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miR-21 regulates tumor progression through the miR-21-PDCD4-Stat3 pathway in human salivary adenoid cystic carcinoma

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miR-21, which is a putative tumor onco-miR and frequently overexpressed microRNA in various tumors, has been linked to tumor progression through targeting of tumor-suppressor genes. In this study, we sought to determine whether miR-21 has any role on tumor progression of salivary adenoid cystic carcinoma (SACC) and the possible mechanisms. We found that the level of miR-21 expression was significantly higher in SACC than that in normal salivary tissues, and it is also higher in tumors with metastasis than that without metastasis. Using an anti-miR-21 inhibitor in an *in vitro* model, downregulation of miR-21 significantly decreased the capacity of invasion and migration of SACC cells, whereas a pre-miR-21 increased the capacity of invasion and migration of SACC cells. To explore the potential mechanisms by which miR-21 regulate invasion and migration, we identified one direct miR-21 target gene, programmed cell death 4 (*PDCD4*), which has been implicated in invasion and metastasis. The suppression of miR-21 in metastatic SACC-LM cells significantly increased the report activity of *PDCD4* promoter and the expression of PDCD4 protein. This subsequently resulted in downregulation of the p-STAT3 protein. The level of miR-21 expression positively related to the expression of PDCD4 protein and negatively related to the expression of p-STAT3 protein in SACC specimens, respectively, indicating the potential role of the STAT3-miR-21-PDCD4 pathway in these tumors. Dysregulation of miR-21 has an important role in tumor growth and invasion by targeting *PDCD4*. Therefore, suppression of miR-21 may provide a potential approach for the treatment of advanced SACC patients.

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Salivary adenoid cystic carcinoma (SACC) is one of the most common malignancy of the salivary gland, accounting for ~10% of salivary gland tumors and 30% of human salivary gland malignancies.[1,2] SACC is characterized by slow but aggressive growth, nerve and blood vessel invasion, multiple recurrences, and distant metastases.[3–5] Lung metastasis and nerve metastasis are the biological characteristics of SACC.[6–8] It has been reported that the incidence of SACC with distant metastasis ranged from 35 to 50% of all cases. Lung was the most common organ of its distant metastasis, and lung metastasis is still the leading death cause of patients with SACC.[4,5] Although the 5-year survival rate is high for patients with SACC, probably because of the slow growth of the tumor, the 10- and 15-year prognoses are poor because of the frequent local recurrences and distant metastases.[6–8] Although multiple genetic and epigenetic alterations have been detected in SACC,[3,9–13] the precise molecular mechanisms of progression and metastasis of SACC remain unknown.

MicroRNAs (miRNAs) are small non-coding RNA molecules, with a length of 20–22 nucleotides, that regulate gene expression by either translational inhibition or mRNA degradation. miRNAs function as either oncogenes or tumor suppressors by inhibiting the expression of target genes, some of which are either directly or indirectly involved with canonical signaling pathways.[13,14] The roles and function of some selected miRNAs in SACC have been reported.[15]

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Liu *et al* found that miR-155 facilitates cell cycle progression and promotes invasion in ACC and that the EGFR/NF-κB pathway might participate in mediating the effects of miR-155.[16] Mitani *et al* reported that the overexpression of the miR-17 and miR-20a were significantly associated with poor outcome in SACC.[17] He *et al* first indicate that miR-181a has an important role in the metastasis of SACC by regulating the MAPK-Snai2 pathway, and may serve as a novel therapeutic target for SACC.[18] Chen *et al* employed microarray technology to identify miRNA expression profiles that are involved in the metastatic progression of SACC. The microarray data revealed that the levels of 38 miRNAs significantly differed between the ACC-M cells and the control ACC-2 cells. Six miRNAs (miR-4487, -4430, -486-3p, -5191, -3131, and -211-3p) were selected to validate the microarray data via qPCR. The expression of two miRNAs (miR-4487 and -4430) was significantly upregulated in the ACC-M cells, whereas the expression of two other miRNAs (miR-5191 and -3131) was significantly downregulated in the ACC-M cells.[19] Sun *et al* reported that miR-320a inhibits metastasis in SACCs by targeting ITGB3 and may serve as a therapeutic target and prognostic marker in salivary cancers.[20] However, until now, nothing has been reported about the relationship between dysregulation of miR-21 and SACC progression. The upregulation of miR-21 has been found in a variety of malignant tumors, which thus could be functioning as an oncogene in tumor developments.[21] Recently, Bera *et al* identify a previously unrecognized signaling node where high miR-21 levels reduce rictor–programmed cell death 4 (*PDCD4*) interaction to increase phosphorylation of Akt and contribute to metastatic fitness of renal cancer cells.[22] The relationship between miRNAs and signaling pathways in SACC is extremely complicated.

As tumor-suppressor genes, *PDCD4* is also implicated in cell migration and invasion,[23] suggesting that miR-21 may also have a role in invasion and metastasis. However, little is known as to how miR-21 affects these processes. The role of *STAT3* in the expression of the onco-mir miR-21 is particularly important because constitutive activation of *STAT3* has been demonstrated in a large number of diverse human tumors, and considerable evidence suggests that constitutive *STAT3* activation actively participates in tumor formation and progression.[24,25] Abnormal STAT3 protein activation has been identified in many cancers of the breast,[26] prostate,[27] and pancreas,[28] as well as cancers of blood-forming cells (leukemia and lymphoma).[29,30] Normally, the STAT3 protein is switched on and off in response to signals that control cell growth and development. A continuously active version of this protein relays messages to the nucleus even in the absence of these chemical signals. Researchers believe that the overactive STAT3 protein instructs cells to continue growing and dividing, and prevents damaged cells from self-destructing (undergoing apoptosis). Excess STAT3 protein may contribute to the growth of cancers by allowing abnormal cells to grow and divide uncontrollably.[31–33] The present study provides the first evidence that miR-21 regulates invasion and metastasis of SACC cells, at least in part, by targeting *PDCD4*. Furthermore, examination of human SACC specimens revealed an inverse correlation of miR-21 with *PDCD4*, *PDCD4* and p-*STAT3*.

MATERIALS AND METHODS

Ethics Statement

De-identified human tissue samples were obtained from the Zhejiang Province Human Tissue Specimen Bank. The use of specimens was approved by the Institutional Review Board at Zhejiang Province Cancer Hospital. Written informed consent was obtained from each patient in accordance with the requirements of our institution's board of ethics. Twenty cases of normal salivary gland tissues (NSGs) from non-cancer patients provided written informed consent. The Institutional Review Board on Medical Ethics of Zhejiang Province Cancer Hospital also approved the method of NSG specimen collection including written informed consent from all patients.

