MYB promotes the growth and metastasis of salivary adenoid cystic carcinoma

LI-HUA XU[1], FEI ZHAO[1], WEN-WEN YANG[1], CHU-WEN CHEN[1], ZHI-HAO DU[1], MIN FU[1], XI-YUAN GE[1,2] and SHENG-LIN LI[1,2]

[1]Central Laboratory, Peking University School and Hospital of Stomatology, National Clinical Research Center for Oral Diseases, National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing Key Laboratory of Digital Stomatology; [2]Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, Beijing 10081, P.R. China

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Abstract. The incidence of recurrent t(6;9) translocation of the *MYB* proto-oncogene to *NFIB* (the gene that encodes nuclear factor 1 B-type) in adenoid cystic carcinoma (ACC) tumour tissues is high. However, *MYB* [the gene that encodes transcriptional activator Myb (MYB)] overexpression is more common, indicating that MYB serves a key role in ACC. The current study aimed to investigate the role of MYB in salivary (S)ACC growth and metastasis. A total of 50 fresh-frozen SACC tissues and 41 fresh-frozen normal submandibular gland (SMG) tissues were collected to measure *MYB* mRNA expression, and to analyse the associations between *MYB* and epithelial-mesenchymal transition (EMT) markers. Compared with normal SMG tissue, SACC tissues demonstrated significantly increased *MYB* expression, with a high expression rate of 90%. Interestingly, *MYB* tended to be negatively correlated with *CDH1* [the gene that encodes cadherin-1 (E-cadherin)] and positively correlated with *VIM* (the gene that encodes vimentin), suggesting that MYB is associated with SACC metastasis. To explore the role of MYB in SACC, the authors stably overexpressed and knocked down *MYB* in SACC cells. The authors of the current study demonstrated that *MYB* overexpression promoted SACC cell proliferation, migration and invasion, whereas its knockdown inhibited these activities. Additionally, when *MYB* was overexpressed, *CDH1* expression was downregulated, and *CDH2* (the gene that encodes cadherin-2), *VIM* and *ACTA2* (the gene that encodes actin, aortic smooth muscle) expression was upregulated. Then, the effect of *MYB* on lung tumour metastasis was investigated *in vivo* in non-obese diabetic/severe combined immunodeficiency mice. *MYB* overexpressing and control cells were injected into the mice through the tail vein. The results revealed that MYB promoted SACC lung metastasis. Collectively, these results demonstrated that *MYB* is aberrantly overexpressed in SACC tissues, and promotes SACC cell proliferation and metastasis, indicating that MYB may be a novel therapeutic target for SACC.

Introduction

Salivary adenoid cystic carcinoma (SACC) is one of the most common malignancies of the salivary glands and is characterized by slow indolent growth, frequent local recurrences, a high incidence of metastasis and a poor prognosis (1-3). A total of 40-60% of patients with SACC suffer distant metastasis even when treated with surgical resection and intensive radiotherapy (4,5). The lung is the most common distant metastasis site, followed by bone, liver and brain (6). However, no effective treatment is available to inhibit or reduce lung metastases (6-8). Thus, there is an urgent need to find novel treatments to suppress SACC tumour metastasis, especially lung metastases.

MYB [the gene that encodes transcriptional activator Myb (MYB)]-*NFIB* (the gene that encodes nuclear factor 1 B-type) fusion occurred in 119/232 (51%) of SACCs, and *MYB* mRNA overexpression was detected in 119/136 (88%) of SACCs (9-15), indicating that MYB may serve an important role in the occurrence and development of SACC. MYB is associated with the development of tumours, including leukaemia, pancreatic cancer, breast cancer and prostate cancer (16-18). However, whether MYB is associated with the development and metastasis of SACC is not clear (19).

Correspondence to: Dr Xi-Yuan Ge or Professor Sheng-Lin Li, Central Laboratory, Peking University School and Hospital of Stomatology, National Clinical Research Center for Oral Diseases, National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing Key Laboratory of Digital Stomatology, 22 South Zhongguancun Avenue, Beijing 100081, P.R. China
E-mail: gexiyuan@bjmu.edu.cn
E-mail: kqshlli@bjmu.edu.cn

Abbreviations: MYB, transcriptional activator Myb; *CCND1*, the gene encoding G1/S-specific cyclin-D1; ACC, adenoid cystic carcinoma; EMT, epithelial-mesenchymal transition; SMG, submandibular gland; *p21*, the gene encoding cyclin-dependent kinase inhibitor 1; *ICAM1*, the gene encoding intercellular adhesion molecule 1; VEGF, vascular endothelial growth factor A

Key words: adenoid cystic carcinoma, transcriptional activator Myb, lung metastasis, epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a typical event in SACC metastasis (20,21). Changes in cellular morphology and phenotypic characteristics facilitate epithelial cell transformation into cells with mesenchymal features, which gain an invasive phenotype and stronger motility (22-24). During EMT, the expression of cell adhesion molecules, such as cadherin-1 (E-cadherin, encoded by *CDH1*), decrease, and the expression of mesenchymal markers, including vimentin (encoded by *VIM*) and cadherin-2 (N-cadherin, encoded by *CDH2*), increase (25). MYB has been reported to enhance breast cancer metastasis by activating the Wnt/catenin β-1 (β-catenin)/Axin-2 signalling pathway, which is one of the most important pathways that leads to EMT activation. However, whether MYB is associated with EMT during SACC metastasis is unclear. Based on the aforementioned studies, the authors of the current study aimed to explore the effect of MYB on the development and lung metastasis of SACC, and to analyse the association of MYB and EMT with SACC metastasis.

Materials and methods

Human tissue samples. A total of 10 human SACC tissues and 10 paired normal submandibular gland (SMG) tissues were used for the microarray analysis. They were collected from patients with SACC at the Peking University School and Hospital of Stomatology (Beijing, China) between August 2015 and April 2016. A total of 50 human SACC and 41 SMG tissues collected between June 2008 and September 2013 at the Peking University School and Hospital of Stomatology were also used to analyse *MYB* expression in tissues. Patients had not undergone radiation therapy or chemotherapy. The tumours were classified according to the histological classification of salivary gland tumours proposed by the World Health Organization (26). The clinicopathological data are summarized in Table I. The study was approved by the Ethics Committee of Peking University School and Hospital of Stomatology (permit no. PKUSSIRB-201522040).

