



PRL-3 promotes migration and invasion and is associated with poor prognosis in salivary adenoid cystic carcinoma

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BACKGROUND: PRL-3 had been found to be involved in tumorigenesis in various malignancies. In this study, we investigated the role of PRL-3 in the development, migration, and invasion of salivary adenoid cystic carcinoma (SACC).

METHODS: Immunohistochemistry (IHC) was used to analyze the role of PRL-3 in the development and prognosis of SACC. Then, we overexpressed or inhibited the expression of PRL-3 in paired SACC cells to analyze the role of PRL-3 in the migration and invasion of SACC. *In vitro* migration and invasion assays were used. Western blotting was used to detect metastasis-related protein levels.

RESULTS: IHC results confirmed that the deregulation of PRL-3 was a frequent event in SACC; the upregulation of PRL-3 was related to clinical stages, vital status, and distant metastasis, which was associated with reduced overall survival and disease-free survival. SACC-LM cells with higher migratory and invasive abilities had more robust PRL-3 protein expression than SACC-83 cells with lower migratory and invasive abilities. PRL-3 overexpression promoted cell migration, invasion, and proliferation, led to simultaneous upregulation of phosphorylated PRL-3, pERK1/2, Slug, vimentin, and downregulation of E-cadherin in SACC-83 cells. However, the inhibition of PRL-3 by PRL-3 inhibitor or PRL-3 siRNA in SACC-LM cells inhibited cell migration, invasion, and proliferation, resulted in simultaneous downregulation of phosphorylated PRL-3, pERK1/2, Slug, vimentin, and upregulation of E-cadherin.

CONCLUSIONS: Our results confirm that PRL-3 plays an important role in the development of SACC and contributes to the migratory and invasive abilities of SACC.

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Keywords: invasion; migration; PRL-3; prognosis; salivary adenoid cystic carcinoma

Introduction

Salivary adenoid cystic carcinoma (SACC) is a common malignant neoplasm that arises within the major and minor salivary glands of the head and neck, accounting for 10–18% of all salivary malignancies (1). The biological properties of SACC include aggressive local growth, frequent local recurrence, nerve invasion, blood vessel invasion, and distant metastasis, which result in poor patient survival. Until now, a great deal of research has focused on the possible biomarkers that may be involved in the progression and metastasis of SACC. Several growth factors, such as epidermal growth factor, nerve growth factor, and transforming growth factor beta (TGF- β), are known to promote the invasion of SACC cells (2–4). Our previous study also confirmed that Bmi-1 deregulation induced metastasis of SACC (5). Some signaling molecules have also been found to be involved in the metastasis of SACC. These include mitogen-activated protein kinase 1/2 (MAPK1/2), snail homolog 2 (Snail2, also known as Slug), and matrix metalloproteinases (MMPs) (6–8). Other non-coding genes, such as microRNAs, have also been implicated in the metastasis of SACC (9). Our previous study found that microRNA-181a suppresses the invasion and metastasis of SACC by targeting the ERK–Slug pathway (9).

PRL-3, which belongs to the phosphatase of regenerating liver (PRL) family, was first found to be highly expressed in liver metastases of patients with colorectal cancer (10). Until now, much literature had suggested that high expression of PRL-3 was considered to be associated with a poor prognosis in many types of malignant tumors (11–15),

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including acute myeloid leukemia (11), gastric cancer (12), ovarian cancer (13), and hepatocellular cancer (14). PRL-3 had been found to be involved in the following biological function: cytoskeleton rebuilding (16); the regulation of cell adhesion to promote invasion and metastasis of cancer cells (17); the activation of the Rho family and MMP-2 to stimulate the invasion and motility of cancer cells (17, 18); tumor growth through the mechanism of epithelial–mesenchymal transformation (EMT) mediated by the AKT, PI3K, and STAT pathways (19–21).

To investigate the role of PRL-3 in the development of SACC, we analyzed and compared the expression of PRL-3 in clinical SACC samples and normal salivary gland tissues using immunohistochemical staining. To determine the function of PRL-3 in the migratory, invasive, and proliferative abilities of SACC, we either overexpressed or inhibited the expression of PRL-3 in paired SACC cell lines (SACC-83/LM). We found that PRL-3 plays a significant role in the development and prognosis of SACC and promotes the migration and invasion of SACC via ERK–Slug pathway.

Materials and methods

Patients and samples

A total of 50 SACC primary tumor samples without preoperative chemotherapy or radiotherapy and 20 normal salivary gland tissue samples were collected from the archives of the Cancer Center, Sun Yat-sen University between 1998 and 2010. All the clinical tumor samples were reconfirmed by an expert ACC pathologist. The clinicopathologic characteristics of normal salivary glands are presented in Supplementary Table S1. The tumor staging was assessed according to the UICC staging system. Survival was calculated from the diagnosis day to the date of latest follow-up (or death). The median duration of follow-up was 64 months (range 12–139 months). The Institute Research Ethics Committee approved the use of these clinical materials for research purposes.

