

PTEN downregulates WD repeat-containing protein 66 in salivary adenoid cystic carcinoma

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Received May 15, 2018; Accepted November 30, 2018

DOI: 10.3892/or.2018.6931

Abstract. Salivary adenoid cystic carcinoma (SACC) is one of the most common types of salivary gland cancer that causes substantial morbidity and mortality. Despite the substantial health burden of SACC, the molecular mechanisms underlying its development and progression remain poorly understood. We previously reported the loss of phosphatase and tensin homolog (PTEN) expression to be common among SACC tumors, and the PTEN deficiency to be correlated with enrichment of epithelial-mesenchymal transition (EMT) genes based on expression array analysis. The aim of the present study was to investigate further the functional function of WD repeat-containing protein 66 (WDR66), one of the enriched EMT genes, in the context of PTEN deficiency and SACC pathogenesis. WDR66 was identified to be required to maintain the EMT phenotype and the expression of cancer stem

cell genes in the context of PTEN deficiency. Furthermore, knockdown of WDR66 decreased cellular proliferation, migration and invasion. Finally, WDR66 expression was identified to be inversely associated with PTEN expression and negatively correlated with the overall survival of patients with SACC. Collectively, the results of the present study revealed a novel function of WDR66 in mediating the progression of PTEN-deficient SACCs, thereby suggesting WDR66 inhibition to be a potential therapeutic approach towards successful management of SACC disease progression, particularly against tumors with decreased PTEN expression levels.

Introduction

Salivary gland tumor (SGT) is one of the most pathologically heterogeneous and least studied tumor types (1). Currently, the molecular mechanisms that cause SGT pathogenesis remain poorly understood. Although rare, the limited treatment options available for patients with SGT make this disease a significant health problem (2-4). SGT is classified into >20 distinct histological subtypes (5). The most common benign SGT is salivary pleomorphic adenoma, and the most common malignant SGTs are salivary adenoid cystic carcinoma (SACC) and mucoepidermoid carcinoma (5).

The incidence of SACC is ~4.5 cases/10⁶ individuals and accounts for 10% of all SGTs (6-8). SACC is characterized by indolent, yet progressive, growth, perineural invasion and a high rate of distant metastasis (8). SACC presents with three different histological growth patterns, i.e. cribriform, tubular and solid patterns. Among them, the solid-pattern histological type represents the most aggressive form of SACC. Solid-pattern SACCs are typically poorly differentiated, higher-grade malignancy with increased rates of metastasis, all of which lead to shorter disease-specific survival times (9).

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Key words: salivary adenoid cystic carcinoma, WD repeat-containing protein 66, phosphatase and tensin homolog, epithelial-mesenchymal transition

Although the 5-year survival rate is ~70% for patients with SACC, the survival rates decrease to 40% at 10 years and 25% at 15 years owing to frequent local recurrence and distant metastasis (3-8). Treatment options for SACC are currently limited, with the standard treatment regimen being local resection with radiation therapy (10). Chemotherapy has also been used in isolated cases, but has failed to improve overall patient survival (10). An improved understanding of the molecular mechanisms of SACC pathogenesis should allow the development of novel and more effective treatment options for patients with SACC (11).

Significant progress has been achieved in the molecular profiling of human SGT, particularly with the application of exome sequencing (12-17). In addition to the commonly observed chromosome translocations (e.g. nuclear factor IB and MYB) (18), the phosphoinositide 3-kinase (PI3K)/phosphatase and tensin homolog (PTEN) signaling pathway has been noted to be frequently altered in SGTs (12-14). Three independent studies identified PTEN mutations in human SGTs, specifically in SACC, salivary ductal carcinoma and carcinoma ex pleomorphic adenoma, but not in mucoepidermoid carcinoma or acinic cell carcinomas (12,19,20). In addition, genetic loss or decreased PTEN expression has been identified in human SGTs (21-24). Previous studies, including ours, have indicated that loss or decreased expression of PTEN (ranging between 20 and 47%) occurs in several human SGT subtypes (21-24).

To investigate the molecular consequences of decreased PTEN expression or PTEN gene loss, we previously performed a gene expression microarray in human SACC cell lines with ectopically induced PTEN knockdown (24). In the present study, the gene expression profile associated with PTEN knockdown was analyzed further and enrichment of genes associated with epithelial-mesenchymal transition (EMT) was identified. Among the EMT genes associated with PTEN downregulation, WD repeat-containing protein 66 (WDR66) was selected for further characterization of its functions in the mediation of EMT, maintenance of cancer stem cells (CSCs). Specifically, basic cellular phenotype implications, including cellular proliferation, migration and invasion, of WDR66 were also assessed in the context of PTEN downregulation. Finally, the clinical relevance of the results was validated as WDR66 gene expression was identified to be inversely correlated with PTEN expression in SACC cell lines and tumor samples from SACC patients, and its expression level was revealed to be negatively associated with the overall survival of patients with SACC.

Materials and methods

Patients and collection of tissues. A total of 20 normal salivary glands (SGs) and 46 SACC tumor tissues, which were surgically resected between January 2001 and December 2013 at Dalian Medical University (Dalian, China), were included in the present study. All patient samples were collected under the approved guidelines provided by the Institutional Review Boards at Dalian Medical University. All collected tissues were clinically examined by oral pathologists and informed consent forms were obtained from all patients. The demographic and clinical pathological information for each patient is listed in Table I.

Table I. Clinicopathological characteristics of patients.

Factor	n
SACC	46
Tubular	12
Cribriform	14
Solid	20
NSG	20
Age, years	
<60	35
≥60	31
Sex	
Male	32
Female	34
Tumor site	
Major salivary glands	32
Minor salivary glands	14

SACC, salivary adenoid cystic carcinoma; NSG, normal salivary gland.