Microarray gene expression analysis. Total RNA was extracted from the 10 human SACC and 10 paired SMG tissues using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the manufacturer's protocol. Then the RNA was purified using the RNeasy Midi kit (Qiagen Sciences, Inc., Gaithersburg, MD, USA). Hybridization was performed by Shanghai Biotechnology Corporation (Shanghai, China) using The Shbio Human ceRNA microarray v1.0 (for mRNA/circular RNA/long non-coding RNA detection) and the SurePrint Human miRNA microarray v21.0 (Agilent Technologies, Inc., Santa Clara, CA, USA), which contained 18,853 mRNA probes per array product. Genes with fold changes >2 and $P<0.05$ were identified as differentially expressed genes, of which 15 were identified.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from tissues or cells with the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The PrimeScript™ RT kit (cat. no. DRR037A; Takara Biotechnology Co., Ltd., Dalian, China) was used to synthesize the cDNA using 1 μg total RNA, according to the manufacturer's protocol. All primer sequences (sense/antisense) were as follows: *MYB*: 5'-GCCAATTATCTCCCGAATCGA-3'/5'-ACCAACGT TTCGGACCGTA-3'; *CDH1*: 5'-GACCGGTGCAATCTTC AAA-3'/5'-TTGACGCCGAGAGCTACAC-3'; *vimentin*: 5'-GACGCCATCAACACCGAGTT-3'/5'-CTTTGTCGTTGG TTAGCTGGT-3'; G1/S-specific cyclin-D1 (encoded by *CCND1*): 5'-GATCAAGTGTGACCCGGACT-3'/5'-TCC TCCTCTTCCTCCTCCTC-3'; cyclin-dependent kinase inhibitor 1 (encoded by *p21*): 5'-TGCCCAAGCTCTACCT TCC-3'/5'-CAGGTCCACATG GTCTTCCT-3'; induced myeloid leukemia cell differentiation protein Mcl-1 (encoded by *MCL1*):5'-TGCATCGAACCATTAGCAGA-3'/5'-TCCTGATG CCACCTTCTAGG-3'; intercellular adhesion molecule 1 (encoded by *ICAM1*): 5'-CACAGTCACCTATGGCA ACG-3'/5'-GTGTCTCCTGGCTCTGGTTC-3'; vascular endothelial growth factor A (VEGF, encoded by *VEGFA*): 5'-CTT GCCTTGCTGCTCTACCT-3'/5'-AGCTGCGCTGATAGACA TCC-3'; matrix metalloproteinase-7 (MMP-7, encoded by *MMP7*): 5'-GTCTCGGAGGAGATGCTCAC-3'/5'-GGAATG TCCCATACCCAAAG-3'; MMP9: 5'-GCCTGGCACATAG TAGGCCC-3'/5'-CTTCCTAGCCAGCCGGCATC-3'; and *GAPDH*: 5'-CCATGGAGAAGGCTGGG-3'/5'-CAAAGTTG TCATGGATGACC-3'. Quantification of mRNA expression was performed with the FastStart Universal SYBR Green Master (ROX) reagent (Roche Diagnostics, Basel, Switzerland) using the ABI 7500 Sequence Detection System (Thermo Fisher Scientific, Inc.). The amplification conditions were as follows: 50˚C for 2 min, 95˚C for 10 min, and 40 cycles at 95˚C for 15 sec and 60˚C for 1 min, followed by a dissociation curve stage to check amplification specificity. The mRNA expression levels of the target genes were normalized to *GAPDH* and calculated using the ΔCT and ΔΔCT methods (27).

Cell lines and transfection. The SACC-83 cell line originated from the ACC tissue of a 26-year-old female patient's sublingual gland in November 1983 (28). The SACC-LM cell line exhibited enhanced lung metastatic behaviour and were isolated *in vivo* after injecting SACC-83 cells into the tail veins of immunodeficient mice (21-23). The SACC-83 and SACC-LM cell lines were collected by SLL and kept at Peking University School and Hospital of Stomatology. The pSMG cells were derived from a 4-year-old boy patient's sublingual gland in November 2016 (29). The pSMG cell line was collected by ZHD and kept at Peking University School and Hospital of Stomatology. SACC-83 and SACC-LM cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; both Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere containing 5% CO_2. The pSMG cells were cultured with DMEM/F12 (1:1 mixture; Gibco; Thermo Fisher Scientific, Inc.) containing 2.5% FBS, trace element mix (Gibco; Thermo Fisher Scientific, Inc.), 20 nM sodium selenite, 5 μg/ml transferrin (Gibco; Thermo Fisher Scientific, Inc.), 1.1 μM hydrocortisone, 0.1 μM retinoic acid, 2.0 nM T3, 8.4 ng/ml cholera toxin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 5 μg/ml insulin, 80 ng/ml epidermal growth factor (both Gibco; Thermo Fisher Scientific, Inc.), extra glutamine (final concentration, 5 mM), 50 μg/ml gentamicin sulfate and 1 μg/ml amphotericin B (Gibco; Thermo Fisher Scientific, Inc.).

Table I. Correlation between clinicopathological variables and MYB expression in patients with salivary adenoid cystic carcinoma.

Variables (n)	Total (n)	MYB expression		χ^2	P-value
		Low (n)	High		
Age (years)				0.786	0.375
<42	18	6	12		
≥42	32	7	25		
Sex				1.708	0.191
Male	23	8	15		
Female	27	5	22		
Tumour size				3.814	0.051
<4 cm	31	11	20		
≥4 cm	19	2	17		
Clinical stage				4.372	0.037
I/II	26	10	16		
I II/IV	24	3	21		
Site				1.418	0.234
Major salivary gland	13	5	8		
Minor salivary gland	37	8	29		
Pathological type				7.031	0.030
Cribriform	13	6	7		
Tubular	25	7	18		
Solid	12	0	12		
Lymph node metastasis				0.191	0.662
Absent	44	11	33		
Present	6	2	4		
Perineural invasion				0.082	0.775
Absent	17	4	13		
Present	33	9	24		
Lung metastasis				5.418	0.020
Absent	33	12	21		
Present	17	1	16		
Local regional recurrence				0.090	0.764
Absent	21	5	16		
Present	29	8	21		

MYB, the gene that encodes transcriptional activator Myb.

The lentiviral vector pHBLV-CMV-GFP-T2A-puro, empty (negative control) lentiviral vector and auxiliary transfection reagent, Polybrene, were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The *MYB* overexpressing lentiviral vector or empty lentiviral vector with a virus titre of $1x10^8$ TU/ml were transfected into SACC-83 cells. The multiplicity of infection was 50. After 72 h of transfection, SACC-83 cells were incubated in RPMI-1640 medium containing 3 μg/ml puromycin for 72 h to select cells that had been successfully transfected, and then the cells were cultured in RPMI-1640 medium containing 1.5 μg/ml puromycin for 2 weeks to obtain *MYB* overexpressing (MYB OE) or negative control (NC) SACC-83 cells. The GFP-positive cells were sorted using BD FACS Aria II (BD Bioscience, San Jose, CA, USA) and cultured in RPMI-1640 medium supplemented with 10% FBS at 37˚C in a humidified atmosphere containing 5% CO_2. Monoclonal cell lines that stably overexpressed *MYB* (M1, M2 and M3 cells) and monoclonal cell lines successfully transfected with the empty lentiviral vector (Vector1, Vector2 and Vector3 cells) were obtained. Untransfected SACC-83 cells served as control (BLK) cells. Cell morphology under bright field and fluorescence was captured using an Eclipse TE2000-U fluorescence microscope (Nikon Corporation, Tokyo, Japan).

To knockdown *MYB* expression, SACC-83 and SACC-LM cells were transfected with 50 nM small interfering (si)RNAs specific for *MYB* (siMYB) or negative control siRNAs (siNC; Guangzhou RiboBio Co., Ltd., Guangzhou, China) for 24 h using riboFECT™ CP Transfection kit (cat. no. C10511-1; Guangzhou RiboBio Co., Ltd.) according to the manufacturer's protocol. The *MYB* siRNA sequences are as follows: 5'-GGT CGAACAGGAAGGTTAT-3' (siMYB-1) and 5'-CAACCGA GAATGAGCTAAA-3' (siMYB-2). The NC siRNA sequence is 5'-TTTCTCCGAACGTGTCACG-3' (siNC).