Immunohistochemical staining

Immunohistochemistry (IHC) was performed on 5-mm sections of formalin-fixed, paraffin-embedded tissue samples as previously described (5). Briefly, the paraffin section was deparaffinized with xylene and dehydrated in alcohol. Antigen retrieval was treated with boiling citrate buffer (pH 6.0), and the endogenous peroxidase activity was blocked by 3% H₂O₂. Then, the section was stained with anti-PRL-3 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C and incubated with the MaxVision™ HRP-Polymer anti-Mouse IHC Kit (Maixin Biotechnology, Fuzhou, China) for 10 min at room temperature. Finally, the section was dyed with the DAB Horseradish Peroxidase Color Development Kit (Maixin) and counterstained with hematoxylin. The degree of immunostaining was scored independently by three observers according to the proportion of positively stained tumor cells and the intensity of staining. The proportion of tumor cells was scored as 0, 1, 2, 3 (0, <30%, 30–50%, and >50% positive tumor cells, respectively). The intensity of staining was graded as 0, 1, 2, 3 (no, weak [light yellow], moderate

[yellow brown], and strong staining [brown or dark brown, respectively]). The staining index was calculated as staining intensity score × proportion of positive tumor cells, and the results are 0, 1, 2, 3, 4, 6, and 9. An optimal cutoff value (median) was identified: The staining index score of ≥4 was used to define tumors as high PRL-3 expression and <4 as low expression of PRL-3.

Cell culture and transfection

The SACC-LM/SACC-83 cell line (a gift from Dr. Shenglin Li), which originated from a patient's sublingual gland SACC cells, has been confirmed as an authentic adenoid cystic carcinoma cell line by short tandem-repeat analysis (STR) and immunostaining (4). The SACC-LM cell line is more aggressive than SACC-83 cell line in terms of lung metastatic rate (4, 22). Cells were maintained in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified incubator with 5% CO₂. For further study, PRL-3 siRNA or control non-targeting siRNA (Genepharma, Suzhou GenePharma Co., Ltd., Suzhou, China), plasmid-containing PRL-3 cDNA (NM_032611) or control plasmid (pDNA3.1) were transfected into the relevant SACC cell lines using Lipofectamine transfection reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions, as previously described (5). PRL-3 inhibitor (1-4-bromo-2-benzylidene rhodanine, CAS 893449-38-2; Santa Cruz Biotechnology), which was dissolved in DMSO (final concentration <1‰), was used to treat the SACC-LM cell line (DMSO as control). The sequence of PRL-3 siRNA used for transfection is CCC UUA UUG AGA GCG GGA UTT. The control siRNA is GCU ACA AAC ACA UGC GCU UTT.

Cell Immunohistochemical staining

Cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. Endogenous peroxidase activity was blocked by 3% H₂O₂ for 20 min, followed by staining with anti-PRL-3 antibody (Santa Cruz Biotechnology) overnight at 4°C. After washing, the sections were incubated with MaxVision™ HRP-Polymer anti-Mouse IHC Kit (Maixin), developed with DAB Horseradish Peroxidase Color Development Kit (Maixin), and counterstained with hematoxylin. Images were captured by the Zeiss image digital camera (Carl Zeiss Microscopy GmbH, Jena, Germany).

In vitro cell migration and invasion assays

Transwell assays were performed to assess cell migration and invasion using BD BioCoat Control Cell Culture Inserts or the BD BioCoat BD Matrigel™ Invasion Chamber (BD Biosciences, San Jose, CA, USA). In brief, cells were seeded in the upper Boyden chambers of the cell culture inserts. After 24 h of incubation, cells remaining in the upper chamber were carefully removed. Cells adhering to the lower membrane were stained with DAPI in the dark, and then imaged and counted using an inverted microscope equipped with the Zeiss Image digital camera. Three random fields were captured at 200× magnification. The number of cells on the bottom surface was compared between the two groups.

Cell proliferation assays

Cell proliferation was measured using the Cell Counting Kit-8 assay (CCK-8). Cells were seeded in 96-well plates at 5×10^3 cells/well (for SACC-LM) or 1×10^4 cells/well (for SACC-83) in 100 ml of culture medium. After 24, 48, or 72 h of incubation, the medium was removed and the CCK-8 reagent (Dojindo, Kumamoto Techno Research Park, Kumamoto, Japan) was added to each well and incubated for 1 h. The absorbance value of each well was assayed using a plate reader at a wavelength of 450 nm, and the OD value was compared between groups.

Western blot analysis

Cells were harvested by scraping into ice-cold RIPA buffer containing PMSF (MP Biomedicals, Solon, OH, USA). The protein concentration was measured with a BCA protein assay kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions. Western blots were

performed as described previously (23), using antibodies specific for PRL-3, ERK (extracellular signal-regulated kinase) 1/2, phosphorylated ERK1/2 (pERK1/2), Slug, E-cadherin, vimentin, and using GAPDH as control (Cell Signaling Technology, Danvers, MA, USA).