Cell Culture and Tumor Specimens

Cell lines originated from Adenoid cystic carcinoma cells with high metastatic potential (SACC-LM) and low metastatic potential (SACC-83) were provided by Dr Sheng-Lin Li, the Peking University School of Stomatology.[34] Both cell lines were cultured in RPMI 1640 complete medium with 10% inactivated FBS, 200 000 U/l penicillin, and 200 000 U/l streptomycin in humidified 5% CO_2-air at 37 °C.

Thirty-seven cases of resected SACC were collected from the Department of Head and Neck Tumor Surgery, Zhejiang Province Cancer Hospital, between May 2009 and September 2010. All 37 samples were immediately placed in liquid nitrogen after removal, and 18 of 37 cases with freezing preservation had paired NSGs. All the resected specimens were also paraffin embedded and histologically examined by hematoxylin and eosin staining. The samples were collected from 16 males and 21 females, aged 35–74 years (median age 57 years). There were 19 cases of cribriform pattern carcinoma, 8 cases of salivary duct carcinoma, and 10 cases of solid carcinoma. According to the general rules of SACC staging suggested by the International Union Against Cancer,[35] there were 18 cases of stage T1-3 and 19 cases of stage T4. Also, 20 cases of NSGs served as controls, in which 7 males and 13 females.

RNA Extraction

All experimental containers were treated with RNase inactivator, and all reagents were prepared in 0.11% diethypyrocarbonate-water. The tumor and their matched NSGs of 37 cases were homogenized by using a TissueLyser II (Qiagen) for 2 min at 18 Hz according to the manufacturer's instructions at the Zhejiang Cancer Research Institute. Total RNA was isolated from the tumor and their matched NSGs using MiRNeasy Mini Kits (Qiagen, USA) and a modified

acidic guanidinium phenol/chloroform method, following the manufacturer's instructions. Total RNA was treated with DNase I (TaKaRa Bio, Otsu, Japan) to remove genomic DNA. The mRNA was purified using a poly(A) purification kit (Oligotex, Qiagen, USA) according to the manufacturer's instructions. The quality of mRNA was assessed by the A260/280 ratio, RNA integrity was checked by denaturing agarose gel electrophoresis, and the contamination of genomic DNA was checked using PCR.

Real Time RT-PCR Analysis

PCR-based detection of mature miR-21 was performed by the TaqMan miRNA assays (ABI, Forest City, CA, USA) as described previously.[36,37] The real-time PCR results, recorded as threshold cycle numbers (Ct), were normalized against an internal control (U6 RNA), and then expressed as fold changes.[36]

For analysis of *PDCD4* and *STAT3* mRNA expression, real-time RT-PCR was performed using SYBR Premix Ex Taq™ (TaKaRa). GAPDH was used to normalize the expression levels of *PDCD4* mRNA. The primer sequences for *PDCD4* mRNA were as follows: (F) 5′-GGGAGTGACGCCCTTAGAAG-3′; (R) 5′-ACCTTTCTTTGGTAGTCCCCTT-3′. The primers for *STAT3*: (F)5′-ACCAGCAGTATAGCCGCTTC-3′; (R) 5′-GCCACAATCCGGGCAATCT-3′. The primers for GAPDH: (F) 5′-GAAGGTGAAGGTCGGAGTC-3′; (R) 5′-GAAGATGGTGATGGGATTTC-3′. All real-time RT-PCR were performed in triplicates.

Transfection

SACC-LM cells (1×10^5) were plated to 50% confluent and were transfected with 50 nmol/l GMR-miR™ miR-21 inhibitor (GenePharma, Shanghai, China) or inhibitor-negative control by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in Opti-MEM (Invitrogen), according to the manufacturer's protocol. The culture medium was changed and transfection efficiency was determined using fluorescent images after transfection for 24 h. Cells were harvested for analysis after transfection for 48 h. All experiments were performed in triplicates.

Luciferase Reporter Assays

The 3′-untranslated region (UTR) of human *PDCD4* mRNA containing the miR-21-binding sites was amplified by PCR from human genomic DNA and inserted into the *Xba*1 site of pGL3 vector (Promega, Madison, WI, USA) and named as pGL3-*PDCD4*-wt. Mutations in the predicted miR-21-binding sites were performed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), with pGL3-*PDCD4*-wt as a template and named as pGL3-*PDCD4*-mut. SACC-LM cells were co-transfected in 24-well plates with wild-type (wt) or mutant reporter plasmid vector by Lipofectamine 2000, and at 6 h after transfection, the cells

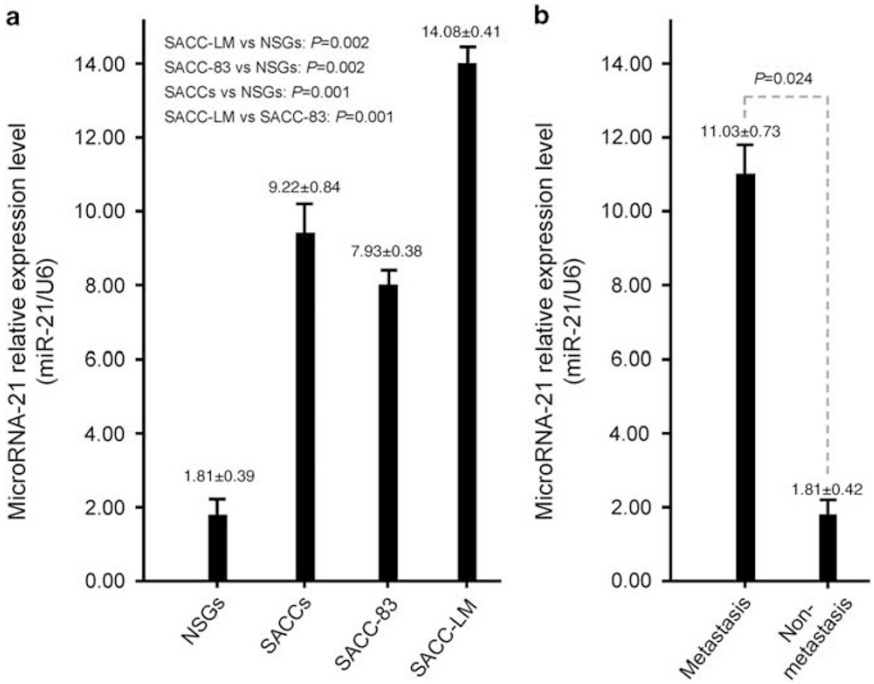

Figure 1 Overexpression of miR-21 in SACC tissues (SACCs) and SACC cell lines (SACC-83 and SACC-LM) as compared with normal salivary gland tissues (NSGs). (**a**) Comparison of expression level of miR-21 between SACCs, SACC-83, SACC-LM, and NSGs, respectively. (**b**) Comparison of expression level of miR-21 between SACC with metastasis and those without metastasis (non-metastasis). Relative expression level of miR-21 was determined by TaqMan RT-PCR, and all data were normalized to U6 RNA. Means ± S are shown. SACC, salivary adenoid cystic carcinoma.