Experimental animals. A total of 13 female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (5-6 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animal experiments complied with the ARRIVE guidelines, and were conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines as well as the EU Directive 2010/63/EU for animal experiments (30-32). These experiments were approved by the Peking University Institutional Animal Care and Use Committee (permit no. LA2015099).

Western blot analysis. SACC-83, NC, MYB OE, siNC, siMYB-1 and siMYB-2 cells were seeded in 100 mm dishes at $2x10^6$ cells/dish and incubated in RPMI-1640 medium supplemented with 10% FBS at 37˚C in a humidified atmosphere containing 5% CO_2 for 48 h. Cells were harvested, washed with ice-cold PBS and dissolved in ice-cold radioimmunoprecipitation assay buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) containing a protease inhibitor cocktail (Applygen Technologies, Inc., Beijing, China). The protein concentration was measured with a Pierce™ Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, Inc.), following the manufacturer's protocol. The protein extracts (40 μg/lane) derived from each sample were separated by SDS-PAGE on 10-15% gels and electroblotted onto polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat milk in TBST (20 mM Tris, 137 mM NaCl and 0.1% Tween-20, pH 7.4) for 1 h at room temperature and then incubated with the following primary antibodies: Anti-MYB (cat. no. ab45150; Abcam, Cambridge,

MA, USA), anti-E-cadherin (cat. no. 3195), anti-N-cadherin (cat. no. 13116), anti-vimentin (cat. no. 5741), anti-actin, aortic smooth muscle (α-SMA, encoded by ACTA2; cat. no. 19245), anti-β-catenin (cat. no. 8480; all Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-β-actin (cat. no. ZM0001; OriGene Technologies, Inc., Beijing, China). Primary antibodies (1:1,000) were incubated for at least 1 h at 4˚C. Then, the membrane was washed extensively with TBST and incubated with secondary antibodies (1:10,000) for 1 h at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit (cat. no. ZB-2301) or anti-mouse (cat. no. ZB-2305; both OriGene Technologies, Inc.) IgG antibodies were used as the secondary antibodies. The immunocomplexes were detected using SuperEnhanced chemiluminescence detection kit (cat. no. P1030-100; Applygen Technologies, Inc.). β-actin was used as the internal control. All bands were quantified using Adobe Photoshop CS5 Extended 12.0.3 x32, which was purchased from Adobe Systems Incorporated (San Jose, CA, USA). The MYB bands of SACC cells used in this experiment exhibited two bands; the two bands were counted in all the statistics. Three independent experiments with three biological replicates each were performed.

Cell proliferation assay. Cell proliferation was assessed by counting viable cells with Cell Counting Kit-8 (CCK8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). SACC-83, NC, MYB OE, Vector3, M1, M2, M3 cells and siMYB-1-, siMYB-2- and control siRNA-transfected cells were seeded into 96-well plates at a density of 3,000 cells/well in 100 μl RPMI-1640 medium. At 24, 48 and 72 h, the absorbance of each well was measured at 450 nm using the ELx808 Absorbance Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). For SACC-83, NC, MYB OE, Vector3, M1, M2, M3 cells, cell proliferation was compared by calculating the relative cell number because the initial density of the seeded cells was difficult to control. Relative cell number = optical density (OD) 450 value per time point / OD 450 values at 0 h. The data presented are representative of one of three replicates.

In vitro wound closure assay. After 12 h of FBS deprivation, confluent cell monolayers of BLK, Vector3, M1, M2 and M3 cells were scratched with a 200-μl pipette tip to create wounded areas with widths of 400-600 μm. Images were captured 0 and 1 h after scratching under an Eclipse TE2000-U fluorescence microscope (Nikon Corporation). The migrated distance was quantified by Integrated Performance Primitives software (version 6.0.0.260; Intel Corporation, Santa Clara, CA, USA). Three independent experiments were performed with three biological replicates each.

Transwell migration and invasion assays. To analyse Transwell migration and invasion, cell inserts (8.0-μm pore size; EMD Millipore, Billerica, MA, USA) were coated with (invasion) or without (migration) Matrigel (BD Bioscience). SACC-83, NC, MYB OE, Vector1, Vector2, Vector3, M1, M2 and M3 cell or siMYB-1-, siMYB-2- and siNC-transfected SACC-83 and SACC-LM cells were seeded at a density of $5x10^4$ cells/well in RPMI-1640 medium without serum in the upper chamber. The lower chamber contained RPMI-1640 medium supplemented with 10% FBS. After 16 h of incubation, the cells on the upper surface of the insert were wiped off. The cells on the lower surface of the insert were fixed with 95% ethanol for 30 min at room temperature, stained with 1% crystal violet for 40 min at room temperature and counted under a BX51 fluorescence microscope (Olympus Corporation, Tokyo, Japan) at a magnification of x20. Every experiment was repeated independently at least three times.

Mouse lung metastasis model. NOD/SCID mice were injected in the tail vein with $4x10^6$ MYB OE (n=7) or NC cells (n=6). After 8 weeks, the mice were sacrificed and their lungs were collected. After the lungs were fixed with 4% paraformaldehyde for 24 h at room temperature, the lungs was embedded in paraffin and then cut into 4-μm-thick sections. The sections were used for hematoxylin and eosin staining.

Briefly, the sections were deparaffinized and rehydrated at 37˚C with xylene I, xylene II and xylene III for 20 min each; then absolute ethanol I and absolute ethanol II for 10 min each; and finally 95, 80 and 70% ethanol for 10, 4 and 2 min, respectively. The sections were wash with deionized water three times for 3 min each time, then stained with hematoxylin for 2-3 min at room temperature. Next, differentiation was performed with hydrochloric acid alcohol for 1 sec, and then the sections were rinsed with deionized water for 10 min and stained with eosin for 8 min at room temperature. Gradient dehydration was performed at room temperature with 70, 80, 90, 95 and 100% ethanol for 2, 2, 3, 3 and 10 min, respectively, then with xylene I, xylene II and xylene III for 10 min each. The sections were sealed with neutral gum and placed in a ventilated room at room temperature overnight. The stained sections were evaluated by an experienced pathologist from the Department of Oral Pathology, Peking University School and Hospital of Stomatology.

Statistical analysis. All statistical analyses were conducted with SPSS (version 19.0; IBM Corp., Armonk, NY, USA). All *in vitro* experiments were performed at least three times and numerical data are presented as mean ± standard deviation of three independent experiments. P-values were calculated by a two-tailed unpaired Student's t-test or one-way analysis of variance with Bonferroni post-test correction. The results were confirmed in at least three independent experiments. $P<0.05$ indicated that the difference between groups was statistically significant. The Pearson correlation coefficient was calculated to evaluate the correlation of MYB with E-cadherin or vimentin.