Cell culture, treatment and transfection. The human SACC cell line SACC83 was generously provided by Dr Shenglin Li (Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, Beijing, China). Cells were subcultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1X penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). All cell cultures were maintained at 37°C in a humidified incubator containing 5% CO₂. To inhibit the PI3K pathway, SACC cells were treated with 1 μM GDC-0941 (LC Laboratories, Woburn, MA, USA) or vehicle (dimethylsulfoxide) control for 4 h. To knock down the expression of WDR66, SACC83 cells were transfected with the WDR66 short interfering RNA (siRNA) or control siRNA using Lipofectamine™ 3000 Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Three siRNAs against WDR66 were tested. The sequence of these three siRNAs were: #1918, 5'-GCGGUGUACCACUUAACAATT-3'; #1566, 5'-GCU GGUUGUCUGGGACAUATT-3'; and #969, 5'-GCAGAG AGUUCUUCUGUAUTT-3'. The sequence for the negative control siRNA was 5'-UUCUCCGAACGUGUCACG UTT-3'. PTEN knockdown was achieved by transfecting with PTEN siRNA (5'-GAUCUUGACAAAGCAAUATT-3'). At between 48 and 72 h post-transfection, SACC83 cells were harvested and the knockdown efficiencies were determined using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting.

Immunohistochemistry (IHC). Formalin-fixed paraffin-embedded (FFPE) slides of SACC biopsies (4-mm thick) from patients and normal HSG tissue sections were used for IHC staining according to a protocol described

Table II. Primer sequences for the quantitative polymerase chain reaction.

Gene	Forward	Reverse
<i>WDR66</i>	5'-TTATGGTTCCCCTATTGAGC-3'	5'-GTCTTATGTGGATTGCCGTC-3'
<i>PTEN</i>	5'-GAGCGTGCAGATAATGACAAGGAAT-3'	5'-GGATTTGACGGCTCCTCTACTGTTT-3'
<i>CDH1</i>	5'-TGCCCAGAAAATGAAAAAGG-3'	5'-GTGTATGTGGCAATGCGTTC-3'
<i>VIM</i>	5'-AATGGCTCGTCACCTTCGTGAAT-3'	5'-CAGATTAGTTTCCCTCAGGTTTCAG-3'
<i>ALDH1</i>	5'-GCACGCCAGACTTACCTGTCCTA-3'	5'-GGCCTTCACTGCCTTGTCAAC-3'
<i>OCT4</i>	5'-GTGCCGTGAAGCTGGAGAA-3'	5'-TGGTCGTTTGGCTGAATACCTT-3'
<i>NANOG</i>	5'-CCTGTGATTTGTGGGCCTGA-3'	5'-CTCTGCAGAAGTGGGTTGTTTG-3'
<i>SOX2</i>	5'-CCAAGATGCACAACCTCGGAGA-3'	5'-CCGGTATTTATAATCCGGGTGCT-3'
<i>ZEB1</i>	5'-TCCATGCTTAAGAGCGCTAGCT-3'	5'-GTATCTTGTCTTTTCATCCTGATTTC-3'
<i>ZEB2</i>	5'-TTCCTGGGCTACGACCATAACC-3'	5'-CAAGCAATTCTCCCTGAAATCC-3'
<i>TGFBI</i>	5'-CGCCAGAGTGTTTATCTTTTGA-3'	5'-CGGTAGTGAACCCGTTGATGT-3'
<i>GAPDH</i>	5'-GCACCGTCAAGGCTGAGAAC-3'	5'-TGGTGAAGACGCCAGTGGA-3'

WDR66, WD repeat-containing protein 66; *PTEN*, phosphatase and tensin homolog; *CDH1*, cadherin 1 (epithelial cadherin); *VIM*, vimentin; *ALDH1*, aldehyde dehydrogenase 1; *SOX2*, sex-determining region Y box 2; *ZEB*, zinc finger E-box-binding homeobox; *TGFBI*, transforming growth factor β 1.

previously (25). Antibody binding was visualized using a Vectastain[®] Avidin-Biotin Complex kit (cat. no. PK-4001 [rabbit immunoglobulin G (IgG)] or PK-4002 (mouse IgG); Vector Laboratories, Inc., Burlingame, CA, USA) and a Diaminobenzidine kit (cat. no. DAB-1031, Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China), according to the manufacturer's protocol, and counterstained with hematoxylin. Images of the slides were captured using a BX43 light microscope (Olympus Corporation, Tokyo, Japan) controlled with CellSens software (version 1.12; Olympus Corporation). The primary antibodies used for IHC were mouse anti-WDR66 (1:200; cat. no. ab83694; Abcam, Cambridge, UK) and mouse anti-PTEN (1:200; cat. no. 18-0652; Zymed; Thermo Fisher Scientific, Inc.). The quantitative analysis of IHC staining followed the criteria we have described previously (24). The staining index was determined as follows: 0-2, low (-); 3-6, medium (+); 7-9, high (++) expression.

Double immunofluorescence (IF) staining. Double IF was performed as described previously (26) using mouse anti-WDR66 (as aforementioned) and rabbit anti-PTEN (1:100; cat. no. 60300; ProteinTech Group, Inc., Chicago, IL, USA) primary antibodies, and Alexa Fluor 488-conjugated goat anti-rabbit IgG [heavy and light chains (H+L)] (1:500; cat. no. ab181448; Abcam, UK) and Alexa Fluor 594-conjugated goat anti-mouse IgG (H+L) (1:500; cat. no. ab150120; Abcam, UK) secondary antibodies. Nuclei were labeled with DAPI (1:200; cat. no. C1005; Beyotime Institute of Biotechnology, Haimen, China).

RNA extraction and RT-qPCR. Total RNA was isolated with TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA samples were reverse-transcribed into cDNA using the PrimeScript[™] RT reagent kit with gDNA Eraser (cat. no. DRR047A; Takara Bio, Inc., Otsu, Japan). qPCR was performed using a

Thermal Cycler Dice Real-Time system (TP800; Takara Bio, Inc.) using SYBR Premix Ex Taq II kit (cat. no. DRR820A; Takara Bio, Inc.), according to the manufacturer's protocol. GAPDH served as an internal control gene. All primers were synthesized by Takara Bio, Inc., and the sequences are presented in Table II.