Table 1 Correlation of miR-21 expression with clinicopathological factors in SACC

Variables	No. of cases	$X \pm S$	P^a
Cases			
Tumor	35	9.35 ± 2.34	0.003
Normal	35	1.95 ± 0.22	
Sex			
Male	16	8.59 ± 3.87	0.366
Female	19	4.70 ± 1.63	
Age at diagnosis (years)			
>57	19	7.15 ± 3.26	0.714
≤57	16	5.67 ± 2.02	
Tumor site			
Minor salivary gland	19	8.83 ± 3.50	0.172
Major salivary gland	16	3.69 ± 0.94	
Histotypes			
Cribriform	20	7.47 ± 3.12	0.623
Tubular	6	8.17 ± 4.91	
Solid	9	3.16 ± 1.07	
Perineural invasion			
Positive	17	5.12 ± 1.86	0.511
Negative	18	7.76 ± 3.44	
Bone invasion			
Positive	8	8.88 ± 5.43	0.517
Negative	27	5.77 ± 2.04	
Lymph node involvement			
N+	5	11.80 ± 5.51	0.278
N0	30	5.59 ± 2.10	
T phage			
T1–T3	17	8.12 ± 3.85	0.548
T4	18	5.30 ± 1.80	
Distant metastasis			
Positive	18	10.81 ± 3.58	0.024
Negative	17	1.90 ± 0.16	

SACC, salivary adenoid cystic carcinoma.
[a]$\chi 2$ test.

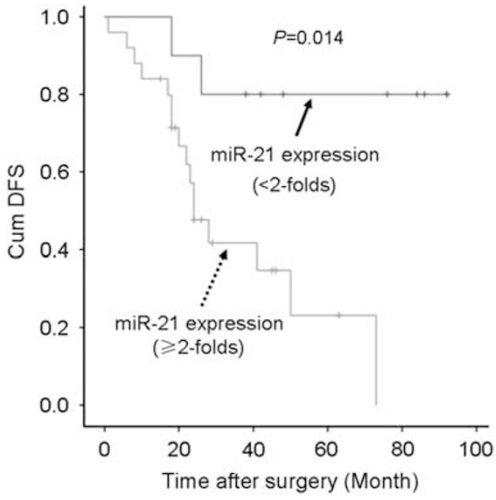

Figure 2 Overexpression of miR-21 in SACC tissues correlated with poor prognosis. Cumulative disease-free survival (Cum DFS) curves are plotted against miR-21 level in SACC tissues. There was significant difference in the median survival (28 *vs* 64 months) between patients with miR-21 overexpression (≥2-folds) and those patients without miR-21 expression (<2-folds; $P = 0.014$). SACC, salivary adenoid cystic carcinoma.

were transfected again with miR-21 inhibitor or negative control. Each transfection was carried out in four wells. Luciferase assays were performed 24 h after transfection using the Dual-Luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

Transwell Migration Assay

SACC-LM cells were grown in RPMI 1640 containing 10% fetal bovine serum to ~60% confluence and 3.0×10^5 cells were transfected with miR-21 inhibitor or negative control. After 24 h, the cells were harvested by trypsinization and washed once with Hanks' balanced salt solution (Invitrogen). To measure cell migration, 8-mm pore size culture inserts (Transwell; Costar, High Wycombe, UK) were placed into the wells of 24-well culture plates, separating the upper and the lower chambers. In the lower chamber, 400 μl of RPMI 1640 containing recombinant human hepatocyte growth factor (20 ng/ml) were added. Hepatocyte growth factor was purchased from R&D Systems (Minneapolis, MN, USA). Then, 1 μl of 10^5 cells were added to the upper chamber. After 24 h of incubation at 37 °C with 5% CO_2, the number of cells that had migrated through the pores was quantified by counting 10 independent visual fields under the microscope (Zeiss) using a ×20 objective, and cell morphology was observed by staining with hematoxylin and eosin.

Construction of Expression Vectors

To construct a vector to express Myc-tagged *PDCD4*, a DNA fragment covering the *PDCD4*-coding region was first amplified from MCF10A cDNA by PCR (forward primer 5′-GAATTCTGGATGTAGAAAATGAGCAGA-3′ and reverse primer 5′-GCGGCCGCTCAGTAGCTCTCTGGTTTAAG-3′)

Table 2 Univariate survival analysis of clinicopathologic data of 35 SACC

Variables	Hazard ratio	95% Confidence interval	*P*-values
Age (<57/≥57 years)	1.457	0.054–3.931	0.458
Gender (female/male)	1.489	0.587–3.778	0.402
Site (major/minor)	1.124	0.444–2.846	0.804
T-stage (1–3/4)	1.731	0.653–4.586	0.27
Histotypes (cribriform/solid/tubular)	1.731	0.613–4.396	0.324
Perineural invasion (+/–)	1.516	0.595–3.864	0.384
Bone invasion(+/–)	0.771	0.243–2.448	0.659
Lymph node involvement (+/–)	2.24	0.627–7.997	0.214
Distant metastasis (+/–)	2.122	0.808–5.571	0.127
miR-21 (≥2.0/<2.0)	6.808	1.481–31.289	0.014[a]

SACC, salivary adenoid cystic carcinoma.
[a]Statistically significant.

Table 3 Multivariate survival analysis of clinicopathologic data of 35 SACC

Variables	*Hazard ratio*	*95% Confidence interval*	P-*values*
Age (<57/≥57 years)	0.712	0.174–2.924	0.638
Gender (female/male)	1.200	0.374–3.847	0.759
Site (major/minor)	0.983	0.275–3.520	0.979
T phage (1–3/4)	2.369	0.628–8.933	0.203
Histotypes (cribriform/solid/tubular)	1.824	0.595–5.590	0.293
Perineural invasion (+/–)	1.643	0.442–6.114	0.459
Bone invasion(+/–)	1.289	0.302–5.503	0.732
Lymph node involvement (+/–)	0.705	0.115–4.328	0.706
Distant metastasis (+/–)	1.171	0.298–4.602	0.822
miR-21 (≥2.0/<2.0)	7.239	1.081–48.460	0.041[a]

SACC, salivary adenoid cystic carcinoma.
[a]Statistically significant.

and was then cloned into pCR8 (Invitrogen). After verification by DNA sequencing, the full-length *PDCD4*-coding fragment was finally cloned into pCMV-Myc (Clontech) at the *Eco*R1 and *Not*1 sites.