Results

MYB is overexpressed in SACC tissue samples and associated with the potential for metastasis in clinical cases. As presented in Fig. 1A, *MYB* was 11th among the top 15 upregulated mRNAs in the 10 human SACC and paired SMG tissues in the microarray analysis. The mRNA expression levels of *MYB* and EMT-associated markers were also measured in 50 fresh frozen SACC tissues and 41 normal fresh frozen SMG tissues using RT-qPCR. *MYB* expression was significantly higher in the SACC tissues compared with the normal SMG tissues, with a high expression rate of 90% (Fig. 1B). The clinicopathological features of the SACC patients are stratified

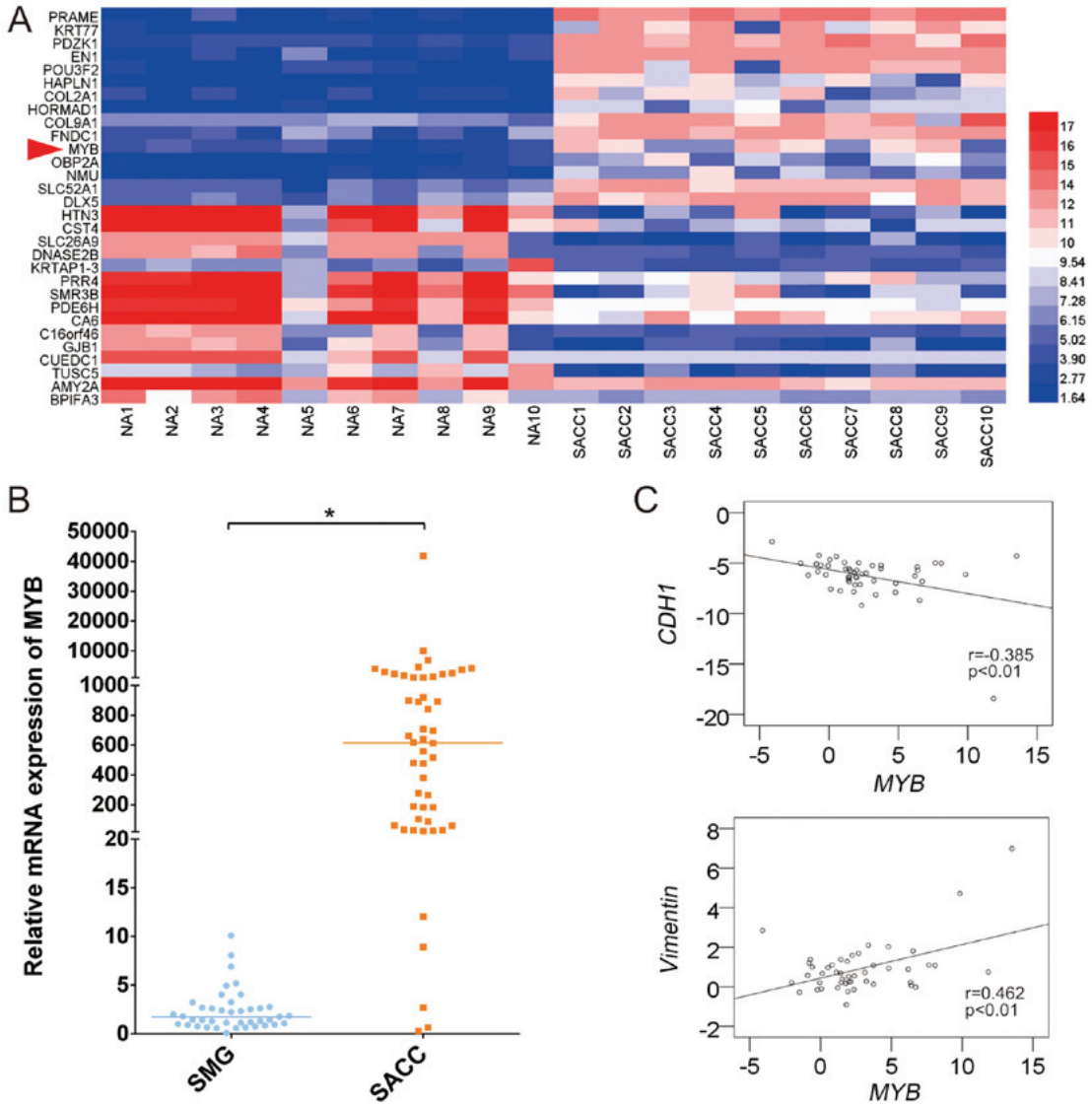


Figure 1. *MYB* is overexpressed in SACC tissues and correlates with the SACC metastatic potential. (A) Microarray screening of differentially expressed mRNAs in 10 human SACC tissues and paired SMG tissues. The key represents relative mRNA expression. The heatmaps presented the 15 most upregulated mRNAs and the 15 most downregulated mRNAs. (B) Reverse transcription-quantitative polymerase chain reaction analysis of *MYB* overexpression in 50 human SACC and 41 SMG tissues. (C) Correlation of *MYB* mRNA expression with *CDH1* and *vimentin* mRNA expression in SACC tissues (n=50). SACC, salivary adenoid cystic carcinoma; SMG, submandibular gland; *MYB*, transcriptional activator Myb gene; *CDH1*, cadherin-1.

in Table I. A total of 50 SACC patients were classified into the high or low *MYB* group depending on the median *MYB* level. High *MYB* expression was correlated with the pathological type, lung metastasis and clinical stage. Although high *MYB* expression did not significantly correlate with tumour size, the P-value was close to the threshold. No correlation was identified between *MYB* expression and the other parameters, including age, sex, site, lymph node metastasis, perineural invasion and local regional recurrence.

CDH1 and *vimentin* expression was also measured by RT-qPCR in these SACC tissues, and the correlation of *MYB* with *CDH1* and vimentin was analysed. *MYB* tended to be significantly and negatively correlated with CDH1, and significantly and positively correlated with *vimentin* (Fig. 1C).

MYB is overexpressed in SACC cells and modulates cell proliferation in vitro. To explore the biological role of *MYB* in SACC cells, *MYB* expression was measured in SACC-83 cells, primary cells derived from a human SMG tissue (pSMG cells) and a subset of SACC cells that exhibited high lung metastasis (SACC-LM cells). *MYB* mRNA expression levels were significantly higher and *MYB* protein expression levels were markedly higher in the SACC-83 and SACC-LM cells compared with in the pSMG cells (Fig. 2A). *MYB* mRNA expression levels were significantly higher and *MYB* protein expression levels were markedly higher in the SACC-LM cells compared with the SACC-83 cells, indicating that MYB is associated with SACC oncogenesis and metastasis.