Western blot analysis. SACC cells were harvested and lysed in radioimmunoprecipitation buffer containing protease/phosphatase inhibitor cocktail (cat. no. KGP250, Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Total proteins (40 μ g) were separated by SDS/PAGE (10% gels) prior to transfer onto nitrocellulose membranes, which were blocked with fat-free milk powder (5%) diluted in Tris-buffered saline containing 0.1% Tween-20 at room temperature for 1 h then incubated with primary antibody at 4°C overnight followed by horseradish peroxidase-conjugated secondary antibodies [HRP-conjugated goat anti-rabbit IgG (H+L); 1:2,000; cat. no. ABC-AS014; and HRP-conjugated goat anti-mouse IgG (H+L); 1:2,000, cat. no. ABC-AS003; both from ABClonal Biotech Co., Ltd., Woburn, MA, USA] for 2 h at room temperature. A Prime Western Blotting Detection Reagent enhanced chemiluminescence kit (cat. no. K-12045-D10; Advansta Inc., San Jose, CA, USA) was used to detect the signals.

Primary antibodies used for the western blotting were: Mouse Anti-WDR66 (1:500; as aforementioned), rabbit anti-epithelial (E-)cadherin (1:2,000; cat. no. 20874; ProteinTech Group, Inc.), rabbit anti-vimentin (1:2,000; cat. no. 10366; ProteinTech Group, Inc.), rabbit anti-aldehyde dehydrogenase 1 (ALDH1; 1:400; cat. no. 15910; ProteinTech Group, Inc.), rabbit anti-Oct3/4 (1:500; cat. no. 11263; ProteinTech Group, Inc.), rabbit anti-Nanog (1:2,000; cat. no. 14295; ProteinTech Group, Inc.), rabbit anti-sex-determining region Y box 2 (Sox2; 1:1,000; cat. no. 3579; Cell Signaling Technology, Inc., Danvers, MA, USA), mouse anti-vinculin (1:10,000; cat. no. ab73412; Abcam) and mouse

anti-GAPDH (1:1,000; cat. no. AG019; Beyotime Institute of Biotechnology).

Co-immunoprecipitation (IP) assay. SACC cells were harvested and were lysed with ice-cold IP buffer. The cell lysates were incubated with Protein G-conjugated sepharose beads at 4°C for 2 h and centrifuged at 1,000 x g for 5 min at 4°C. The extract was incubated with pre-coupled antibodies bound to Protein G-conjugated beads at 4°C overnight. Samples were resolved by SDS-PAGE (10% gels) and transferred onto polyvinylidene fluoride membranes. Primary antibodies against PTEN (1:100; as aforementioned) and WDR66 (1:100; as aforementioned) were used for IP.

Proliferation assay. Cell proliferation was evaluated using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly, cells were seeded in a 96-well plate at a density of 0.5×10^4 cells/well in RPMI-1640 with 10% FBS. Cells were harvested from day 1 to day 6. CCK-8 reagent was added to each well and incubated at 37°C for 4 h before each time point of harvest. The absorbance values were determined at a wavelength of 450 nm.

Migration and invasion assay. Cells at 6×10^4 cells/well in serum-free RPMI-1640 medium were added to the upper chamber of a 6.5-mm Transwell with 8.0- μ m pore polyester membrane inserts (Corning Incorporated, Corning, NY, USA) and the inserts were placed in 24-well plates with RPMI-1640 containing 10% FBS in the lower chamber. For invasion assays, the Transwell membranes were pre-coated with 50 μ g/ml Matrigel solution (BD Biosciences, San Jose, CA, USA), whereas Matrigel was not used for cell migration assays. After 48 h of incubation at 37°C, cells that had invaded to the reverse side of the Transwell membrane were fixed with methanol and stained with 0.1% crystal violet at room temperature for 20 min. For each filter, five randomly selected fields were analyzed under an inverted light microscope at x20 magnification.

Wound healing assay. SACC cells ($\sim 1 \times 10^6$) were seeded onto 6-well plates at 37°C for 24 h. The plated cells were then scratched with a 200 μ l sterile pipette tip. Images of the wounded areas were captured under a microscope at x10 magnification after 0, 12 and 24 h of incubation at 37°C, and the distances between the wound edges were determined at each time point.

TCGA database search. To investigate the gene status of WDR66 in human cancer, a cross-cancer genomic analysis of WDR66 was performed using The Cancer Genome Atlas (TCGA) database (www.cbioportal.org/public-portal) using WDR66 as a key word.

Statistical analysis. All statistical analyses were performed using GraphPad Prism (version 6; GraphPad Software, Inc., La Jolla, CA, USA) and SPSS software (version 19.0; IBM Corp., Armonk, NY, USA). All data were acquired from at least three independent experiments and are presented as the mean \pm standard error of the mean. For two group-designed experiments, comparisons were performed using unpaired

Student's t-test; for multiple comparisons, analysis of variance (ANOVA) with two-way ANOVA followed by Dunn's post hoc test or two-way repeated-measures ANOVA followed by Bonferroni's post hoc test was performed. Additionally, the correlations of PTEN and WDR66 were assessed using Spearman rank correlation coefficients. Survival curves were estimated using the Kaplan-Meier method and differences in survival distributions were evaluated by the log-rank test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

WDR66 is identified as one of the candidates for EMT in the context of PTEN deficiency. We previously reported a gene expression profile by microarray analysis using human SACC83 cells with ectopic knockdown of *PTEN* (24). Initial review of the microarray data revealed a total of 244 mRNAs to be differentially expressed (fold change >3) between SACC83 cells and SACC83 cells with *PTEN* knockdown (24). Following reanalysis of these microarray data for the present study, EMT genes were identified to be particularly enriched in SACC83 cells with *PTEN* knockdown (Fig. 1A). Selected genes were validated by RT-qPCR. Decreases in *CDH1* (E-cadherin) and increase in *VIM* (vimentin) RNA expression levels were determined in SACC83 cells with *PTEN* knockdown (Fig. 1B), suggesting that loss of PTEN may promote EMT in SACC cells. In addition, the transcription factor of EMT, zinc finger E-box-binding homeobox 1 (27), and a confirmed EMT promoter, transforming growth factor β 1 (28), were also validated to reveal increased expression levels in SACC83 cells with *PTEN* knockdown (Fig. 1B). Among the validated genes, *WDR66* (Fig. 1A and B) was selected for further functional investigation as it was identified to be the least-studied EMT gene candidate in the context of cancer development and maintenance.