Immunohistochemical (IHC) Analysis

IHC was performed as described previously.[38] Avidin–biotin–peroxidase method was used for IHC assay. All sections were deparaffinized with xylene and dehydrated with gradient alcohol followed by inactivation of endogenous peroxidase activity by 0.5% H_2O_2 in methanol for 10 min. Non-specific binding was blocked by incubation with 10% normal goat serum in phosphate-buffered saline for 1 h at room temperature. Then, slides were incubated with primary antibodies: anti-PDCD4 (Abcam, ab80590, Cambridge), anti-total STAT3 (Abcam ab32500, Cambridge), and anti-phospho-STAT3 (phospho Y705, Abcam, ab76315, Cambridge) antibody, respectively, at 4°C overnight, followed by biotinylated goat antimouse IgG (1:400; Sigma, St Louis, MO, USA) or goat anti-rabbit IgG (1:400; Sigma) for 1 h at room temperature. Then streptavidin–biotin–peroxidase complex assay was performed. A brown color was indicative of protein-positive expression. Peroxidase activity was developed by incubating with 0.1% 3,3-diaminobenzidine (Sigma) in phosphate-buffered saline with 0.05% H_2O_2 for 5 min at room temperature. The IHC staining score is determined by three independent pathologists based on combining staining frequency and intensity as previous described.[38]

Western Blot Analysis

Tumor specimens and their matched NSGs of 4 cases were randomly selected from 37 SACC patients for western blot analysis. Total protein was extracted and then quantified using the Lowry method.[39] Western blot analysis was performed using anti-PDCD4, anti-total STAT3, and anti-phospho-STAT3 monoclonal antibodies according to the previous report[9] and normalized to GAPDH (Abcam, Cambridge) protein expression.

Statistical Analysis

SPSS 17.0 statistical software (SPSS, Chicago, IL, USA) was adopted for data analysis. Counting data comparisons between groups were subjected to the $\chi 2$ test or Fisher's exact test. miR-21 expression was shown as $X \pm S$. $P<0.05$ was considered statistically significant. The correlation of miR-21 and *PDCD4* or *p-STAT3* was analyzed by Spearman Rank Correlation, with double-sided $P<0.05$ as statistically significant. Survival analysis was computed by means of the Kaplan–Meier method and significance were assessed by means of the log-rank test. A univariate analysis of the Cox regression model was used to determine prognostic factors.

RESULTS

miR-21 Is Aberrantly Overexpressed in SACC Tissue Samples and Cell Lines

TaqMan quantitative real-time PCR analysis of miR-21 expression was succeeded in 35 (94.6%) of 37 samples. miR-21 is expressed at a significantly higher level ($P=0.003$; 4.8-fold on average) in SACC tissue samples than that in normal tissue samples (Figure 1a). A significant overexpression of miR-21 is also observed in two SACC cell lines (SACC-83 and SACC-LM) relative to that in normal tissue samples (SACC-83 *vs* NSGs, SACC-LM *vs* NSGs, both $P=0.002$). And the significant overexpression of miR-21 is found in high-metastasis-potential SACC-LM cells than that in low-metastasis-potential SACC-83 cells (Figure 1a;

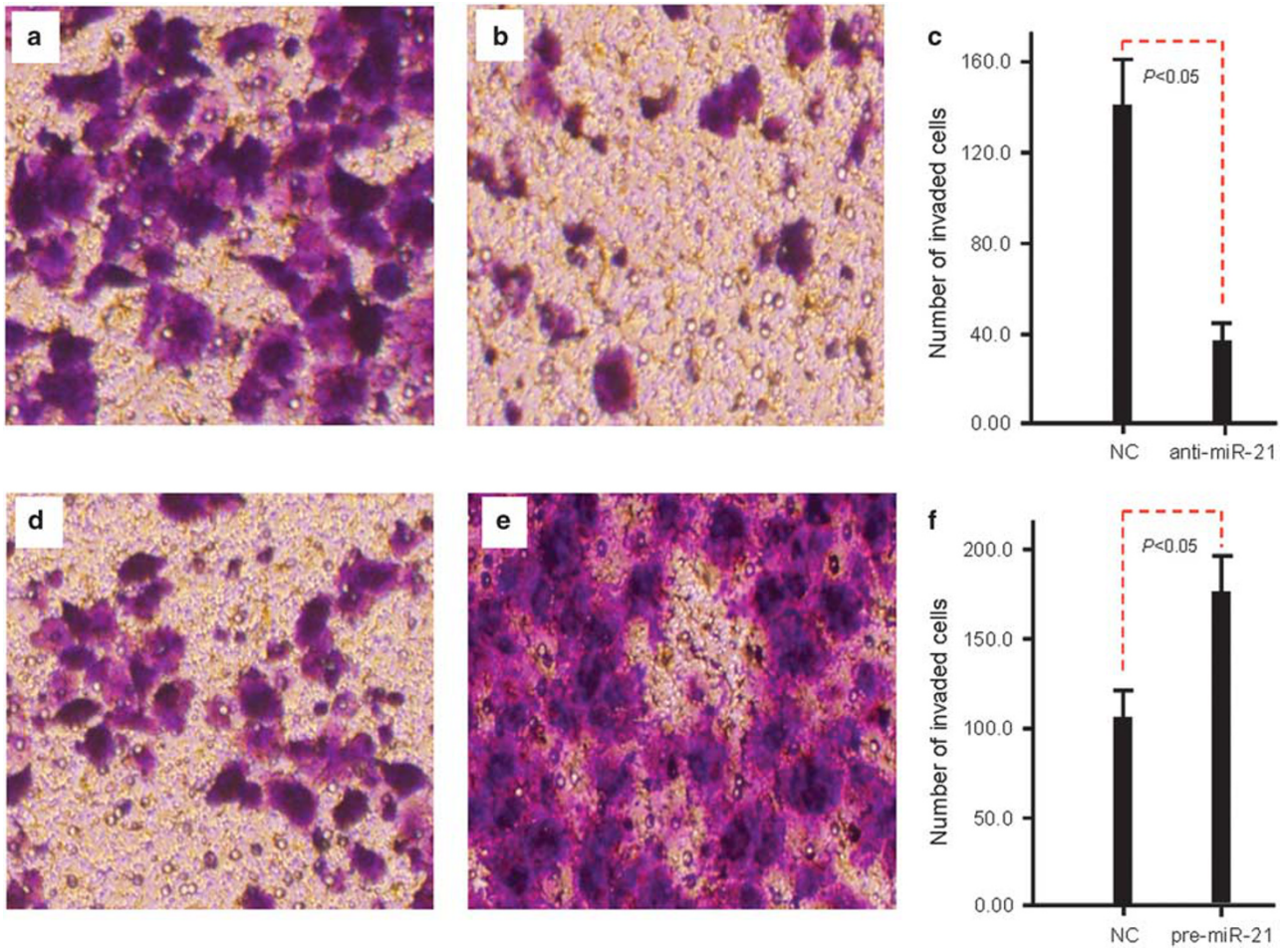

Figure 3 miR-21 regulates cell invasion ability in SACC cells. SACC-LM cells invaded through Matrigel-coated membrane after transfection with negative control miRNA (**a**) and transfection with an anti-miR-21 inhibitor (**b**). (**c**) Quantification of SACC-LM cells that invaded through Matrigel-coated membrane after transfection with anti-miR-21 inhibitor or negative control. (**d**) SACC-83 cells that invaded through Matrigel-coated membrane after transfection with negative control vector and (**e**) transfection with MSCVpuro-miR-21 (pre-miR-21). (**f**) Quantification of SACC-83 cells that invaded through Matrigel-coated membrane after transfection with MSCVpuro-miR-21 or negative control. SACC, salivary adenoid cystic carcinoma.