MYB mRNA expression levels were significantly higher in the MYB OE cells compared with NC and BLK cells, demonstrating that *MYB* overexpressing vector transfection was stable (Fig. 2B). *MYB* protein expression levels were markedly higher in the MYB OE cells compared with NC and BLK cells. The *MYB* mRNA expression levels were significantly higher in the M1, M2 and M3 cells compared with the Vector3 cells, while protein expression levels were

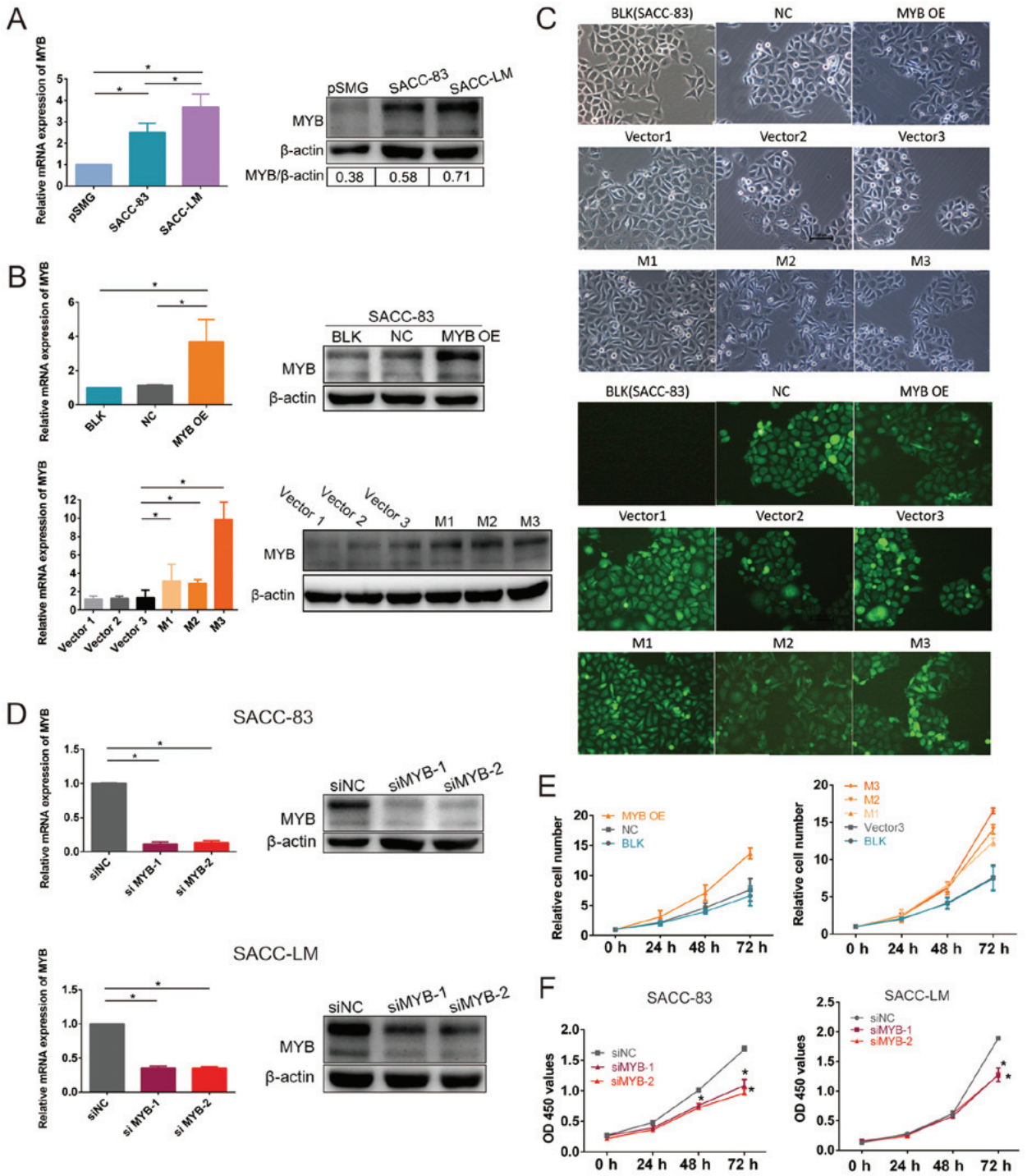


Figure 2. MYB is overexpressed in SACC cells, which promotes SACC cell proliferation, and its knockdown inhibits SACC cell proliferation. (A) RT-qPCR analysis of *MYB* mRNA expression and western blot analysis of MYB protein expression in pSMG, SACC-83 and SACC-LM cells (*P<0.05 as defined). (B) RT-qPCR analysis of *MYB* mRNA expression and western blot analysis of MYB protein expression in transfected SACC-83 cells (*P<0.05 as defined). (C) Fluorescence microscopy in transfected SACC-83 and BLK cells. (D) RT-qPCR analysis of *MYB* mRNA expression and western blot analysis of MYB protein expression in siNC-, siMYC-1- and siMYC-2-transfected SACC-83 and SACC-LM cells (*P<0.05 as defined). (E) Cell proliferation of the transfected SACC-83 cells, including BLK, NC, MYB OE, Vector3, M1, M2 and M3 cells. Cell proliferation was assessed using the Cell Counting Kit-8 assay and compared by calculating the relative cell number. Relative cell number = OD 450 values per time point / OD 450 values of 0 h. (F) Proliferation of siNC-, siMYC-1- and siMYC-2-transfected SACC-83 and SACC-LM cells was assessed using the Cell Counting Kit-8 assay. Data are presented as mean ± standard deviation of three independent experiments (*P<0.05 vs. siNC). RT-qPCR, reverse transcription-quantitative polymerase chain reaction analysis; pSMG, primary cells derived from the human submandibular gland; SACC, salivary adenoid cystic carcinoma; *MYB*, transcriptional activator Myb gene; NC, cells transfected with an empty vector; MYB OE, *MYB* overexpressing cells; M, cells derived from *MYB* overexpressing cells; Vector, cells derived from NC cells; si, small interfering RNA; siNC, cells transfected with a negative control siRNA; siMYB, cells transfected with a siRNA against MYB; BLK, untransfected cells; OD, optical density.

markedly higher in the M1, M2 and M3 cells compared with the Vector3 cells. Fibroblastic morphological changes of the cells undergoing EMT were identified in *MYB* overexpressing cells. Microscopic observation revealed the cobblestone-like