To investigate whether the inverse association between PTEN and WDR66 is due to PTEN's regulation of the PI3K signaling pathway (29), the SACC83 cells were treated with a PI3K inhibitor, GDC-0941 (1 μ M), to inhibit PI3K activity. However, PI3K inhibitor treatment failed to cause the expected decrease in WDR66 expression (Fig. 1C), suggesting that the inverse association between PTEN and WDR66 expression levels may not depend entirely on the function of PTEN in regulating the PI3K signaling pathway. WDR66 belongs to the WD-repeat containing family of proteins, which collectively functions in the formation of protein-protein complexes in a variety of signaling pathways (30). Thus, a co-IP experiment between WDR66 and PTEN was performed, and the results suggested that there may be an interaction between PTEN and WDR66 in SACC83 cells (Fig. 1D).

To further determine the effect of the PTEN-WDR66 interaction on SACC pathogenesis, double IF staining was performed to examine the intracellular distribution of WDR66 in the control and under PTEN-deficient conditions. As presented in Fig. 1E, WDR66 expression (green) was inversely associated with PTEN expression (red).

WDR66 is essential for EMT and regulates the expression of genes associated with CSCs in the context of PTEN deficiency

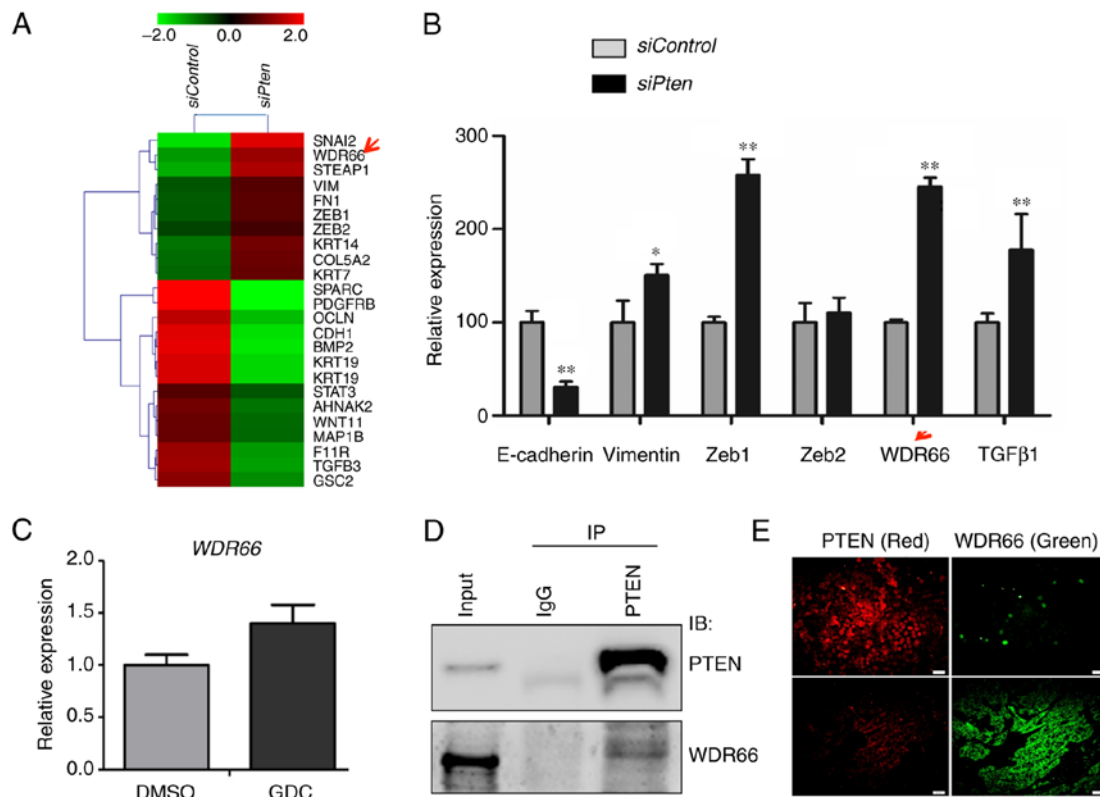


Figure 1. WDR66 is identified as one of candidates for EMT in the context of PTEN deficiency. (A) Heatmap of the EMT-associated genes in the microarray data of the SACC83 cell with *PTEN* knocked down (siPten) in comparison with control (siControl). Red represents an increase; green represents a decrease. (B) Validation of the expression of EMT-associated genes from the microarray data using RT-qPCR. *P<0.05; **P<0.01 vs. Control (independent sample t-test). (C) Expression of *WDR66* following treatment with a phosphoinositide 3-kinase inhibitor, GDC, determined using RT-qPCR. (D) Co-IP of PTEN and WDR66 protein in the SACC83 cell line. (E) Double immunofluorescence staining of PTEN (red) and WDR66 (green) in tissues of human salivary adenoid cystic carcinoma samples. Scale bar, 100 μm. WDR66, WD repeat-containing protein 66; EMT, epithelial-mesenchymal transition; PTEN, phosphatase and tensin homolog; si, short interfering RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GDC, GDC-0941; DMSO, dimethylsulfoxide; IP, immunoprecipitation; IB, immunoblot; E-cadherin, epithelial cadherin; Zeb, zinc finger E-box-binding homeobox; TGFβ1, transforming growth factor β1; IgG, immunoglobulin G.

in SACC. To facilitate the functional studies of WDR66, *WDR66* mRNA was knocked down using three siRNAs, and siRNA #1918 was selected on the basis of its optimal knockdown efficacy as determined using RT-qPCR (Fig. 2A). Knockdown efficacy of siRNA #1918 was validated by western blotting (Fig. 2B). siRNA #1918 was used to determine the function of WDR66 in mediating the EMT phenotype induced by PTEN deficiency. Downregulation of *CDH1* and upregulation of *VIM* mRNAs were confirmed in the SACC83 cells with *PTEN* knockdown (Fig. 2C). However, simultaneous knockdown of WDR66 mRNA with *PTEN* mRNA knockdown led to a complete reversal of the effect of *PTEN* siRNA on decreasing the *CDH1* expression level (Fig. 2C). Similar observations were made at the protein expression level on the basis of western blot results for E-cadherin and vimentin (Fig. 2D). Collectively, these results suggest that WDR66 may be essential for the maintenance of the EMT phenotype induced by PTEN deficiency in SACCs.