$P=0.001$). These results indicate that miR-21 expression is frequently upregulated in SACC cells, and miR-21 may have a pivotal role in SACC metastasis.

miR-21 Overexpression Correlates with the Metastasis of SACC Patients

The association of miR-21 expression in tumor tissues with the clinical pathologic factors of the 35 SACC patients were analyzed and are displayed in Table 1. Significant higher level of miR-21 expression was observed in the SACC of the 18 patients with metastasis than those in patients without metastasis (Figure 1b, $P=0.024$). No significant differences were found in miR-21 expression with respect to gender, age at surgery, tumor site, histotypes, perineural invasion, bone invasion, lymph node involvement, T phage, and distant metastasis (Table 1, all $P>0.05$).

miR-21 Expression Is an Independent Predictor of Survival in SACC Patients

Kaplan–Meier analysis was used to analysis the association between miR-21 expression and patients survival. As shown in Figure 2, miR-21 expression in tumor tissues was an unfavorable predictor for the survival of SACC patients after surgery. Patients with miR-21 overexpression (≥2-folds) had significantly lower disease-free survival as compared those with lower miR-21 expression (<2-folds; Figure 2 and Table 2). There was significant difference in the median survival (28 *vs* 64 months) between patients with miR-21 overexpression (≥2-folds) and those patients with lower miR-21 expression (<2-folds; $P=0.014$). Retrospective analysis using Cox regression models indicated that miR-21 overexpression in tumor tissues is a promising independent predictor of survival in SACC patients ($P=0.041$; Table 3). The risk ratio after adjustment for competing risk factors, sex,

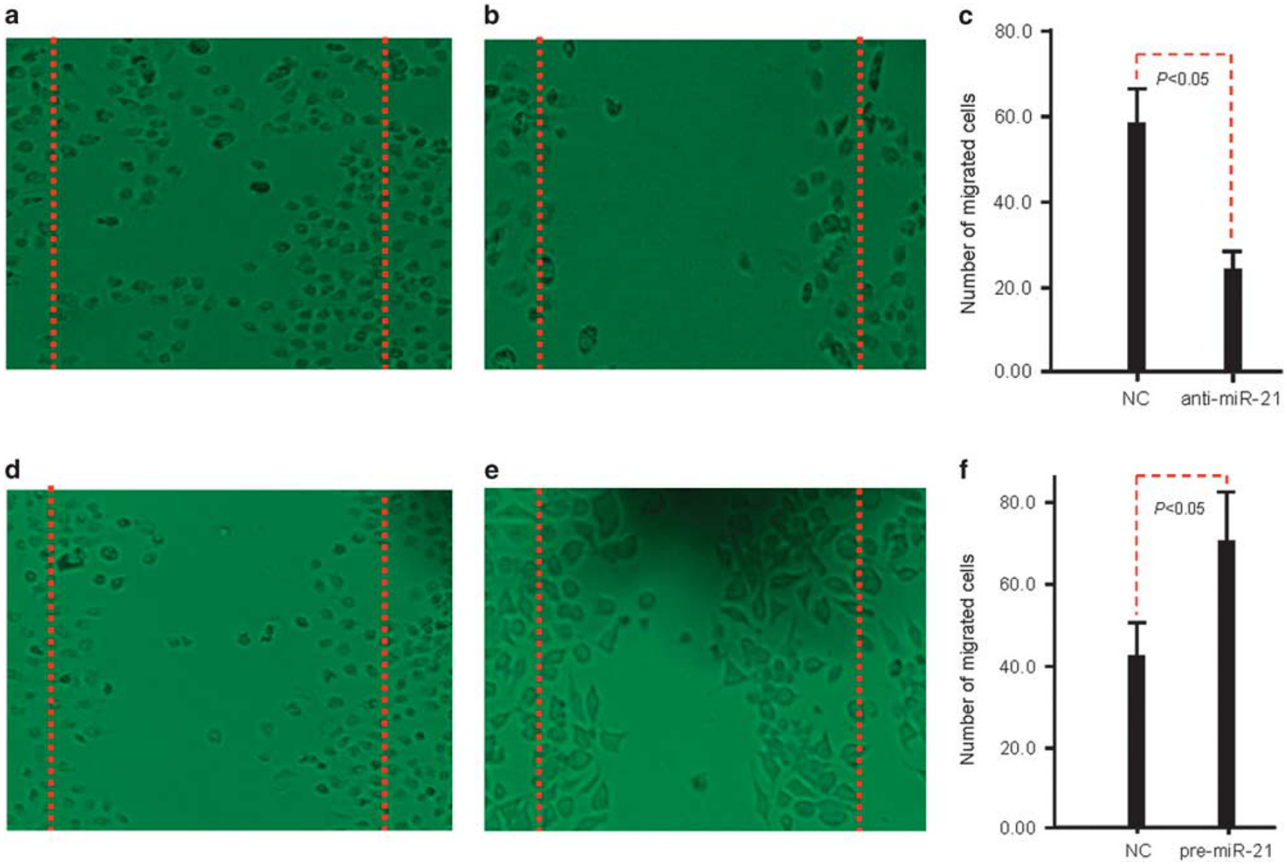

Figure 4 miR-21 affects SACC cell migration ability. Scratch assay was used to evaluate the cell capacity of migration. (**a**) Migration of SACC-LM cells transfected with negative control miRNA and (**b**) transfected with anti-miR-21 inhibitor. (**c**) Quantification of SACC-LM cells that migrated from the edge of scratch in cells transfected with anti-miR-21 inhibitor or negative control. (**d**) Migration of SACC-83 cells transfected with negative control vector and (**e**) transfected with MSCVpuro- miR-21 (pre-miR-21). (**f**) Quantification of SACC-83 cells that migrated from the edge of scratch after transfection with MSCVpuro-miR-21 or negative control. SACC, salivary adenoid cystic carcinoma.

age, and stage of disease was found to be 7.239 (95% confidence intervals, 1.081–48.460). The results showed that increases in miR-21 expression had a greater impact on the prognosis than tumor clinical stage (Table 3).

miR-21 Regulates SACC Cell Invasion and Migration *In Vitro*

In an *in vitro* cell invasion assay, cell invasion was significantly enhanced by transfection of MSCVpuro-miR-21 (pre-miR-21 group, 174 ± 22 cells per high-power field (HPF); negative control group, 116 ± 18 cells per HPF; Figure 3d–f). In contrast, cell invasion was significantly suppressed ($P < 0.05$; ~4-fold) by transfection of an anti-miR-21 inhibitor (anti-miR-21 group, 34 ± 12 cells per HPF; negative control group, 141 ± 21 cells per HPF; Figure 3a–c). Similar to cell invasion assay, in cell scratch wound-healing test, overexpressed miR-21 in SACC-LM cells resulted in significant more number of the migrated cells (72 ± 11 cells per HPF) than negative control cells (43 ± 7 cells per HPF; Figure 4d–f; $P < 0.05$). However, the number of SACC-LM cells migrated to the scratched area was significantly fewer ($P < 0.05$; ~2.5-fold) in cells transfected with anti-miR-21 inhibitor (23 ± 5 cells per HPF) than in those transfected with negative control (58 ± 8 cells per HPF; Figure 4a–c). These results indicate that miR-21 enhances cellular capacity of invasion and migration that are associated with tumor metastasis.