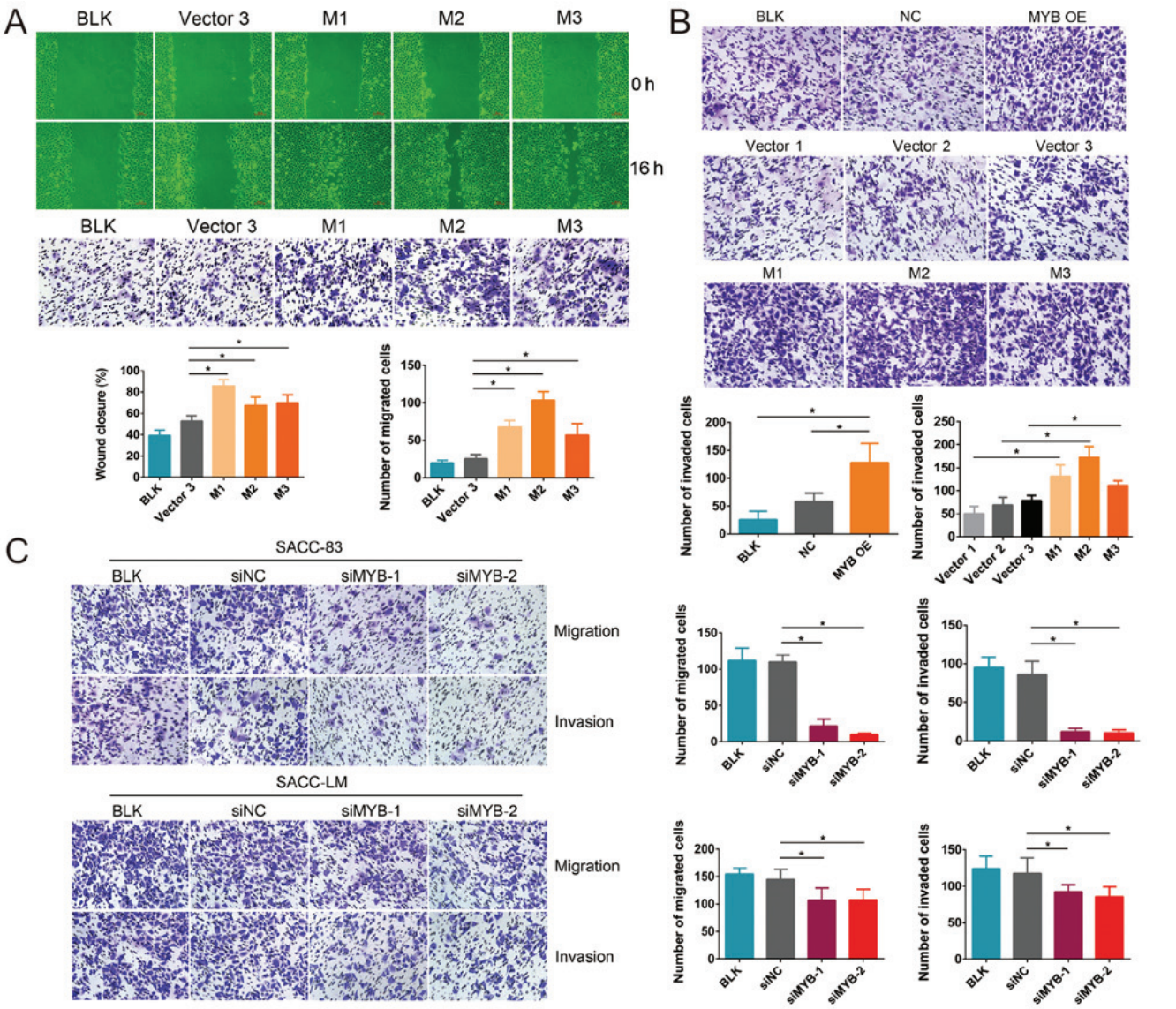


Figure 3. *MYB* overexpression promotes and *MYB* knockdown inhibits the migration and invasion of SACC cells. (A) Wound closure and Transwell migration assays of M1-, M2-, M3- and Vector3-transfected, and BLK SACC-83 cells. (B) Transwell invasion assay of transfected SACC-83 cells and BLK SACC-83 cells. (C) Transwell migration and invasion assays of siNC-, MYB-1- and MYB-2-transfected, and BLK SACC-83 cells. Data are presented as mean ± standard deviation of three independent experiments (*P<0.05). SACC, salivary adenoid cystic carcinoma; *MYB*, transcriptional activator Myb gene; NC, cells transfected with an empty vector; M, cells derived from *MYB* overexpressing cells; Vector, cells derived from NC cells; si, small interfering RNA; siNC, cells transfected with a negative control siRNA; siMYB, cells transfected with a siRNA against MYB; BLK, untransfected cells.

structure of BLK, NC, Vector1, Vector2 and Vector3 cells. MYB OE, M1, M2 and M3 cells exhibited varying degrees of fibroblastic changes (Fig. 2C). Approximately 90% of MYB OE, M1 and M2 cells and ~40% of M3 cells lost their original polygonal phenotype and acquired a fibroblastic phenotype. *MYB* expression was knocked down in the SACC-83 and SCC-LM cell lines using siMYB-1 and siMYB-2 (Fig. 2D). The *MYB* mRNA expression levels were significantly lower and *MYB* protein expression levels were markedly lower in the siMYB-1 and siMYB-2 cells compared with the siNC cells.

The proliferation ability of MYB OE, M1, M2 and M3 cells was markedly higher compared with control cells at 72 h (Fig. 2E). The cell proliferation assay demonstrated that SACC cell growth was inhibited in the two cell lines (Fig. 2F). The proliferation was significantly attenuated in siMYB-1 and siMYB-2 cells compared with the siNC cells. Together, these findings support a growth-promoting role for MYB in SACC cells.

MYB promotes SACC cell migration and invasion in vitro. To investigate the effect of *MYB* expression on the aggressiveness of SACC cells, the effect of *MYB* on cell migration and invasion was studied. The wound healing and Transwell migration assays demonstrated that the migration of M1, M2 and M3 cells was significantly higher compared with the Vector3 cells (Fig. 3A). Additionally, the Transwell invasion assay demonstrated that the invasive ability of the MYB OE cells was significantly greater compared with that of the NC and BLK cells (Fig. 3B). Similarly, the M1, M2 and M3 cells had significantly higher invasive abilities compared with the Vector1, Vector2 and Vector3 cells, respectively.