Statistical analysis

Data were expressed as the mean \pm SD, and all experiments were performed in triplicate. All statistical analyses were carried out using the statistical software package for the Social Science (SPSS 13.0, Chicago, IL, USA). Student's *t*-test was used to compare the difference between groups. The chi-squared test was used to analyze the correlation between gene expression and the clinical pathologic characteristics. Survival curves were plotted using the Kaplan–Meier method and compared with the log-rank test. Cox regression (forward model) was used for both univariate and multivariate analysis. $P < 0.05$ in all cases was considered statistically significant.

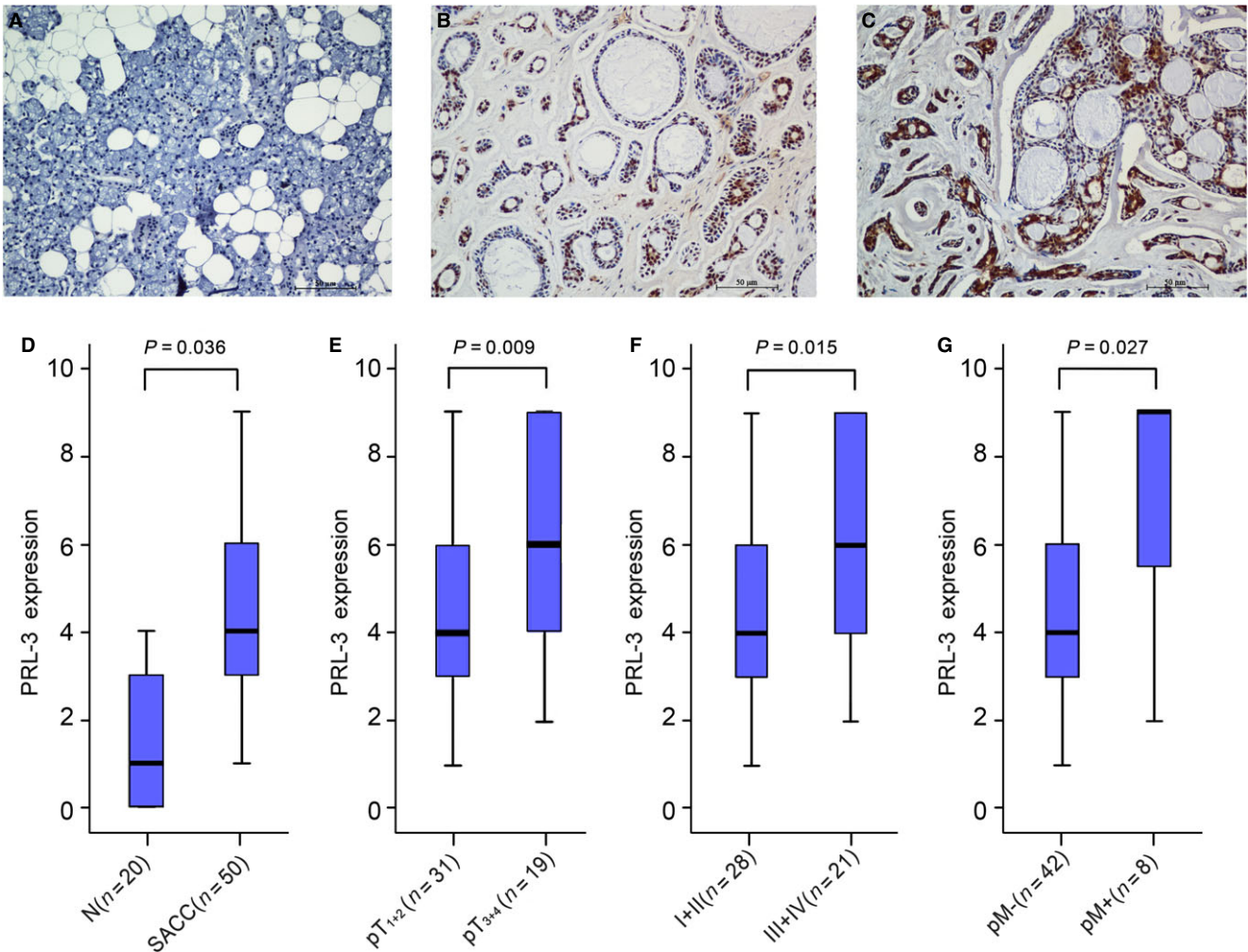


Figure 1 Immunohistochemistry (IHC) analyses of PRL-3 expression in normal salivary gland and salivary adenoid cystic carcinoma (SACC) tissue samples. IHC analyses for PRL-3 were performed as described in the Material and Methods on A: normal salivary gland; B: salivary gland adenoid cystic carcinoma without lung metastasis; C: salivary gland adenoid cystic carcinoma with lung metastasis. Scale bar: 50 μm. The expression level of PRL-3 in SACC tissue is shown in D–G. Box plots are presented for comparing the expression of PRL-3 between normal salivary gland and SACC cases (D), and in SACC cases with different tumor stages (E), different clinical stages (F), with or without distant metastasis (G). The boxes represent the 25th to 75th percentile of the observations, and the lines in the middle of the box represent the median.

