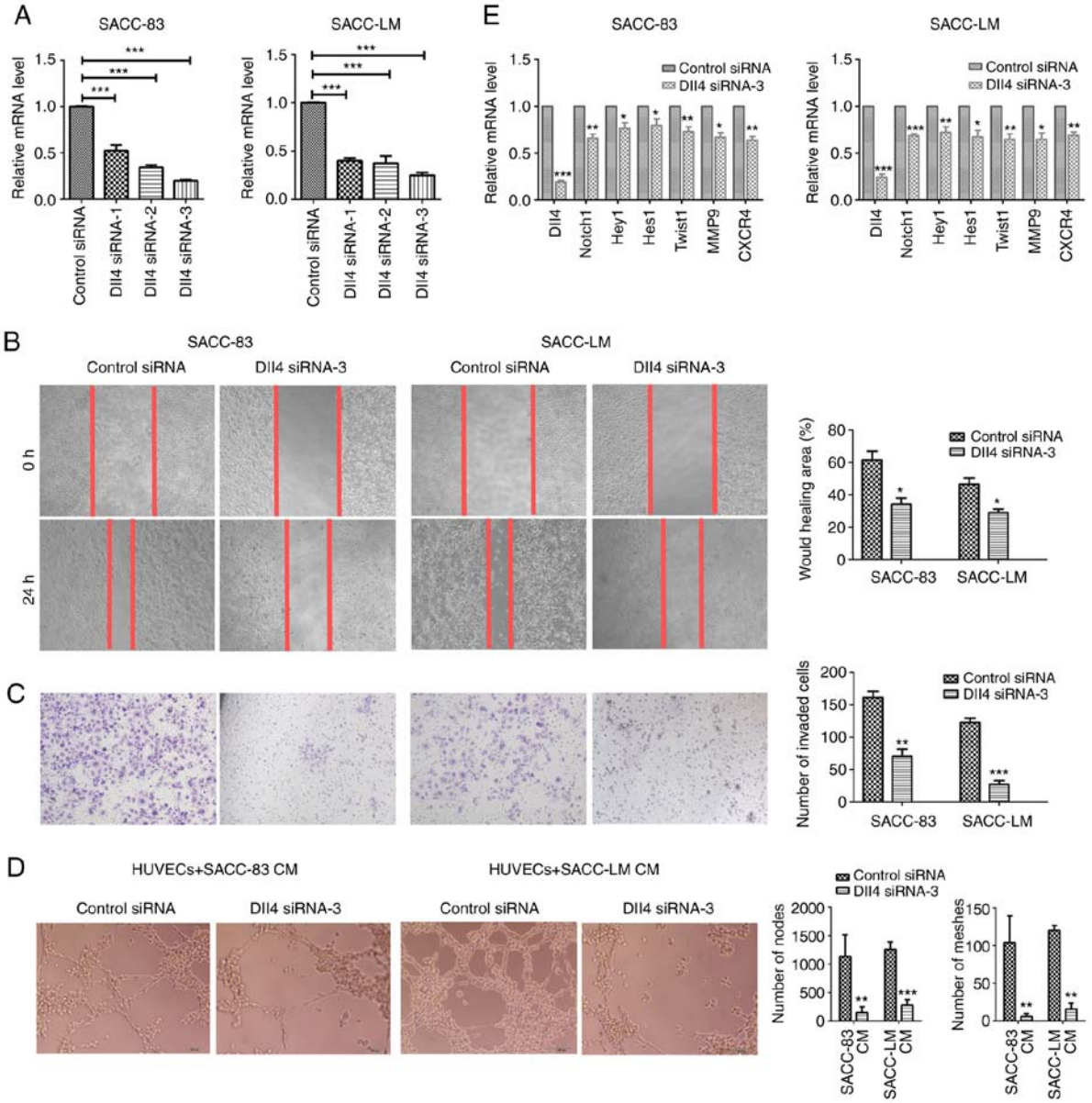

Figure 4. Dll4 knockdown inhibits the migration and invasion of SACC cells. (A) The relative Dll4 mRNA expression in transfected cells was evaluated using RT-qPCR. (B) The migration of SACC cells transfected with Dll4 siRNA-3 or control siRNA was determined using a wound healing assay (magnification, x100). (C) The invasion of SACC cells transfected with Dll4 siRNA-3 or control siRNA was measured using a Transwell assay (magnification, x100). (D) The tube formation ability of HUVECs treated with CM of SACC cells was quantified by measuring the number of nodes and the number of meshes (magnification, x100). (E) In SACC cells, RT-qPCR revealed that Dll4 knockdown was associated with the downregulation of Dll4, Notch1, Hey1, Hes1, Twist1, MMP9 and CXCR4 mRNA expression. The data are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. control siRNA or as indicated. SACC, salivary adenoid cystic carcinoma; Dll4, δ-like ligand 4; CM, conditioned medium; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; siRNA, small interfering RNA; HUVECs, human umbilical vein endothelial cells; Hey1, hairy/enhancer-of-split related with YRPW motif protein 1; Hes1, transcription factor HES-1; Twist1, Twist-related protein 1; CXCR4, C-X-C chemokine receptor type 4.