Since EMT has been suggested to confer CSC-like properties on neoplastic cells (31), the function of WDR66 in regulating the expression of genes associated with cancer cell stemness in the context of PTEN deficiency was also investigated. Similar to the gene changes in EMT, the embryonic stem cell genes *NANOG* and *OCT4*, and the putative CSC genes *ALDH1* and *SOX2* in SG cancer (32) were all

increased following *PTEN* knockdown in SACC cells. The expression of *NANOG* and *OCT4* genes was decreased following *WDR66* knockdown with or without *PTEN* knockdown (Fig. 3A). Similar observations were made at the protein expression level on the basis of western blot results for *NANOG* and *OCT4* (Fig. 3B). Collectively, these results suggest that WDR66 may be required to maintain the expression of genes associated with CSCs in the context of PTEN deficiency.

Knockdown of WDR66 decreases cell proliferation, migration and invasion in the context of PTEN deficiency. To determine the cellular effects of WDR66 in the context of PTEN deficiency, cellular proliferation assays were performed in SACC83 cells carrying *WDR66* knockdown with or without *PTEN* knockdown. As expected, knockdown of *PTEN* promoted cellular proliferation rates, but this effect was fully nullified with *WDR66* knockdown (Fig. 4A). Similarly, knockdown of *PTEN* accelerated wound closure, but simultaneous knockdown of WDR66 led to the attenuation of this effect to the point that there was no significant difference between the siPten + siWDR66 group and the siControl group (Fig. 4B). Finally, the migratory and invasive abilities that were enhanced by *PTEN* knockdown were also decreased with *WDR66* knockdown (Fig. 4C).

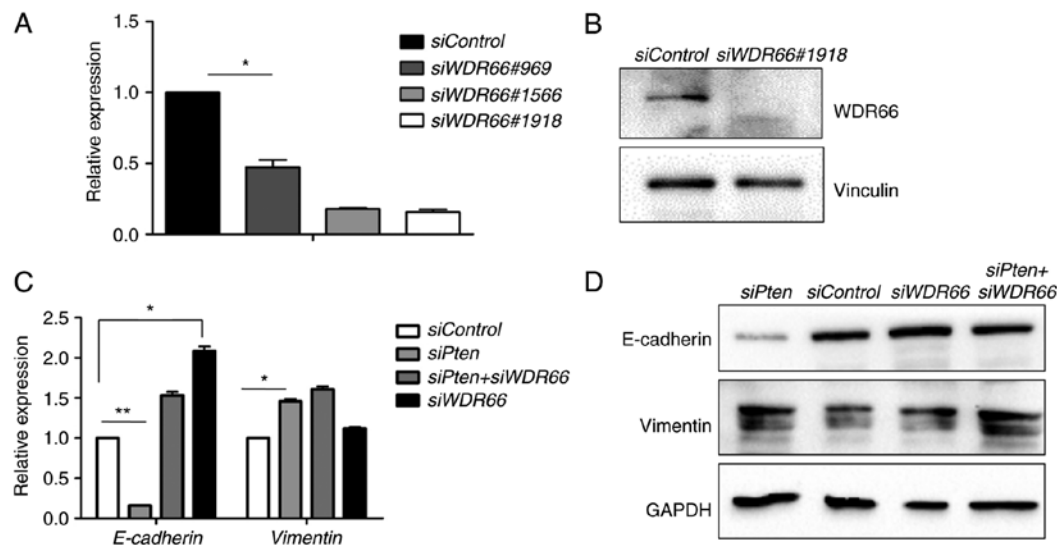


Figure 2. WDR66 is required to maintain the EMT phenotype in the context of PTEN deficiency. (A) RT-qPCR analysis of *WDR66* expression in SACC83 cells transfected with different siRNAs. (B) Western blot analysis of WDR66 expression in SACC83 cells transfected with the siWDR66 #1918. (C) RT-qPCR analysis of E-cadherin and vimentin expression in the SACC83 cells 4 days after transfection of siPten, siWDR66 and siPten + siWDR66. * $P < 0.05$, ** $P < 0.01$ (two-way analysis of variance followed by Dunn's post hoc test). (D) Western blot analysis of E-cadherin and vimentin expression in the SACC83 cells 4 days after transfection of siPten, siWDR66 and siPten + siWDR66. WDR66, WD repeat-containing protein 66; EMT, epithelial-mesenchymal transition; PTEN, phosphatase and tensin homolog; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; si, short interfering RNA; E-cadherin, epithelial cadherin.

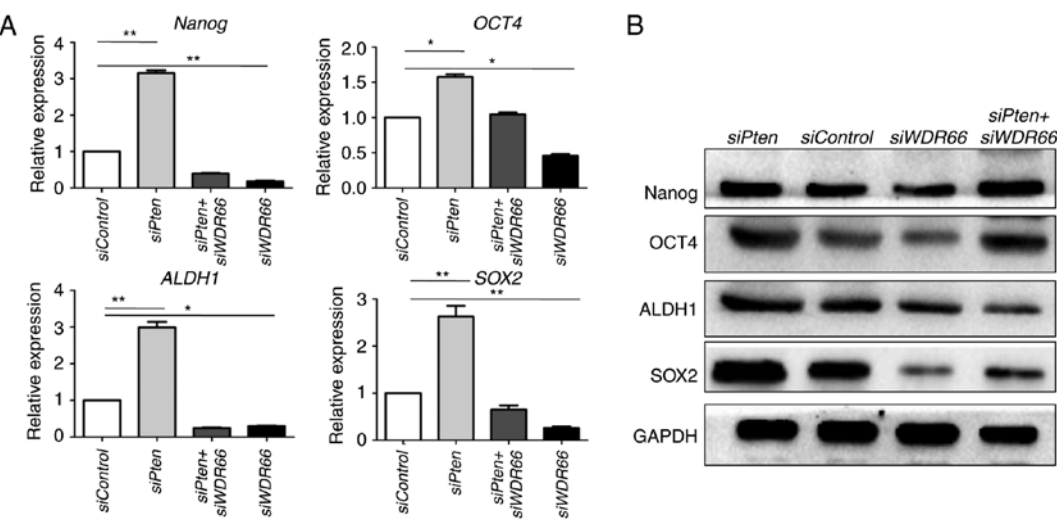


Figure 3. WDR66 is required to maintain expression of genes associated with cancer stem cells in the context of PTEN deficiency. (A) Reverse transcription-quantitative polymerase chain reaction analysis of genes associated with cancer stem cells in SACC83 cells transfected with siPten, siWDR66 and siPten + siWDR66. * $P < 0.05$, ** $P < 0.01$ (two-way analysis of variance followed by Dunn's post hoc test). (B) Western blot analysis of genes associated with cancer stem cells in SACC83 cells transfected with siPten, siWDR66 and siPten + siWDR66. WDR66, WD repeat-containing protein 66; PTEN, phosphatase and tensin homolog; si, short interfering RNA; ALDH1, aldehyde dehydrogenase 1; SOX2, sex-determining region Y box 2.