Results

PRL-3 deregulation in the development of SACC

To confirm the relationship between PRL-3 and SACC, PRL-3 expression was examined by IHC in 50 cases of SACC and 20 normal salivary gland tissues. As illustrated in Fig. 1, PRL-3 was detected both in the cytoplasm and the cell nucleus. PRL-3 was less detectable in normal tissues (Fig. 1A), but there was pronounced enhanced expression of PRL-3 in SACC samples from patients without (Fig. 1B) or with (Fig. 1C) lung metastasis. PRL-3 expression was significantly increased in primary cancer tissues compared with normal tissues (Fig. 1D). Among SACC cases, PRL-3 levels were significantly higher in: pT3 + 4 vs. pT1 + 2; late clinical stage (stage III and IV) vs. early clinical stage (stage I and II) (Fig. 1E,F). Statistically significant increased PRL-3 expression was also observed in SACC samples with positive distant metastasis status (pM+) compared with those with negative status (pM-) (Fig. 1G).

Correlations between PRL-3 expression and clinicopathologic characteristics of patient with SACC

The correlation between PRL-3 expression and clinicopathologic variables of patients with SACC is shown in Table 1. These observations show that high levels of PRL-3 were associated with tumor stages ($P = 0.009$), clinical stages ($P = 0.015$), distant metastasis ($P = 0.027$), tumor site ($P = 0.046$), recurrence ($P = 0.031$), and vital status ($P = 0.009$). No relationship was found between PRL-3 expression and gender ($P = 0.153$) or age ($P = 0.643$).

Table 1 Correlations between PRL-3 expression and clinicopathologic characteristics of patients with SACC

Characteristics	PRL-3 expression		P-value*
	Low	High	
Gender			
Male	9	10	0.153
Female	21	10	
Age (years)			
≤46	17	10	0.643
>46	13	10	
Clinical stage			
I + II	21	7	0.015
III + IV	9	13	
Tumor stage			
T ₁ + 2	23	8	0.009
T ₃ + 4	7	12	
Distant metastasis			
Negative	28	14	0.027
Positive	2	6	
Recurrence			
Negative	27	12	0.031
Positive	3	8	
Tumor site			
Parotid	16	7	0.046
Submandibular	10	13	
Sublingual	4	0	
Vital status			
Alive	29	13	0.009
Death (tumor-related)	1	7	

*Chi-square test.