bladder cancer. Another study revealed that leader cells could be identified based on Dll4 expression using a computational model, and that creating a cell-free region reduced mechanical stress, induced Dll4 expression, and induced leader cell formation during collective cell migration (15). These results suggested that the Dll4/Notch1 signalling pathway may play an important role in the collective invasion of SACC, and that Dll4/Notch1 expression might be useful for predicting the prognosis of patients with SACC.

3D models of SACC collective invasion were created using agarose suspension culture, hanging drop culture, and ultra-low adhesion 96-well plate suspension culture. The IFC results revealed high expression of Dll4 in the leader cells and high expression of Notch1 in the follower cells. Furthermore, silencing of Dll4 using siRNA reduced the migratory, invasive and pro-angiogenic abilities of SACC-83 and SACC-LM cells. Another study suggested that downregulating Dll4 notably decreased the invasion, migration and metastatic properties of oesophageal cancer cells via the PI3K/Akt/E-cadherin pathway (28). Downregulation of Dll4/Notch4 has also been reported to reduce endocrine therapy resistance and metastasis in breast cancer (29). Moreover, it was observed in the present study that knockdown of Dll4 downregulated the expression of Hey1, Hes1, MMP9, CXCR4 and Twist1, which are downstream

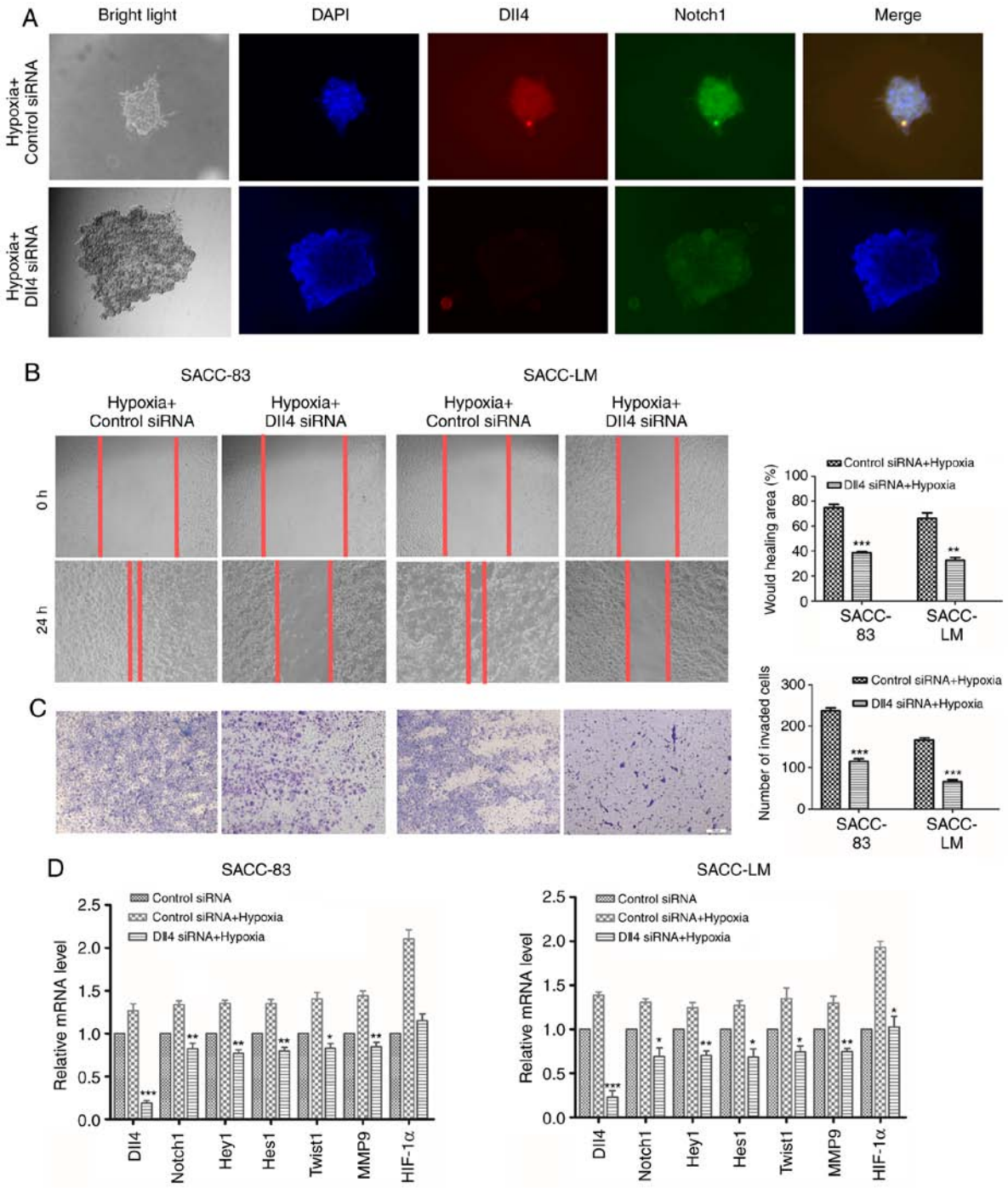