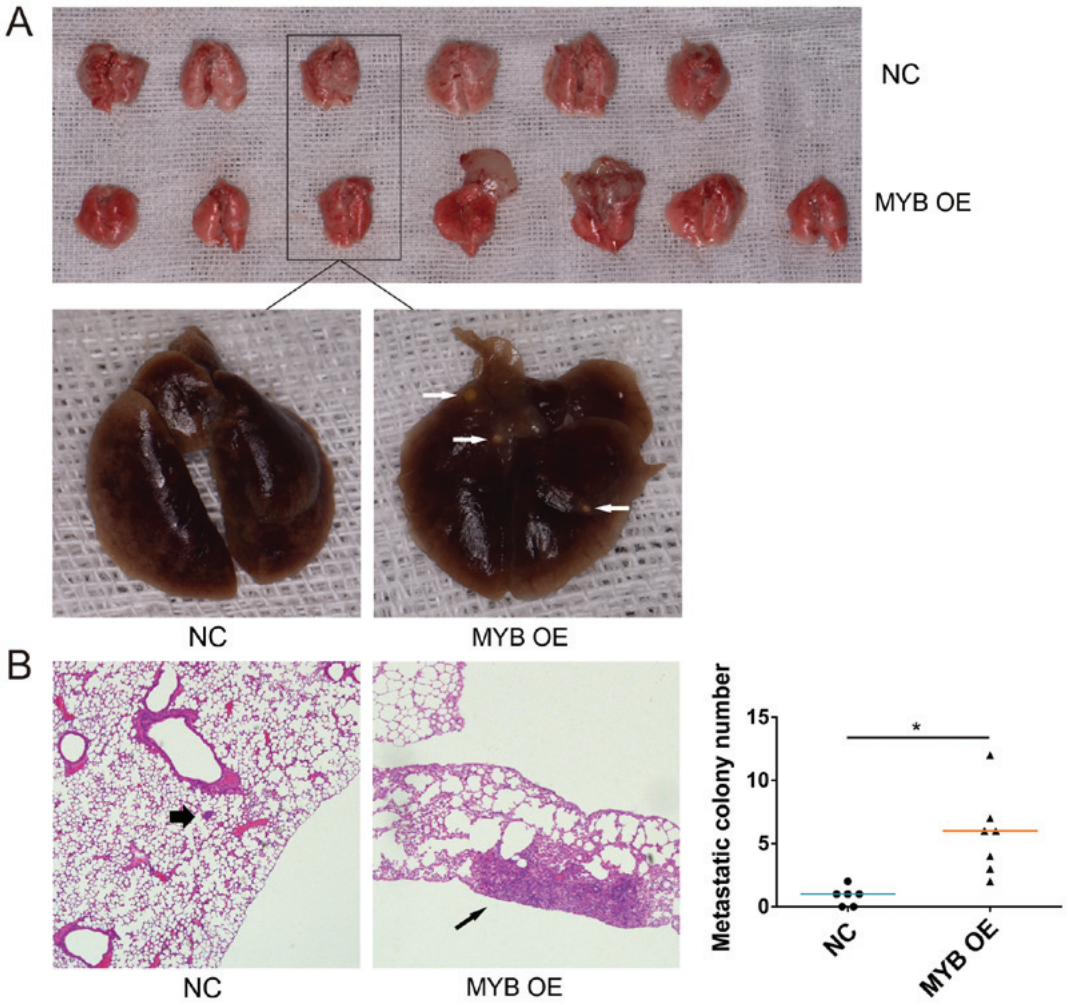


Figure 4. *MYB* promotes salivary adenoid cystic carcinoma lung metastasis *in vivo*. Lung tissues from mice injected intravenously with NC and MYB OE cells were removed after 8 weeks and observed. (A) Images of xenograft model mice. The arrows indicate metastatic tumour nodules. (B) Images of haematoxylin and eosin staining metastatic tumour nodules in the lung tissues and quantification of metastatic colony number (*P<0.05). NC, mice injected with negative control-transfected cells; MYB OE, mice injected with transcriptional activator Myb overexpressing cells.

In contrast, the knockdown of *MYB* by siMYB-1 and siMYB-2 in the SACC-83 and SACC-LM cells significantly inhibited cell migration and invasion compared with the siNC cells (Fig. 3C). These results indicated that *MYB* overexpression promoted SACC cell migration and invasion, whereas *MYB* knockdown inhibited SACC cell migration and invasion.

MYB promotes SACC lung metastasis in vivo. To investigate the effect of MYB on SACC lung metastasis, MYB OE and NC cells were injected into NOD/SCID mice through the tail vein. The lung tissues were collected from all mice after 8 weeks. The mice injected with MYB OE cells had visible tumour nodules, which were not observed in the NC cell-injected mice (Fig. 4A). The number of tumour nodules in the lungs of the mice injected with MYB OE cells was significantly greater compared with that of the NC cells based on hematoxylin and eosin staining of the lung tissues (Fig. 4B). These results indicated that MYB promoted SACC lung metastasis *in vivo*.

MYB modulates the expression of genes associated with proliferation and metastasis. To explore the molecular mechanism underlying MYB-induced tumour promotion and metastasis, genes associated with tumour progression and metastasis were examined in NC, MYB OE, siNC and siMYB-1 cells by RT-qPCR. Significant changes were identified in five of the 22 genes (data not shown). The expression of *CCND1* and *MCL1*, which are associated with cell proliferation (33,34), was upregulated following *MYB* overexpression, whereas *p21* expression was downregulated (Fig. 5A). *ICAM1, VEGFA, MMP7* and *MMP9*, which are involved in cell migration and invasion (24,25,35,36), were upregulated following *MYB* overexpression. In addition, *CCND1, MCL1, ICAM1, VEGFA, MMP7* and *MMP9* were downregulated, whereas *p21* was upregulated following MYB knockdown (Fig. 5B). Taken together, these findings indicate that *MYB* promotes the growth, migration and invasion of SACC cells by regulating the expression of genes involved in proliferation and metastasis.

MYB regulates EMT in SACC cells. As mentioned previously, MYB tends to be negatively correlated with the epithelial marker, E-cadherin, and positively correlated with the mesenchymal marker, vimentin. The expression of E-cadherin