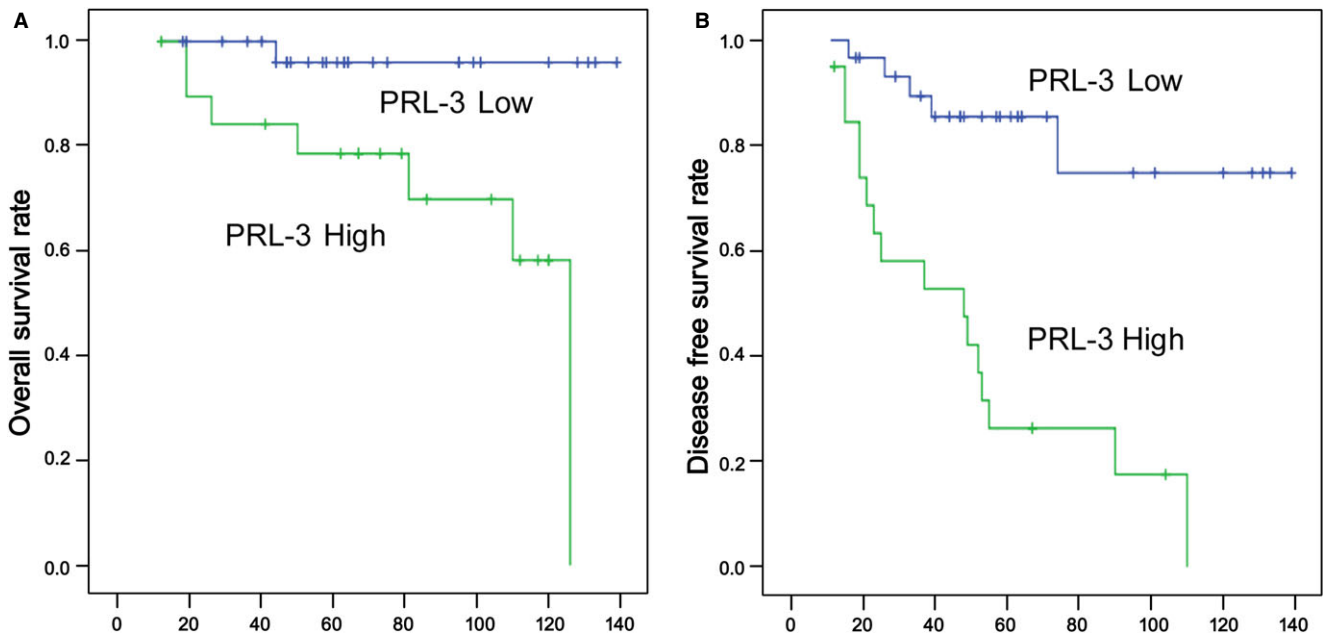


Figure 2 The effects of PRL-3 expression on prognosis. Kaplan-Meier plots of 5-year overall survival (OS) (A) and 5-year disease-free survival (B) in patient groups defined by PRL-3 immunohistochemistry. A statistically significant difference in survival was associated with 5-year OS and PRL-3 expression ($P = 0.005$), 5-year disease-free survival, and PRL-3 expression ($P = 0.000$). In both A and B, the cases number for PRL-3 high group and PRL-3 low group were 20 and 30, respectively. A: The number censored for group of high PRL-3 expression or low PRL-3 expression was 13 and 29, respectively. B: The number censored for group of high PRL-3 expression or low PRL-3 expression was 4 and 25, respectively.

Table 2 Univariate and multivariate analyses for 5-year overall survival (OS) and 5-year disease-free survival in patients with SACC

	Univariate analysis		Multivariate analysis	
	P	Regression coefficient (SE)	P	Relative risk (95% CI)
OS				
Clinical stage	0.156	1.187 (0.837)	–	–
Expression of PRL-3	0.025	2.432 (1.084)	0.048	9.207 (1.020–83.137)
T classification	0.079	1.479 (0.840)	0.635	–
Distant metastasis	0.074	1.372 (0.767)	0.660	–
Disease-free survival				
Clinical stage	0.116	0.694 (0.442)	–	–
Expression of PRL-3	<0.001	1.937 (0.516)	0.106	–
T classification	0.061	0.822 (0.438)	0.916	–
Distant metastasis	<0.001	1.867 (0.470)	0.006	6.507 (1.701–24.898)
Gender	0.642	–0.215 (0.464)	–	–
Age	0.843	–0.090 (0.453)	–	–
Recurrence	<0.001	1.994 (0.471)	<0.001	1.090 (2.444–20.569)

Cox regression (forward model) was used for both univariate and multivariate analysis. All statistical analyses were carried out using the statistical software package for the Social Science (SPSS 13.0). When the *P* value of factors was <0.1 in the univariate analysis, the factors were then analyzed by the multivariate analysis.

The prognostic value of PRL-3 deregulation in patients with SACC

To elucidate the prognostic role of PRL-3 expression in patients with SACC, we examined the relationship between PRL-3 expression and patient outcome with long-term follow-up. As illustrated in Fig. 2A, a striking difference in overall survival (OS) was observed between the high PRL-3 expression group (mean survival = 74.6 months) and the low PRL-3 expression group (mean survival = 70.0 months) (*P* = 0.005). A statistically significant difference in survival was also associated with disease-free survival (PFS) and PRL-3 expression (*P* < 0.001) (Fig. 2B).

To further evaluate the impact of PRL-3 expression and clinicopathological factors on the prognosis of patients with SACC, univariate and multivariate analyses were carried out. As illustrated in Table 2, univariate analysis indicated that PRL-3 expression, T classification, and distant metastasis were independent prognostic factors for 5-year OS, and only PRL-3 expression was associated with poor prognosis in the multivariate analysis. However, for 5-year disease-free survival, both univariate and multivariate analyses indicated that only distant metastasis and recurrence were independent prognostic factors. Thus, our findings indicate that PRL-3 expression level is significantly correlated with the prognosis of SACC.

PRL-3 overexpression promotes the migration and invasion of SACC

As shown in Fig. 3A, the protein level of PRL-3 and phosphorylated PRL-3 were higher in SACC-LM cells than

in the SACC-83 cells as detected by Western blotting (Fig. 3A) and cell IHC (Fig. 3B). To further investigate whether PRL-3 promotes the migration and invasion of SACC, we overexpressed PRL-3 in SACC-83 cells (Fig. 3C). SACC-83 cells overexpressing PRL-3 displayed increased phosphorylated PRL-3 (Fig. 3C), migration, and invasion compared with the control plasmid-transfected cells (Fig. 3D,E). The protein levels of pERK1/2, Slug, and vimentin were increased, and the protein levels of ERK1/2 and E-cadherin were decreased (Fig. 3C). Furthermore, PRL-3 overexpression increased the proliferation rate of SACC-83 cells compared with that of controls (Fig. 3F).