PDCD4 Is a Target of miR-21

To identify the potential targets of miR-21, we searched TargetScan,[40] Miranda,[41] PicTar,[42] and MAMI (http://mami.med.harvard.edu/), for theoretical target genes whose downregulation could mediate the observed effects of miR-21. We found that *PDCD4*, a tumor-suppressor gene, was predicted by all the four programs as a target of miR-21. To validate whether *PDCD4* is a direct target of miR-21, we constructed luciferase-reporter plasmids that contain the wild-type (wt) or mutant 3'-UTR segments of *PDCD4* (Figure 5a). In addition, we mutated the miR-21-binding site in the 3'UTR of *PDCD4* (in the reporter plasmid). The wt or mutant reporter plasmid was co-transfected into SACC-LM cells along with miR-21 inhibitor or negative control. Compared with negative control, miR-21 inhibitor significantly increased the relative

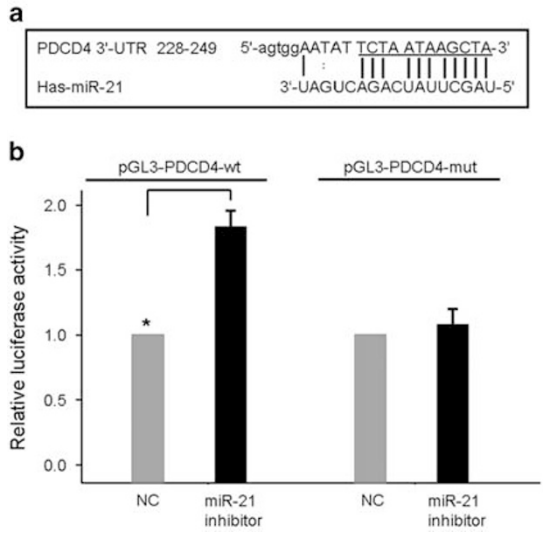

Figure 5 The 3′-UTR of *PDCD4* mRNA is a target for miR-21. (**a**) Predicted miR-21-binding sites within the 3′-UTR of *PDCD4* mRNA. The *PDCD4* UTR sequence reveals a putative *mir-21*-binding site (http://www.mirbase.org/index.shtml). The underlined sequence denotes a deletion in Luc-*PDCD4*-UTR-d. (**b**) The wild-type (wt) or mutant (mut) reporter plasmid was co-transfected into SACC-LM cells with miR-21 inhibitor or negative control (NC). The normalized luciferase activity in the control group was set as relative luciferase activity 1. Luciferase activity of pGL3-*PDCD4*-wt was significantly increased by miR-21 inhibitor (*$P<0.05$). However, luciferase activity of pGL3-*PDCD4*-mut was not affected by miR-21 inhibitor ($P>0.05$). 3′-UTR, 3′-untranslated region.

luciferase activity when co-transfected with the wt reporter plasmid. However, miR-21 inhibitor-mediated increase of luciferase activity was abolished in the mutant reporter plasmid (Figure 5b). These results indicate that *PDCD4* is a direct target of miR-21 and that miR-21 suppresses *PDCD4* by direct binding to the 3'-UTR of *PDCD4*.

Then we assessed whether the down-expression of miR-21 affected PDCD4 protein expression in SACC cells. SACC-LM cells were transiently transfected with miR-21 inhibitor. The expression of PDCD4 protein was significantly increased when endogenous miR-21 was knocked down by the miR-21 inhibitor. Because *Stat3* has been reported as a downstream target of *PDCD4*, therefore, we checked the protein expression of the phosphorylated STAT3 expression and found that the expression of the phosphorylated STAT3 was concomitantly decreased by miR-21 inhibitor (Figure 6a,b and d1). However, *PDCD4* and *STAT3* mRNA were almost not affected by the alternation of miR-21 levels (Figure 6c). Interestingly, the expression of PDCD4 protein was significantly increased, and the expression of miR-21 levels was concomitantly decreased when the knockdown of endogenous phosphorylated STAT3 expression by RNA interference, indicating that there may be a feedback loop among miR-21-PDCD4-p-Stat3 (Figure 6d2,e and f).

PDCD4 Protein Level Negatively Correlates with miR-21 and p-STAT3 Protein Expression in Tumor Specimens

The PDCD4 and p-STAT3 protein in all 35 paraffin-embedded samples were analyzed by IHC staining. In comparison with their matched NSGs, tumor tissues expressed significantly lower levels of PDCD4 protein ($P<0.0001$, χ^2-test), and significantly higher levels of p-STAT3 protein ($P<0.0001$, χ^2-test). The expression of PDCD4 and p-STAT3 protein was validated by western blotting, and representative examples were shown in Figure 6a. The miR-21 levels negatively correlated to PDCD4 protein and positively correlated to p-STAT3 protein (Figure 7a and b). To determine the exact association, we used the Pearson's method for statistical analysis. We confirmed that the inverse correlation between miR-21 levels and PDCD4 protein ($r=-0.556$, $P=0.001$, Figure 7c), positive correlation between miR-21 levels and p-STAT3 protein ($r=0.661$, $P=0.000$, Figure 7d), in these SACC tissues. We also confirmed the inverse correlation between PDCD4 protein and p-STAT3 protein in 35 SACC specimens ($r=-0.972$, $P=0.000$, Figure 7e).

In a word, we found that high levels of miR-21 in SACC samples correlate with low level of PDCD4 protein and high level of p-STAT3 protein.

DISCUSSION

Metastasis is a major cause of cancer-related death. Previous research elucidated that miRNAs have an important role in the occurrence, development, and metastasis of SACC.[16–20] Although miR-21 do not be included these upregulated miRNAs identified by Chen *et al*,[19] it is possible that bounded by selected cell line. The identification of miRNAs in highly metastatic SACC cells may provide an improved understanding of the mechanisms involved in metastatic progression, which would aid in the development of novel strategies for the treatment of SACC. Hence, identifying the role of miR-21 in invasion and metastasis has direct clinical implications. Although it is now known that miR-21 might has a key role in cancer development, the underlying mechanism is largely unknown. We show here that miR-21 is significantly upregulated in SACC and the dysregulation of it may be associated with tumor invasion, migration, and metastasis. And this effect of miR-21 in SACC probably through downregulation of its direct target of *PDCD4* and this results in subsequent activation of p-Stat3.