WDR66 expression is inversely associated with PTEN expression and is negatively associated with overall survival of patients with SACC. To search for genetic alterations of *WDR66* in human malignancies, a cross-cancer genomic analysis of *WDR66* was performed using The Cancer Genome Atlas (TCGA) database. Somatic point mutations and gene amplification were the most common genetic alterations in *WDR66* across various types of cancer. Notably, amplification of *WDR66* was the only genetic alteration noted in patients with SACC (Fig. 5). Next, WDR66 expression levels were determined in patient samples and associated with

PTEN expression in SACCs via IHC. A total of 46 SACC and 20 normal SGs were included. WDR66 was weakly expressed in normal SGs, and the majority of the tubular and cribriform SACCs, but it was markedly expressed in tumor cells of solid-pattern SACCs (Fig. 6A). This staining pattern of WDR66 was the opposite to the staining pattern of PTEN, where it was markedly expressed in normal SGs, tubular and cribriform SACCs, but was lost from the solid pattern SACCs (Fig. 6A). This inverse association of WDR and PTEN expression is exemplified in their opposite staining pattern in the same fields of tumor cells, as presented in Fig. 6A. The

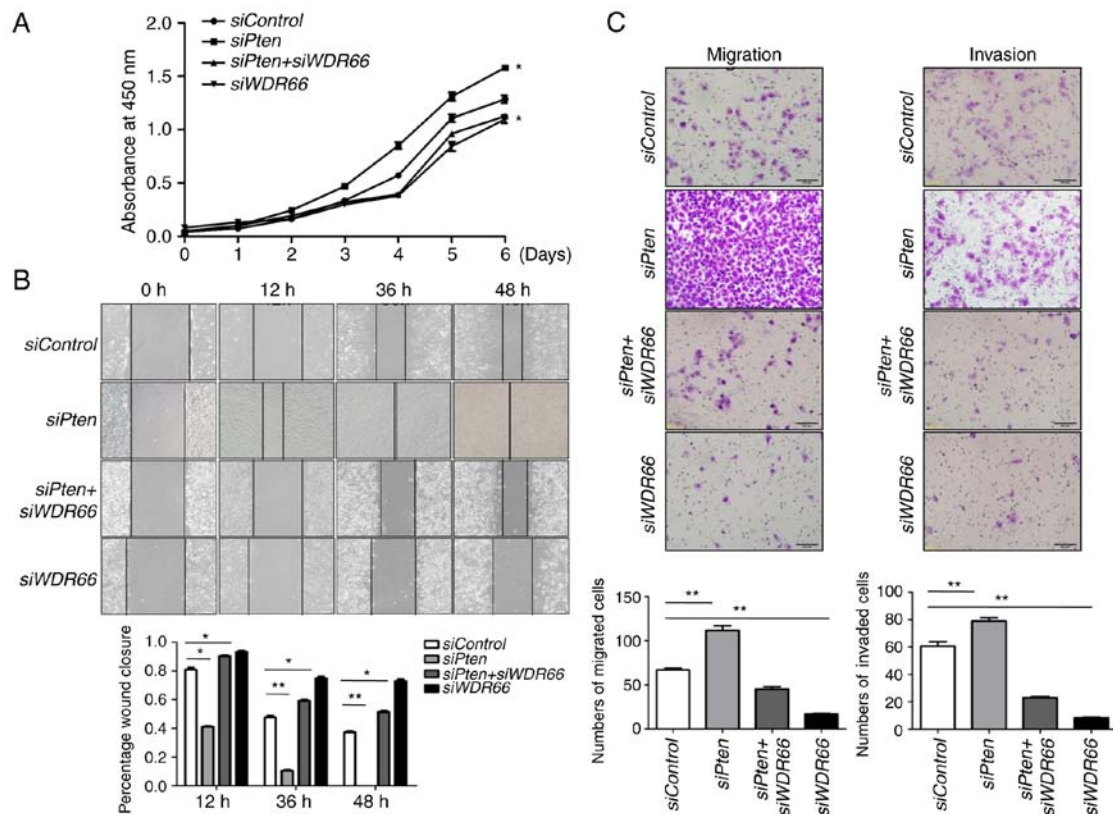


Figure 4. Knockdown of *WDR66* decreases cell proliferation, migration and invasion in the context of PTEN deficiency. (A) Cell proliferation determined using a Cell Counting Kit-8 assay in the SACC83 cell line transfected with siPten and/or siWDR66. * $P < 0.05$ vs. siControl (two-way repeated-measures ANOVA followed by Bonferroni's post hoc test). (B) Wound healing assays in the SACC83 cell line transfected with siPten and/or siWDR66. Images are representative. * $P < 0.05$, ** $P < 0.01$ (two-way ANOVA followed by Dunn's post hoc test). (C) Migration (left) and invasion (right) assays in the SACC83 cell line transfected with siPten and/or siWDR66. Images are representative. ** $P < 0.01$ (two-way ANOVA followed by Dunn's post hoc test). *WDR66*, WD repeat-containing protein 66; PTEN, phosphatase and tensin homolog; si, short interfering; ANOVA, analysis of variance.