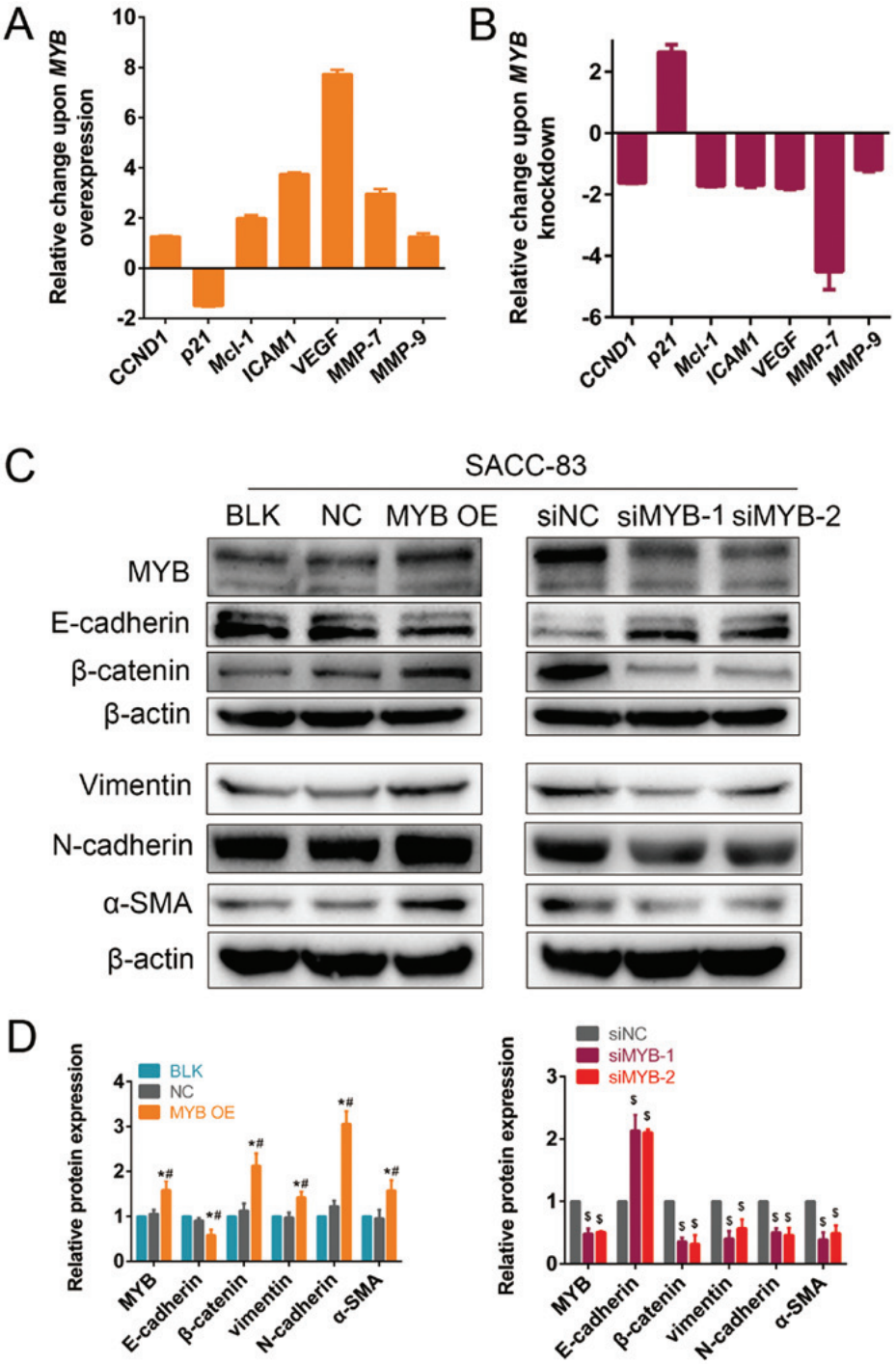


Figure 5. MYB modulates the expression of genes associated with proliferation and metastasis. Quantification of RT-qPCR analysis expression following *MYB* (A) overexpression and (B) knockdown in SACC-83 cells. (C) Western blotting of transfected and BLK SACC-83 cells. (D) Quantification of protein bands in transfected and BLK SACC-83 cells. Data are presented as mean ± standard deviation of three independent experiments (*P<0.05 vs. NC; $^{\#}$P<0.05 vs. BLK; $^{\$}$P<0.05 vs. siNC). SACC, salivary adenoid cystic carcinoma; *MYB*, transcriptional activator Myb gene; MYB, transcriptional activator Myb protein; NC, cells transfected with an empty vector; si, small interfering RNA; siNC, cells transfected with a negative control siRNA; siMYB, cells transfected with a siRNA against MYB; BLK, untransfected cells; *CCND1*, G1/S-specific cyclin-D1 gene; *p21*, cyclin-dependent kinase inhibitor 1 gene; *MCL1*, myeloid leukemia cell differentiation protein Mcl-1 gene; *ICAM1*, intercellular adhesion molecule 1 gene; *VEGFA*, vascular endothelial growth factor A gene; *MMP*, matrix metalloproteinase gene; E-cadherin, cadherin-1; β-catenin, catenin β-1; N-cadherin, cadherin-2; α-SMA, actin, aortic smooth muscle.

in the MYB OE group was significantly downregulated following *MYB* overexpression compared with the BLK and NC groups, whereas N-cadherin, vimentin and α-SMA expression was significantly upregulated (Fig. 5C and D). β-catenin, which is the key molecule of the canonical Wnt signalling pathway (18), was also significantly upregulated following *MYB* overexpression compared with the BLK and NC groups, suggesting that MYB may regulate EMT via the Wnt signalling pathway. Additionally, E-cadherin was significantly upregulated, and N-cadherin, vimentin, α-SMA and β-catenin were significantly downregulated in the siMYB-1- and siMYB-1-transfected cells compared with the siNC group.

Discussion

In the current study, *MYB* expression was measured in 50 fresh frozen SACC tissues and 41 fresh frozen SMG tissues, and the clinicopathological features of the SACC patients were stratified. In particular, it was determined that MYB was closely associated with lung metastasis and was associated with the pathological tumour type in patients with SACC (14,18). *MYB* expression was significantly greater in SACC tissues compared with normal SMG tissues, with an expression rate of 90%. This result was consistent with that of previous studies (12,14), but the proportion of *MYB* overexpression seemed to be higher in the current study. These results evidently suggest that MYB serves an important role in SACC.

To explore the effect of MYB in SACC, overexpression and knockdown of MYB in SACC cells was performed and cell proliferation was explored using CCK8. The results demonstrated that upregulation of *MYB* increased the relative number of cells, which was consistent with previous findings in prostate and pancreatic cancer (37,38). Knockdown of *MYB* significantly inhibited cell proliferation, which was similar to findings for acute myeloid leukaemia, chronic myeloid leukaemia and colon cancer (39,40). Mechanistically, previous studies demonstrated that *MYB* induced the growth of tumour cells through the promotion of cell cycle progression, which was associated with cell proliferation (41,42). It has been suggested that MYB is capable of regulating various genes responsible for tumorigenesis in multiple cancers (17,35,43,44). Using RT-qPCR, the authors of the current study revealed that MYB may improve cell proliferation by enhancing cell cycle progression through *CCND1* upregulation and *p21* downregulation. Additionally, MYB may decrease apoptosis by upregulating *MCL1* expression, which encodes proteins belonging to the Bcl-2 family (34).

Apart from its effect on tumour cell growth, MYB also has been revealed to promote a variety of phenotypes associated with the migration and invasion of cancer cells (18,41,42,45,46). Similarly, in the current study, MYB significantly enhanced SACC cell migration and invasion in *in vivo* and *in vitro* experiments. Previous studies demonstrated that VEGF promoted metastasis via angiogenesis (24,25,47). The current study demonstrated that *VEGFA* was upregulated in *MYB* overexpressing SACC cells, indicating that MYB could increase the metastasis of SACC cells by increasing *VEGFA* expression. In addition, MYB was demonstrated to increase the *ICAM1* level, which encodes intercellular adhesion molecules that can promote tumour metastasis (48). *MMP7* and *MMP9* are involved in angiogenesis and tumor metastasis (45,49). The current study revealed that MMP-7 and MMP-9 were upregulated in *MYB* overexpressing SACC cells, indicating that MYB could increase the metastasis of SACC cells by increasing *MMP7* and *MMP9* expression. However, the corresponding protein levels of these RNAs were not assessed in the current study, which is a limitation.

Tumour metastasis depends on the occurrence of EMT, including decreased expression of cell adhesion molecules, such as E-cadherin, and increased expression of mesenchymal markers, including N-cadherin and vimentin (22-24). In SACC tissues, *MYB* tended to be negatively correlated with *CDH1* and positively correlated with *vimentin*. Additionally, the authors revealed that, in SACC cells, MYB upregulated EMT-associated markers, including vimentin, N-cadherin and α-SMA, which was similar to the results of previous reports investigating breast cancer (18,50). These results indicated that MYB regulated SACC metastasis by promoting the EMT.

In conclusion, MYB regulated proliferation-associated molecules, including *CCND1, p21* and *MCL1*, in SACC cells to enhance cell proliferation. MYB also regulated I*CAM1, VEGFA, MMP7, MMP9* and EMT-associated markers, including E-cadherin, vimentin, N-cadherin and α-SMA, in SACC cells to increase metastasis. In addition, MYB promoted SACC lung metastasis in a xenograft mouse model. Specifically, it was determined that MYB was closely associated with lung metastasis and the pathological tumour type in patients with SACC, which has rarely been reported. The results of the current study suggest that MYB may be an important therapeutic target for SACC.

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Availability of data and materials

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

Authors' contributions

LHX, XYG and SLL conceived and designed the experiments. LHX and FZ performed the experiments and acquired the data. LHX, WWY and CWC analysed and interpreted the data. LHX, ZHD and MF performed the statistical analysis. LHX wrote the manuscript. All authors edited the final manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The collection and use of human tissue samples was approved by the Ethics Committee of Peking University School and Hospital of Stomatology (Beijing, China; permit no. PKUSSIRB-201522040). All animal experiments complied with the ARRIVE guidelines and were performed in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines and the EU Directive 2010/63/EU for animal experiments. These experiments were approved by the Peking University Institutional Animal Care and Use Committee (Beijing, China; permit no. LA2015099). Written informed consent was provided by all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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