PRL-3 downregulation inhibits the migration and invasion of SACC

To demonstrate whether inhibiting the expression of PRL-3 inhibits the migration and invasion of SACC-LM cells, we knocked down the expression of PRL-3 by RNA interference or treated the cells with PRL-3 inhibitor, which had been verified to have anticancer effect in esophageal squamous cell carcinoma (24). The protein level of PRL-3 and phosphorylated PRL-3 was obviously decreased in SACC-LM cells after transfection with PRL-3 siRNA or treatment with PRL-3 inhibitor (Fig. 4A or Fig. 5A). SACC-LM cells transfected with PRL-3 siRNA or treated with PRL-3 inhibitor displayed decreased migration and invasion abilities compared with the control siRNA-transfected cells (Fig. 4B,C) or DMEM control-treated cells (Fig. 5B,C). After inhibiting the expression of PRL-3 in SACC-LM cells, pERK1/2, Slug, and vimentin were obviously decreased, and the protein levels of ERK1/2 and E-cadherin were obviously increased (Fig. 4A or Fig. 5A). Furthermore, PRL-3 downregulation resulted in a reduction of the cell proliferation rate (Fig. 4D and Fig. 5D).

Discussion

PRL-3, also known as PTP4A3, belongs to the family of tyrosine phosphatases, which play a fundamental role in regulating protein phosphorylation balance (25). PRL-3 expression has been evaluated in various human cancers, and it was found to be associated with invasion, metastasis, and poor prognosis (12, 15). Wang et al. found that PRL-3 protein was upregulated in many types of human cancer (26). In this study, we found that PRL-3 expression was increased in SACC tissues compared with normal salivary gland tissues. We further demonstrated that higher levels of PRL-3 expression were associated with pT and distant metastasis. Patients with high-level PRL-3 expression have significantly poorer OS compared with those with low-level expression. Taken together, these data suggest that PRL-3 plays an important role in SACC and is also an important prognostic factor for SACC. Although PRL-3 may play an important role in SACC, it is not known to be mutated in SACC, which was confirmed by Stephens (27).

PRL-3 expression had been found to be associated with metastasis in many types of tumors (12, 15). In the present study, we found that patients with high-level expression of PRL-3 have a high risk of lung metastasis.