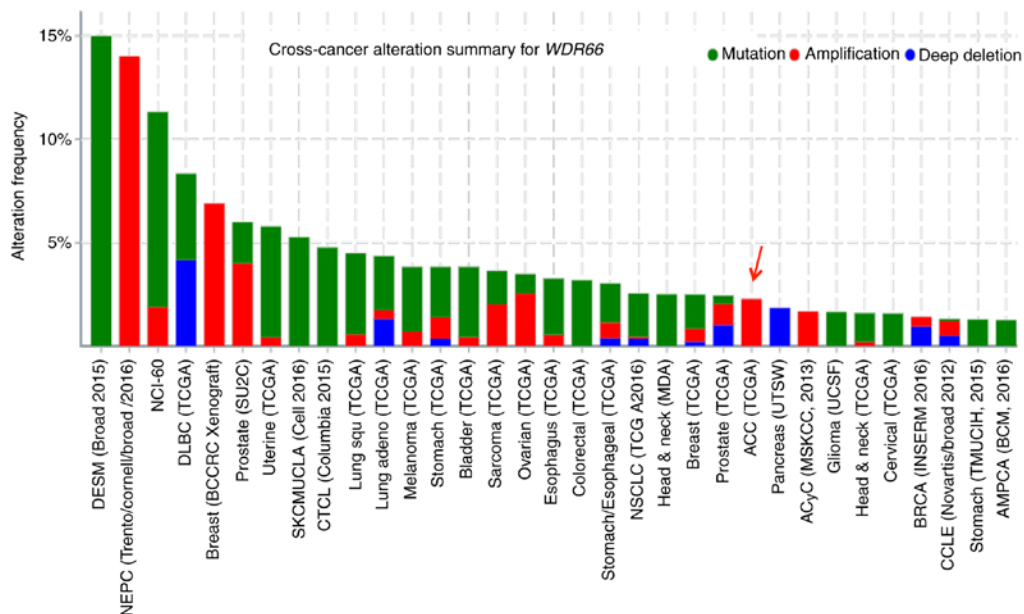


Figure 5. Genetic alterations of the *WDR66* gene in a variety of human malignancies. Data were extracted from The Cancer Genome Atlas database (cBioportal). *WDR66*, WD repeat-containing protein 66.

IHC staining results were summarized in Fig. 6B, and revealed a statistically significant inverse correlation between *WDR66* and PTEN expression in patient tissues ($P = 0.044$). Finally,

the significance of *WDR66* on prognosis of the 46 patients with SACC was investigated. Kaplan-Meier analysis demonstrated that the patients with positive *WDR66* expression

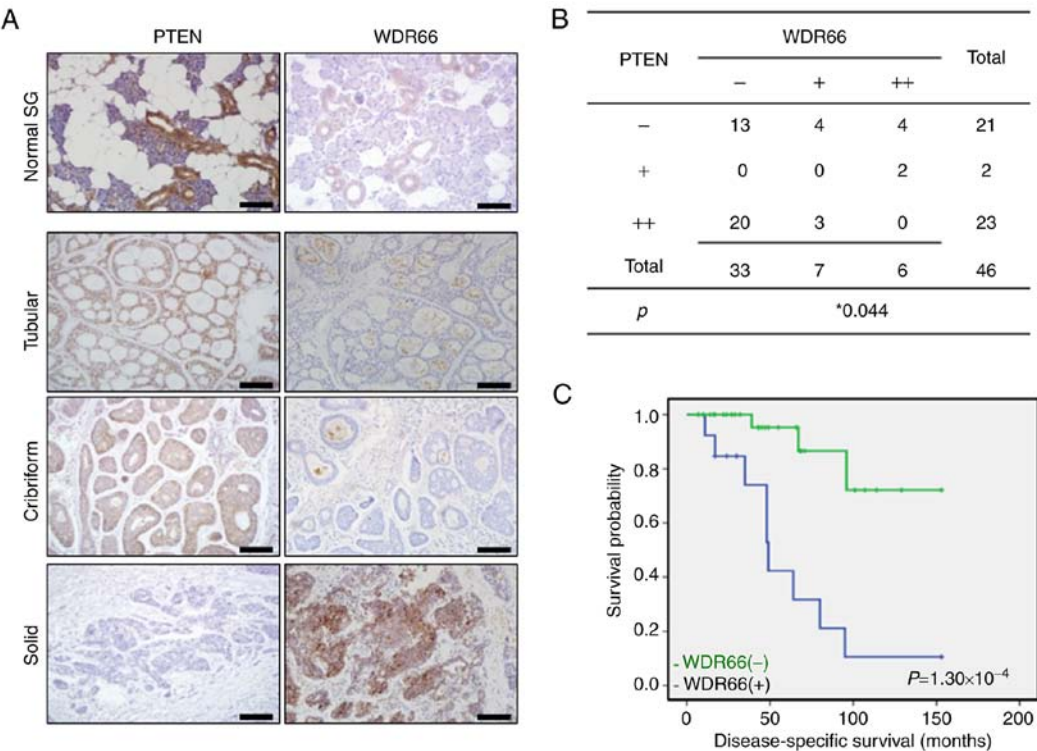


Figure 6. WDR66 expression is inversely associated with PTEN expression and is associated with the survival of patients with SACC. (A) IHC staining of PTEN and WDR66 in normal human salivary glands and three subtypes of SACC. Scale bar, 100 μ m. (B) Summary of PTEN and WDR66 IHC results. Spearman rank correlation analysis. (C) Kaplan-Meier survival curve of the patients with SACC with and without WDR66 expression. WDR66, WD repeat-containing protein 66; PTEN, phosphatase and tensin homolog; SACC, salivary adenoid cystic carcinoma; IHC, immunohistochemistry; SG, salivary gland.

had significantly decreased overall survival times compared with patients with negative WDR66 expression (70.1 months vs. 121.8 months, $P=1.30 \times 10^{-4}$; log-rank test; Fig. 6C), which revealed that WDR66 expression was negatively associated with the overall survival periods of patients with SACC.

Discussion

We have demonstrated previously that the loss of PTEN or decreased PTEN expression is common in human SACCs, particularly in the most aggressive solid pattern form (24). To further understand the molecular mechanism of SACC pathogenesis in the context of PTEN deficiency, a gene expression profile array was performed in the SACC cells with ectopic *PTEN* knockdown (24). Although the knockdown approach using siRNA may not be able to completely eliminate PTEN expression, it represents at least a significant portion of patients with SACC who exhibited decreased PTEN expression. A complete knockout of PTEN using the clustered regularly interspaced short palindromic repeats system should provide additional information for patients with SACC with complete loss of PTEN in future studies. In the present study, the array results were further analyzed, specifically for genes associated with the EMT process which was one of the enriched signatures in SACC cells with *PTEN* knockdown. Individual gene expression validation of EMT regulators and EMT-associated transcription factors confirmed the microarray data analysis, and one EMT-associated gene, *WDR66*, was selected to be the focus of the present study due to the relative novelty of the function of WDR66 in human cancer and interaction with PTEN.