In this study, we found that miR-21 expression was significantly higher in metastatic SACC than that in non-metastatic SACC samples. We also found that miR-21 remarkably increased invasion and migration in SACC cells, these results are consistent with previous findings from other cancers.[21,22,43,44] In addition, we showed that knockdown of miR-21 could dramatically decrease cell invasion and migration in SACC cells. Overexpression of miR-21 has been reported in various human cancers. All these results including our data indicate that miR-21 has multiple functions in diverse cancers and could be recognized as an important onco-miR.[14,21]

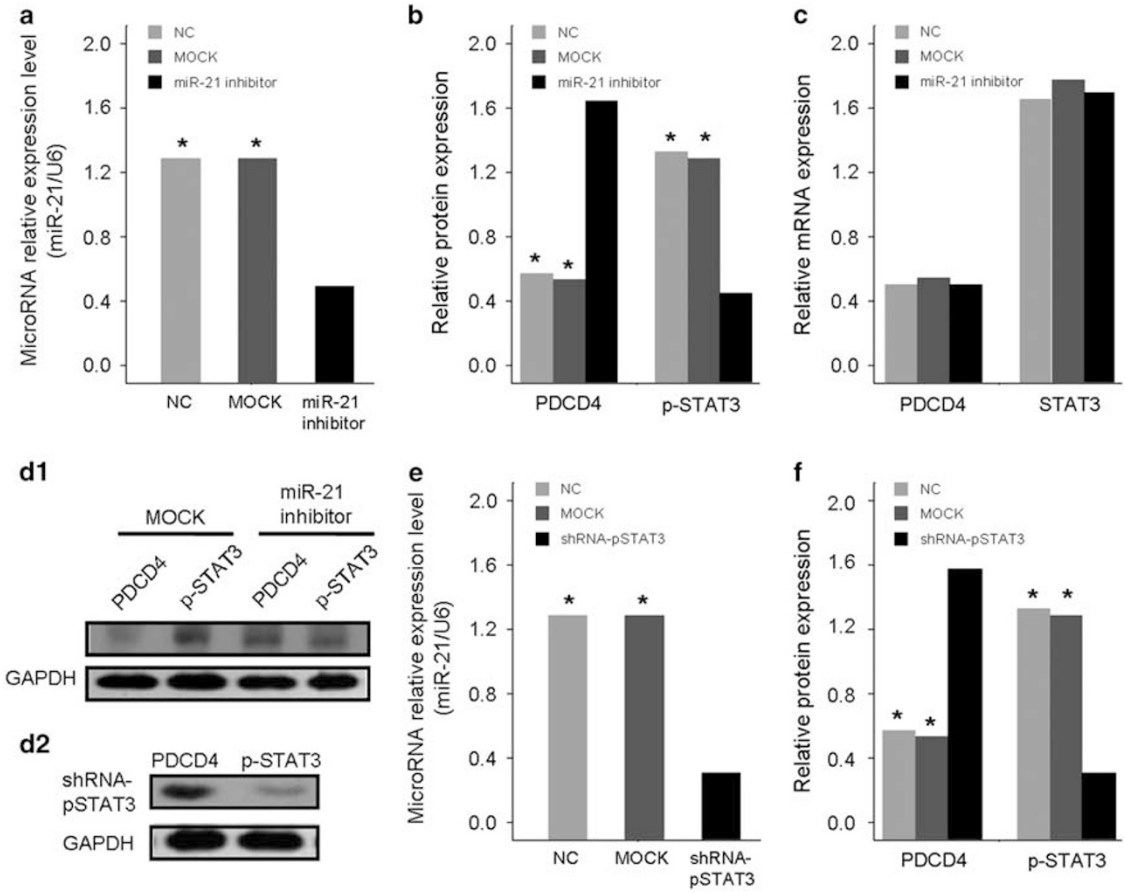

Figure 6 miR-21 regulates PDCD4 expression at the post-transcriptional level and influences the phosphorylation of STAT3. (**a**) Cells were transfected with miR-21 inhibitor, inhibitor-negative control (NC), or blank control culture medium (MOCK). Cell lysates were obtained after 48 h for analysis. miR-21 expression was detected by TaqMan RT-PCR. The results were normalized to U6 expression and expressed as fold change relative to the corresponding NC (*$P<0.05$). (**b**, **d**) The expression of PDCD4 and phosphorylated STAT3 were examined by western blot analysis. The results were normalized to GAPDH protein expression and expressed as fold change relative to the corresponding NC (*$P<0.05$). (**c**) The expression of *PDCD4* and *STAT3* mRNA were examined by real-time RT-PCR. The results were normalized to GAPDH mRNA expression and expressed as fold change relative to the corresponding NC. (**e**) The silencing of p-*STAT3* regulates miR-21 expression. Cells were transfected with shRNA-p-*STAT3*, shRNA-NC, or blank control culture medium. Cells were obtained after 48 h for analysis. miR-21 expression was detected by TaqMan RT-PCR. The results were normalized to U6 expression and expressed as fold change relative to the corresponding NC (*$P<0.05$). (**f**) The expression of PDCD4 and phosphorylated STAT3 was examined by western blot analysis after cells treated with shRNA-p-*STAT3*. The results were normalized to GAPDH protein expression and expressed as fold change relative to the corresponding NC (*$P<0.05$). **d1:** PDCD4 protein was significantly increased and phosphorylated STAT3 protein was concomitantly decreased when endogenous miR-21 was knocked down by the miR-21 inhibitor; **d2:** PDCD4 protein was significantly increased and phosphorylated STAT3 protein was concomitantly decreased when interfered by shRNA-p-STAT3. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Therefore, targeting miR-21 may be a potential therapeutic strategy for these tumors.