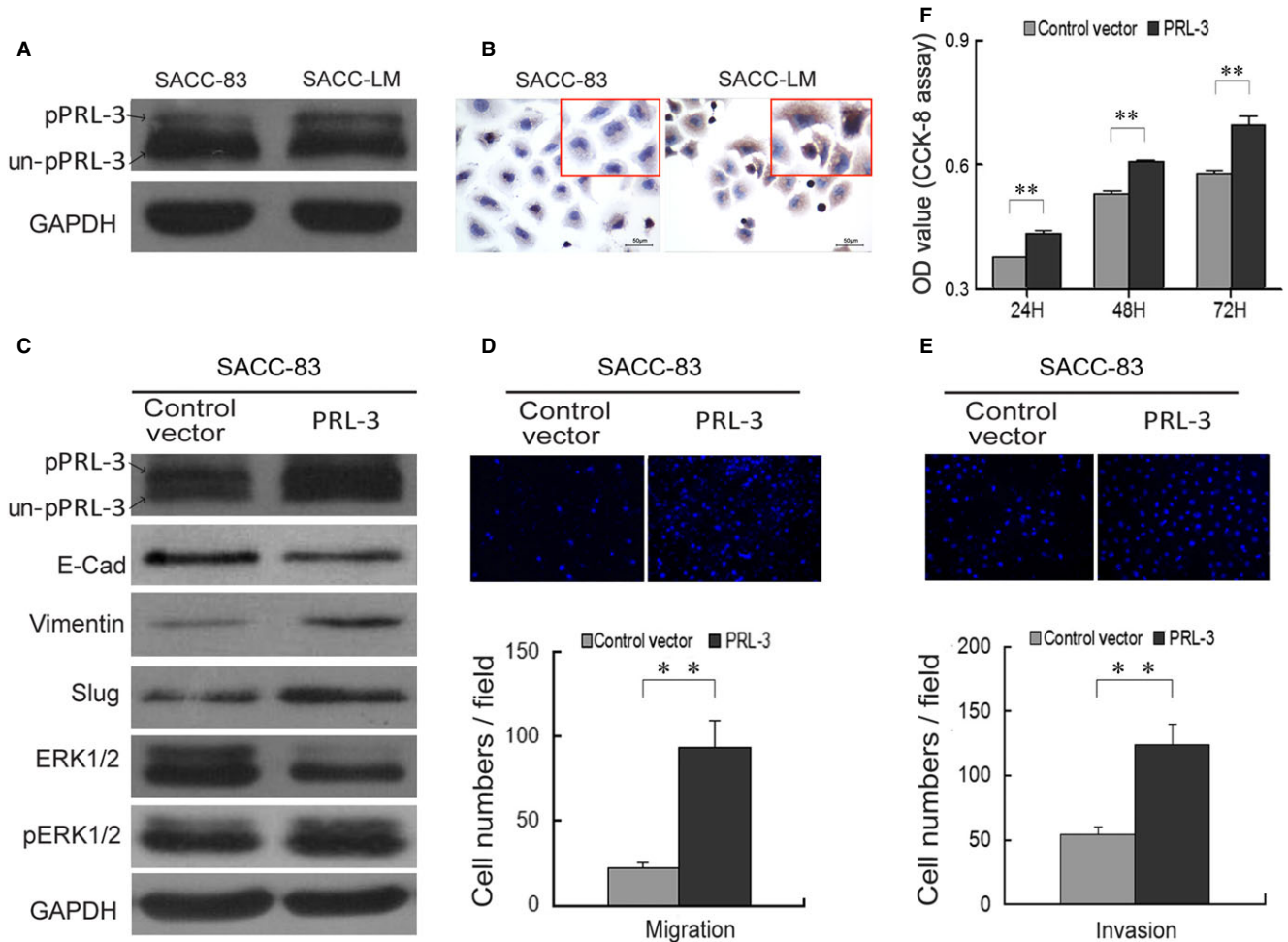


Figure 3 PRL-3 overexpression promotes the migration and invasion of salivary adenoid cystic carcinoma (SACC). (A, B) PRL-3 protein level and phosphorylated PRL-3 in SACC-LM cells was higher than that in SACC-83 detected by Western blot (A) and Immunohistochemistry (IHC) (B, Magnification 400 \times). An amplified image containing representative cells in a small square was present for each cell line (B). (C) Western blotting shows the protein levels of pERK1/2, Slug, and vimentin were obviously increased and the protein levels of ERK1/2 and E-cadherin were obviously decreased in SACC-83 cells after overexpression of PRL-3. (D, E) The migratory and invasive abilities of SACC-83 were significantly enhanced after overexpression of PRL-3. * $P < 0.05$. (F) The cell proliferation rate of SACC-83 was significantly higher than that of the control group after transfection. ** $P < 0.01$.

To further investigate whether PRL-3 is related to the metastasis of SACC, we examined the migratory and invasive abilities of SACC by overexpressing or inhibiting the expression of PRL-3 in SACC cell lines. We found that SACC-LM (highly migratory and invasive cell line) had higher PRL-3 expression than SACC-83 (lower ability of migration and invasion). The overexpression of PRL-3 in SACC-83 resulted in significantly elevated migratory and invasive abilities of these cells. However, inhibiting the expression of PRL-3 in SACC-LM cells using siRNA or treatment with PRL-3 inhibitors blocked the migratory and invasive abilities. These data indicate that PRL-3 contributes to the increased aggressive behavior of SACC.

Epithelial-mesenchymal transition (EMT) is a process by which the epithelial cells undergo remarkable morphological changes, characterized by a transition from the epithelial phenotype to the elongated fibroblastic phenotype and accompanied by increased cell motility and invasion (28). To date, multiple complexes signaling systems have

been found to induce EMT (29), including TGF- β signal pathway, transcription factors, and microRNAs. Our previous study had suggested that PRL-3 regulated the migration and invasion of cancer cells involving EMT through the Akt pathway (30). Liu et al. had also presented evidence for the involvement of PRL-3 in EMT via cadherin-related signaling pathway (31). In our study, we found that the overexpression of PRL-3 in SACC-83 cells increases the expression of mesenchymal markers (Slug and vimentin) and decreases the expression of epithelial markers (E-cadherin). Downregulating the expression of PRL-3 in SACC-LM cells decreases the expression of mesenchymal markers (Slug and vimentin) and increases the expression of epithelial markers (E-cadherin). Furthermore, we also found that the protein level of PRL-3 was positively correlated with the pERK1/2, which can mediate cell proliferation, cell cycle arrest, apoptosis, and metastasis (8, 32, 33). Our previous study had found that ERK-Slug pathway plays an important role in SACC metastasis; Slug is a downstream target of ERK2; siRNA-mediated