WDR66 belongs to the WD-repeat containing family of proteins which functions in the formation of protein-protein complexes in a variety of signaling pathways (30), and affects a wide variety of physiological and pathological conditions, including cancer. Perhaps the best example of a tumor regulator within this family is WDR7, which is an E3 ubiquitin ligase involved in human cancer through multiple mechanisms (33,34). Compared with WDR7, reports of oncogenic relevance of other WD repeat domain-containing protein family members is relatively limited. Overexpression of WDR5 has been identified to be correlated with the aggressiveness of head and neck squamous cell carcinoma (35), whereas WDR79 has been suggested to promote the proliferation of lung cancer cells through mediation of the p53 pathway (36). To date, the best-known function of WDR66 has been in the determination of mean platelet volume (37). In the oncology field, to the best of our knowledge, the only previous study on WDR66 has suggested its association with EMT in esophageal tumors (38).

SACC is characterized as a long indolent growth, but it is a disease that is manifested with a disproportionately high rate of hematogenous spread compared with the relatively low rate of local node metastasis (9). The EMT process has been well-established as the primary mechanism for neoplastic cells to invade blood vessels, survive in the harsh circulatory system and regenerate new tumors by the acquisition of CSC properties (39,40). PTEN deficiency and WDR66 overexpression have been reported individually in colon cancer (41) and esophageal cancer (38), respectively. However, to the best of our knowledge, there have not been any studies that suggest the connection of PTEN and WDR66 in the EMT process within a

single tumor context. The results of the present study indicated that PTEN loss significantly decreased E-cadherin expression at the mRNA and protein levels. In contrast, knocking down WDR66 expression increased the *CDH1* mRNA level, but no significant increase of E-cadherin at the protein level was identified. It is possible that the protein level changes may take longer in WDR66 compared with those of PTEN. Nonetheless, the results indicated an association of EMT/CSC with the expression levels of PTEN and WDR66. More importantly, the attenuation of *PTEN*-knockdown-induced EMT/CSC phenotypes upon *WDR66* knockdown highlighted the functional dependence on WDR66 status of the PTEN-deficiency phenotype in SACCs. The present study was not able to fully elucidate the molecular mechanisms underlying the interaction between PTEN and WDR66 in the context of the EMT process in SACCs. It may be useful to investigate the mechanism of WDR66 in mediating platelet volume (37). Platelets have been revealed to affect the maintenance of the EMT phenotype (42,43), therefore improved understanding the function of WDR66 in platelet volume may lead to an improved understanding of its function in EMT.

The inverse association between PTEN and WDR66 expression has been convincingly documented in the IHC analysis of patient tissues. A question is whether this association is dependent on the enzyme activity of PTEN in the PI3K signaling pathway. In contrast with the predicted decrease of WDR66 expression if the inverse correlation between PTEN and WDR66 depends on PI3K activity, it was identified that WDR66 expression is paradoxically increased. The reason for this increase is unclear, but the results of the present study suggested at least that this inverse correlation may not depend on PI3K activity. One of the primary functions of the WD repeat-containing protein family is forming protein-protein complexes as a structural protein (30). The physical interaction between WDR66 and PTEN demonstrated by the co-IP experiment suggested potential mechanisms associated with physical interactions between WDR66 and PTEN molecules. One limitation of the present study is that there are no data from SACC cells overexpressing WDR66, which will be helpful to improve understanding of the function of WDR66 in mediating PTEN deficiency-associated SACC pathogenesis. This represents a direction for future studies.

Despite the limitations, the present study revealed several translational effects for patients with SACC. First, the study identified WDR66 to be a key mediator of oncogenic effects caused by PTEN deficiency, suggesting WDR66 to be a novel therapeutic target in SACC tumors with PTEN deficiency. WDR66 gene amplification was noted in SACCs via analysis of TCGA database. Furthermore, the inverse association of WDR66 and PTEN expression in patient tissue samples suggested that WDR66 can also be increased in tumors with PTEN deficiency. Whether this negative association between WDR66 and PTEN expression exists in other types of cancer is a question to investigate further. However, the mutational status of PTEN in the patients of the present study is unknown, and patients with wild-type PTEN are unable to be selected for survival comparison. Future study of PTEN mutation status in the patient population used will provide additional information to further address PTEN deficiency in SACC pathogenesis, although the results of the present study have identified that the

survival of patients with SACC with PTEN loss or decreased expression is significantly poorer compared with those with normal PTEN expression level (24). Secondly, the independence of the PI3K signaling pathway for this negative association between WDR66 and PTEN suggested that in PTEN-deficient SACC tumors, targeting the PI3K signaling pathway may not be a good option, particularly in controlling tumor progression and metastasis. Instead, WDR66 itself may appear as a novel target for targeted therapy, particularly in PTEN-deficient human cancer, which includes a certain proportion of SACCs in the present study, and more broadly for expanding to other types of human cancer with PTEN deficiency (44). Thus, targeting WDR66 may be more effective in controlling tumor progression and metastasis in patients with cancer with PTEN deficiency.

Acknowledgements

Not applicable.

Funding

The present study was supported by the China Postdoctoral Science Foundation (grant nos. 2017M621178 and 2015M580227), the National Institutes of Health (grant no. R01DE021788) and Cancer League of Colorado.

Availability of data and materials

All data and materials used in the present study are available from the corresponding authors upon request.

Authors' contributions

YC, HL, SLL and JX conceived and designed the study. YC, HL, SLX, XZ, HB, QY and LG performed the experiments. JL, FJ and MJW contributed to the study design, analysis and interpretation of data. YC, HL, SLL and JX wrote the paper. SLL and JX supervised the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All patient samples were collected under the approved guidelines provided by the Institutional Review Boards at Dalian Medical University (Dalian, China). 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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