Figure 5. Hypoxia regulates SACC collective invasion via the Dll4/Notch1 signalling pathway. (A) Immunofluorescence results for Dll4 and Notch1 expression in SACC cells with or without Dll4 knockdown under hypoxia (magnification, x40). (B) The migration of SACC cells transfected with Dll4 siRNA-3 or control siRNA under hypoxia (magnification, x100). (C) The invasion of SACC cells transfected with Dll4 siRNA-3 or control siRNA under hypoxia (magnification, x100). (D) The relative mRNA expression levels of Dll4, Notch1, Hey1, Hes1, Twist1, MMP9 and HIF1-α in SACC cells transfected with Dll4 siRNA-3 or control siRNA under hypoxia. The data are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. control siRNA + Hypoxia. SACC, salivary adenoid cystic carcinoma; Dll4, δ-like ligand 4; siRNA, small interfering RNA; Hey1, hairy/enhancer-of-split related with YRPW motif protein 1; Hes1, transcription factor HES-1; Twist1, Twist-related protein 1; HIF1-α, hypoxia-inducible factor 1-α.

factors in the Dll4/Notch1 signalling pathway. These results also indicated that Dll4 contributed to the collective invasion of SACC cells.

A hypoxic environment induces tumour invasion and metastasis in several cancer types, including SACC (30,31). Thus, it was evaluated whether the Dll4/Notch1 signalling pathway promoted SACC collective invasion under hypoxic conditions. The results indicated that hypoxic conditions increased and elongated the long fusiform and crab claw-like invasive processes of leader cells, which suggested that hypoxic conditions promoted the collective invasion of SACC cells. Furthermore, the migratory and invasive abilities of SACC-LM were enhanced under hypoxic conditions, while silencing of Dll4 using siRNA partially mitigated the pro-invasive effects of the hypoxic conditions. Co-culture with HUVECs also upregulated Dll4/Notch1 expression in the leader and follower