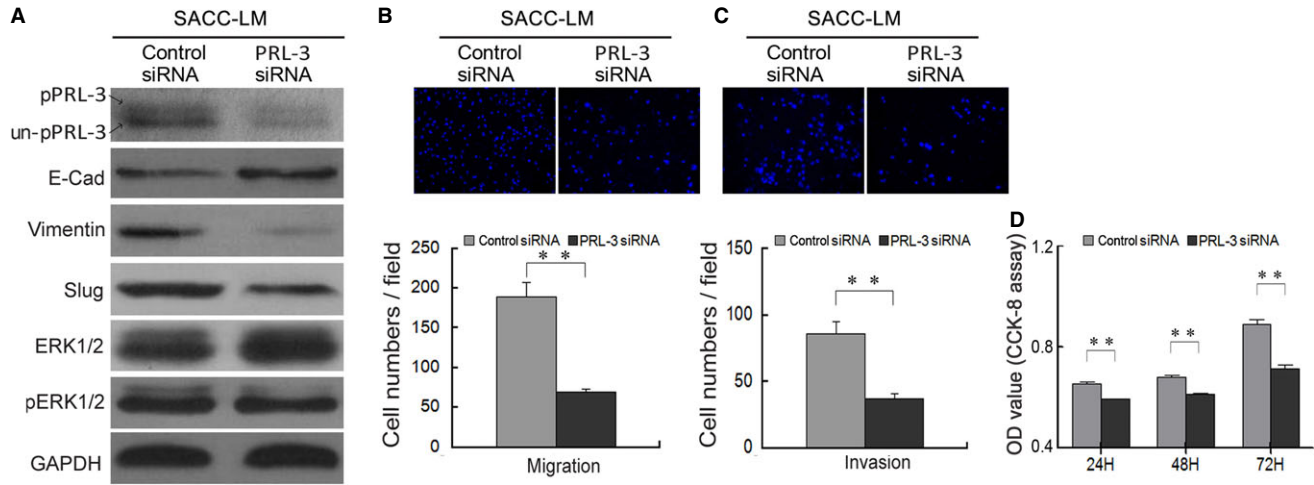


Figure 4 PRL-3 knockdown by siRNA inhibits the migration and invasion of salivary adenoid cystic carcinoma (SACC). (A) Western blotting shows the protein levels of pERK1/2, Slug, and vimentin were obviously decreased and the protein levels of ERK1/2 and E-cadherin were obviously increased in SACC-LM cells after knockdown of PRL-3. (B, C) The migratory and invasive abilities of SACC-LM cells were significantly inhibited after knockdown of PRL-3. $*P < 0.05$. (D) The cell proliferation rate of SACC-LM was significantly lower than that of the control group after transfection. The inhibition rate was about 7.25% (24 h), 11.99% (48 h), 19.69% (72 h), respectively. $**P < 0.01$.

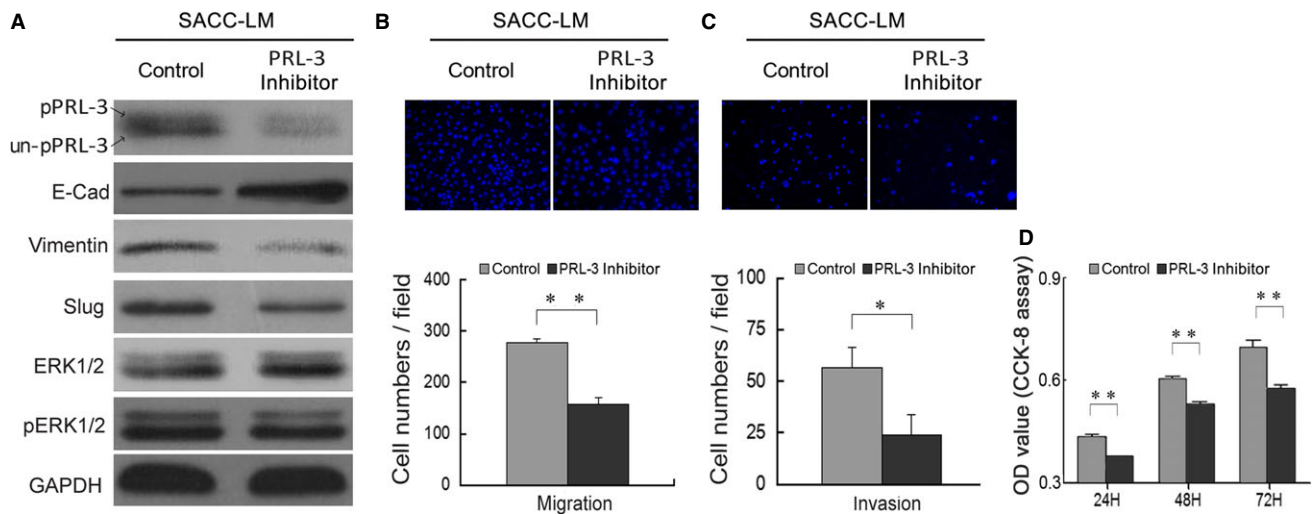


Figure 5 PRL-3 inhibitor inhibits the migration and invasion of SACC. (A) Western blotting shows the protein levels of pERK1/2, Slug, and vimentin were obviously decreased, and the protein levels of ERK1/2 and E-cadherin were obviously increased in SACC-LM cells after treated with PRL-3 inhibitor. (B, C) The migratory and invasive abilities of SACC-LM cells were significantly inhibited after treated with PRL-3 inhibitor. $*P < 0.05$. (D) The cell proliferation rate of SACC-LM was significantly lower than the control group after transfection. The inhibition rate was about 5.86% (24 h), 15.27% (48 h), 29.02% (72 h), respectively. $**P < 0.01$.

ERK2-knockdown suppressed the Slug gene promoter activity and reduced the Slug protein level in SACC cells (9). Thus, PRL-3 regulated the migration and invasion of SACC may be involved EMT through the ERK–Slug pathway.

Conclusions

Our study confirmed that PRL-3 plays an important role in the development of SACC and is correlated with poor prognosis. PRL-3 contributes to the migration and invasion of SACC, possibly through its involvement in EMT via the ERK–Slug pathway.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Clinicopathologic characteristics of normal salivary gland tissues.

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Conflict of interests

The authors declare that there are no conflict of interests.