It has been suggested that *PDCD4* is a potential target for miR-21,[45] and *PDCD4* expression might have an essential role in the progression of SACC.[46] We confirmed that *PDCD4* is a direct target for miR-21 through the miR-21-binding site at the 3′-UTR. *PDCD4* has been reported as a tumor-suppressor gene in various tumors.[22,47,48] It performs an important function in tumor invasion and metastasis through inhibition of *STAT3* activation, which are known to be involved in cancer progression.[22,47–49] Our data suggested that *PDCD4* expression has a negative correlation with the expression of miR-21 in SACC tissues and cell lines at protein level. Applying specific inhibitor of miR-21 can rescue *PDCD4* expression, negatively regulated SACC cell invasion and metastasis. The results suggest that upregulation of miR-21 was attributive to, at least in part, the downregulation of metastasis-related tumor suppressors *PDCD4*, although more samples may be required to make a firm conclusion. Because constitutive activation of *STAT3* has been demonstrated in a large number of diverse human tumors, and considerable evidence suggests that constitutive *STAT3* activation actively participates in tumor formation and progression,[24,25] we also studied the expression of p-stat3 in our *in vitro* model and in SACC samples. We found that p-Stat3 expression is significantly upregulated in SACC samples and its expression was inversely correlated with *PDCD4* expression but positively correlated with miR-21 level. In addition, silencing of

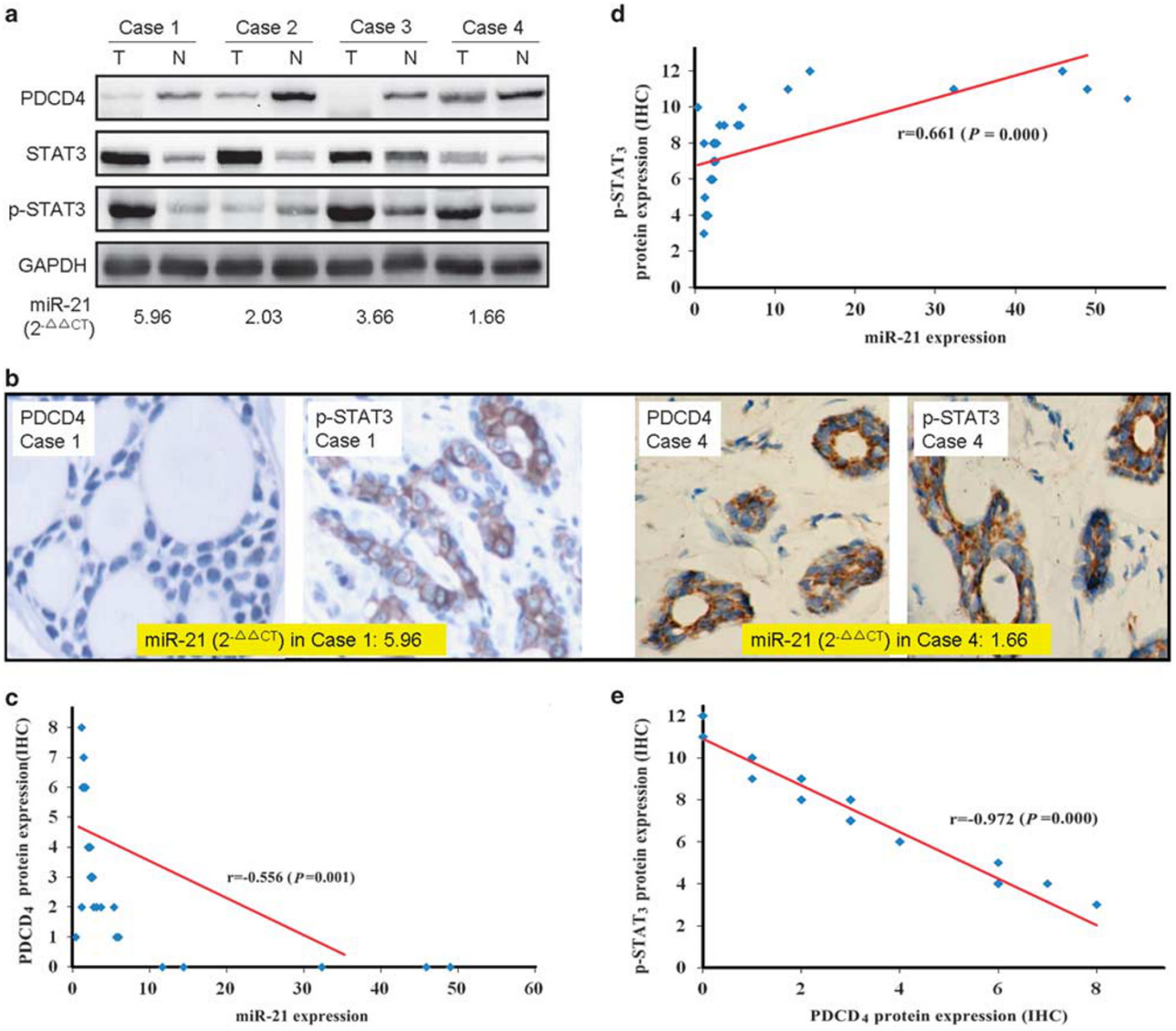

Figure 7 Expression of PDCD4, p-STAT3, and miR-21 in matched SACC tumor specimens. (**a**) Expression of PDCD4, STAT3, and p-STAT3 protein in four pairs of matched SACC tissues and normal tissues, as detected by western blotting and normalized to β-actin protein expression. The specimen number is shown above each pair comprising tumor (T) and normal (N). The miR-21 expression folds of the four cases were shown under the western blotting panels. (**b**) Relative expression of PDCD4 and p-STAT3 protein in SACC tissues were detected by immunohistochemistry. Original magnification ×200. Case 1: Absent of PDCD4 protein expression and high expression of p-STAT3 protein in a SACC tissue with miR-21 overexpression. Case 4: a representative positive, positive expression of PDCD4 and p-STAT3 protein in a SACC tissue without miR-21 overexpression. (**c**) Correlation between miR-21 expression and PDCD4 protein levels in SACC tissues (Pearson correlation, $r = -0.556$, $P = 0.001$). (**d**) Correlation between miR-21 expression and p-STAT3 protein levels in SACC tissues (Pearson correlation, $r = 0.661$, $P = 0.000$). (**e**) Correlation between PDCD4 protein and p-STAT3 protein levels in SACC tissues (Pearson correlation, $r = -0.972$, $P = 0.000$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SACC, salivary adenoid cystic carcinoma.

p-*STAT3* downregulated the expression of miR-21 and upregulated the expression of PDCD4 protein and resulted in the inhibition of invasion and metastasis in SACC-LM cells. We verified *in vitro* that miR-21 negatively regulates *PDCD4* and positively regulates p-*STAT3*, which in turn alters miR-21 expression, suggesting that the STAT3-miR-21-PDCD4 feedback regulatory network may have an important role in SACC pathogenesis and progression. Therefore, the decrease of miR-21 with miR-21 inhibitor in SACC-LM cells could target *PDCD4* gene and in turn enhanced PDCD4 protein expression, then suppressed p-STAT3 protein expression, further feedback reduced miR-21 expression, and the final leading to the inhibition of cell invasion and migration.

miR-21 is overexpressed in SACC cells, and an aberrant expression of miR-21 can alter multiple biological processes of human SACC cells, such as proliferation, invasion, and migration, probably through regulating *PDCD4* and other critical target genes. miR-21 can serve as a biomarker for patients with SACC, and an inhibitory strategy against the STAT3-miR-21-PDCD4 pathway will have a strong rationale for the treatment of SACC patients.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declared no conflict of interest.

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