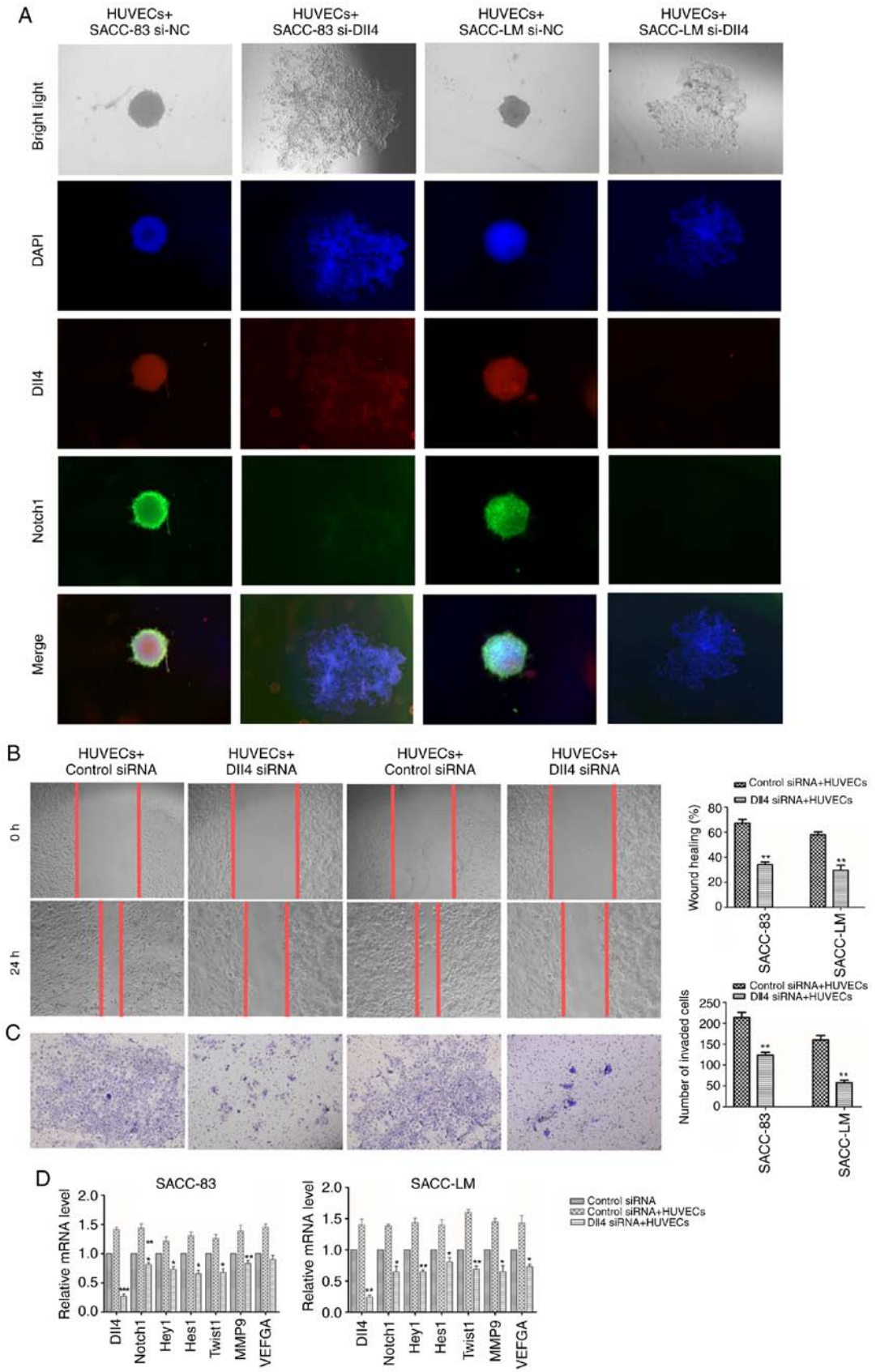

Figure 6. HUVECs regulates SACC collective invasion via the Dll4/Notch1 signalling pathway. (A) Immunofluorescence results for Dll4 expression (red) and Notch1 expression (green) in SACC cells transfected with Dll4 siRNA-3 or control siRNA during co-culture with HUVECs (magnification, x40). (B) The migration of SACC cells transfected with Dll4 siRNA-3 or control siRNA during co-culture with HUVECs (magnification, x100). (C) The invasion of SACC cells transfected with Dll4 siRNA-3 or control siRNA during co-culture with HUVECs (magnification, x100). (D) The relative mRNA expression levels of Dll4, Notch1, Hey1, Hes1, Twist1, MMP9 and VEGFA in SACC cells transfected with Dll4 siRNA-3 or control siRNA during co-culture with HUVECs. The data are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. control siRNA + HUVECs. SACC, salivary adenoid cystic carcinoma; Dll4, δ-like ligand 4; siRNA, small interfering RNA; HUVECs, human umbilical vein endothelial cells; Hey1, hairy/enhancer-of-split related with YRPW motif protein 1; Hes1, transcription factor HES-1; Twist1, Twist-related protein 1.

cells, while knockdown of Dll4 decreased the crab-like invasive processes of leader cells in the HUVEC co-culture. Using a two-dimensional cell culture system, SACC cell migration and invasion was markedly enhanced during HUVEC co-culture, although Dll4 silencing partially inhibited the SACC cells' enhanced migration, invasion and expression of VEGFA. Mendonça *et al* (32) demonstrated that mice with endothelial-specific Dll4 loss-of-function mutations had increased tumour hypoxia, but also a significantly decreased the number and burden of macro-metastases, while Dll4/Notch signalling appeared to mediate the tumour hypoxia-driven increase in EMT. When considered with the present results, it appears that hypoxia and HUVECs might promote SACC collective invasion via the Dll4/Notch1 signalling pathway.

In conclusion, Dll4 and Notch1 were highly expressed in leader and follower cells, and appeared to play important roles in SACC collective invasion. Furthermore, increased extracellular matrix stiffness, hypoxia and co-culture with HUVECs appeared to promote SACC collective invasion by upregulating Dll4. These results suggested that Dll4 expression may define the leader cells in SACC and may be required for collective cell invasion, which also involves Notch1. Therefore, Dll4 might be a novel target for SACC treatment. Further studies should be performed using more surgical specimens from a range of clinic centres to improve the validity of the present results.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KW performed most of the experiments and wrote the manuscript. HYF, XP and MZ assisted in experiments. XHY, JSW, BJC and JJ analyzed the data, assisted in manuscript writing and confirmed the authenticity of all the raw data. XHL and YLT conceived the study and performed the final corrections. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

The samples and clinical data were obtained with informed consent from the patients, and the protocols were approved by the Institutional Ethics Committee of the West China Medical Center, Sichuan University (Chengdu, China; approval no. WCHSIRB-D-2017-208).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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